Use of Antibiotics in Greek Mariculture

Panagiotis Christofilogiannis

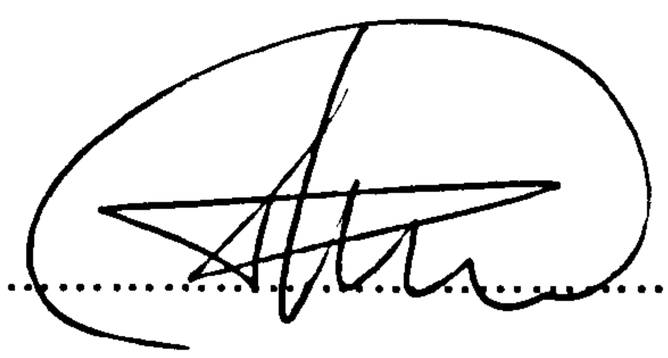
PhD Thesis

May 2002

University of Stirling – Institute of Aquaculture

Declaration

I hereby declare that this thesis has been composed by myself, that it has not been accepted in any previous application for a higher degree, that the work of which it is a record has been performed by myself, and that all sources have been specifically acknowledged.



....

Panagiotis Christofilogiannis Date

....

Randolph H. Richards (supervisor) Date

ACKNOWLEDGEMENTS

The work reported in this study would not have been possible without the support and encouragement of the persons mentioned below:

Professor Randolph Richards is thanked for providing the opportunity to undertake this study. Dr. Maria Alexis is thanked for the support and supervision throughout the experimental period and Mrs Maria Giagnisi, Mrs Maria Koutsodimou, Mrs Argyro Andriopoulou are thanked for long time support in the bacteriological survey of this study. I would like to thank all the stuff in NCMR for making my stay and study easy and fruitful. I would also like to thank my wife Joanna for the long time support during the experimental part, the statistical analysis and the final presentation of this thesis. I would like to thank my brother Dennis for the effective support during difficult periods of this study with large amounts of work at limited time. I would like to thank Dr. Gavin Barker for his critical help in the first steps of the bacteriological survey and Dr. Dionisios Tyrpenou for his help in the Oxytetracycline HPLC method. I would also like to thank my family for the long support and belief in me, and especially my son Yiannis who is the best person to share the joy of concluding this study.

Finally I would like to thank Mr. John Stephanis and the board of directors of the Federation of Greek Maricultures who granted the funding of this project. This study was co-funded by the Y.P.E.R programme of the General Secretariat for Research and Development – Greek Ministry of Development.

P. Christofilogiannis

ABSTRACT

Bacteriological survey of the fish pathogens in Greek mariculture between 1994-1997 was followed by analysis of prevalence in sea bass, sea bream, sharpsnout bream and common Dentex and discussion of the impact of various fish pathogens. In addition antibiotic resistance profiles and frequencies were studied using quantitative antibiogram and MIC analysis for the two most commonly used antibiotics Oxolinic acid and Oxytetracycline and clinically relevant MIC breakpoints were extrapolated for different fish species and main fish pathogens.

The kinetics of the above antimicrobials were analysed in eight experiments where two fish species namely sea bass and sea bream as well as two water temperatures were employed. Muscle, liver, serum, skin samples were analysed by two HPLC methods and two bioassay methods were developed. The relative importance and significance of these findings was evaluated in the general context of pharmacokinetic studies in fish. Kinetic data were compared to clinical data and practical implications were evaluated. Issues like antibiotic resistance and its implications, the implications of residues and resistance in human health and the environment were analysed in order to put this study in context. Conclusions tackled important aspects of antimicrobial chemotherapy and future work was suggested.

CONTENTS

1 GENERAL INTRODUCTION

1.1 Mediterranean mariculture industry

Supply of Mediterranean fish species depended a few decades ago on coastal fisheries and traditional extensive culture in coastal lagoons. In the 70's market demand increased leading to extreme intensification of coastal fisheries and lagoon management. In the 80's the EU supported extensive research projects as well as a development program in order to organise Mediterranean mariculture in the prototype of the Japanese and European models of salmon culture. The first phase of development of the sector («research phase») started in France and Italy in 1969-1970 and expanded to the rest of the Mediterranean in the 80's. The second phase («pro-development») started in the mid '70s and finished in the first half of the 80's. During this period the first companies were created in France, Spain, Greece and Italy. Many of the sector's major problems were started at that period with strong R&D motivation and development of common EU fisheries policy. In the following years (1987-1993) large scale production («development phase») was initiated in Spain, France, Italy, Greece, Turkey, Cyprus, Malta, Tunisia, Morocco and Croatia. The last period (1993-1998) was a period of a new expansion of the industry («pro-maturity phase») with groups of major companies with multidisciplinary vertical structure dominating the market. These companies are selling know-how and investing into new areas like the Arabian Gulf countries (Kuwait, Saudi Arabia) or in South East Asia (Singapore and Indonesia). Since 1998 the industry has been facing a maturity phase with supply segmentation, structural inefficiencies, lack of defined image and ever drifting prices of the final product.

Mediterranean mariculture in 1994 comprised of 408 production units with 60 of them being vertically developed with their own fry supply. In 2000, among 642 fish farms in the Mediterranean 41,9% were located in Greece, 27,3% in Turkey and the rest in

South Europe and North Africa (Figure 1). In recent years Mediterranean sea bass and sea bream production has increased 9 times with estimated production in 1992 at around 14.700 tons and 112.000 tons in 2000 (Tables 1,2). Greece is the leading producer in the sector with 59.000 tons in 2000 produced from 269 units and 200 million fry. (Figure 2, Table 3) Greek production exceeds 50% of the European market and almost 60% of it is being exported. In the last half of the 90's the mariculture industry was facing many problems. Problems included marketing in new markets with supply segmentation and market congestion resulting in drifting prices (Figures 3 & 4). Optimisation of production process, cost cutting management policies coupled with increased consumed awareness and strict monitoring on quality and safety are some of the sector characteristics. Great interest and effort is devoted in the Quality assurance protocols in order to develop products of high quality, low lipid content, and nutritional value lacking bacterial human pathogens, antimicrobial agents and dioxin residues in order to preserve the natural identity of the product that is not only required but is being demanded by the customer. At the same time extensive research on new species development is coupled with advances in diagnostic techniques and more effective protocols for the prevention and treatment of new pathological conditions in order to minimize cost of production, decrease the incidence of diseases and reduce the use of antibioticswhilst also ensuring fish welfare.

Figure 1: Distribution of Sea bass and Sea bream production units in Mediterranean Sea. (2000) – Total number of farms: 642.

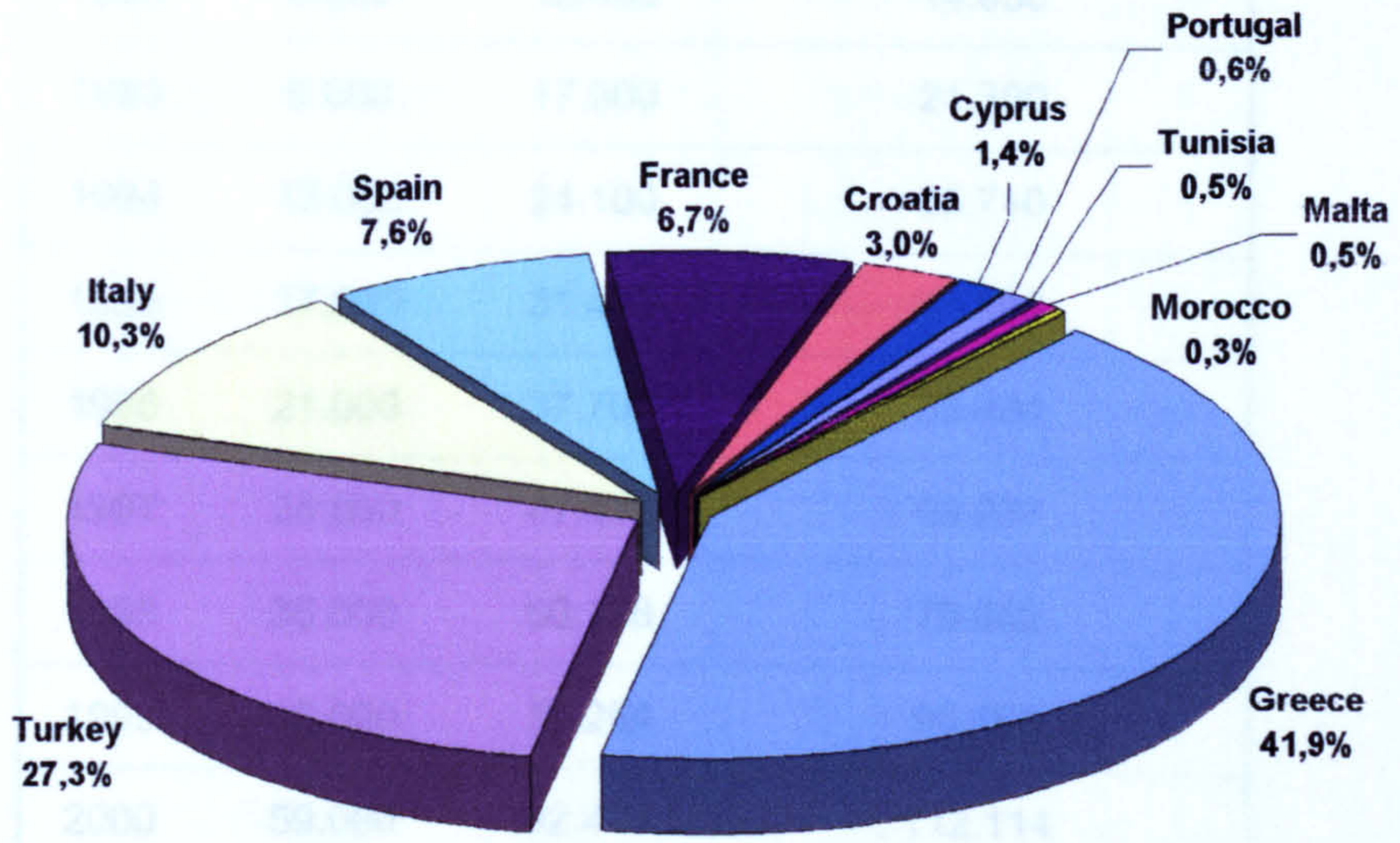
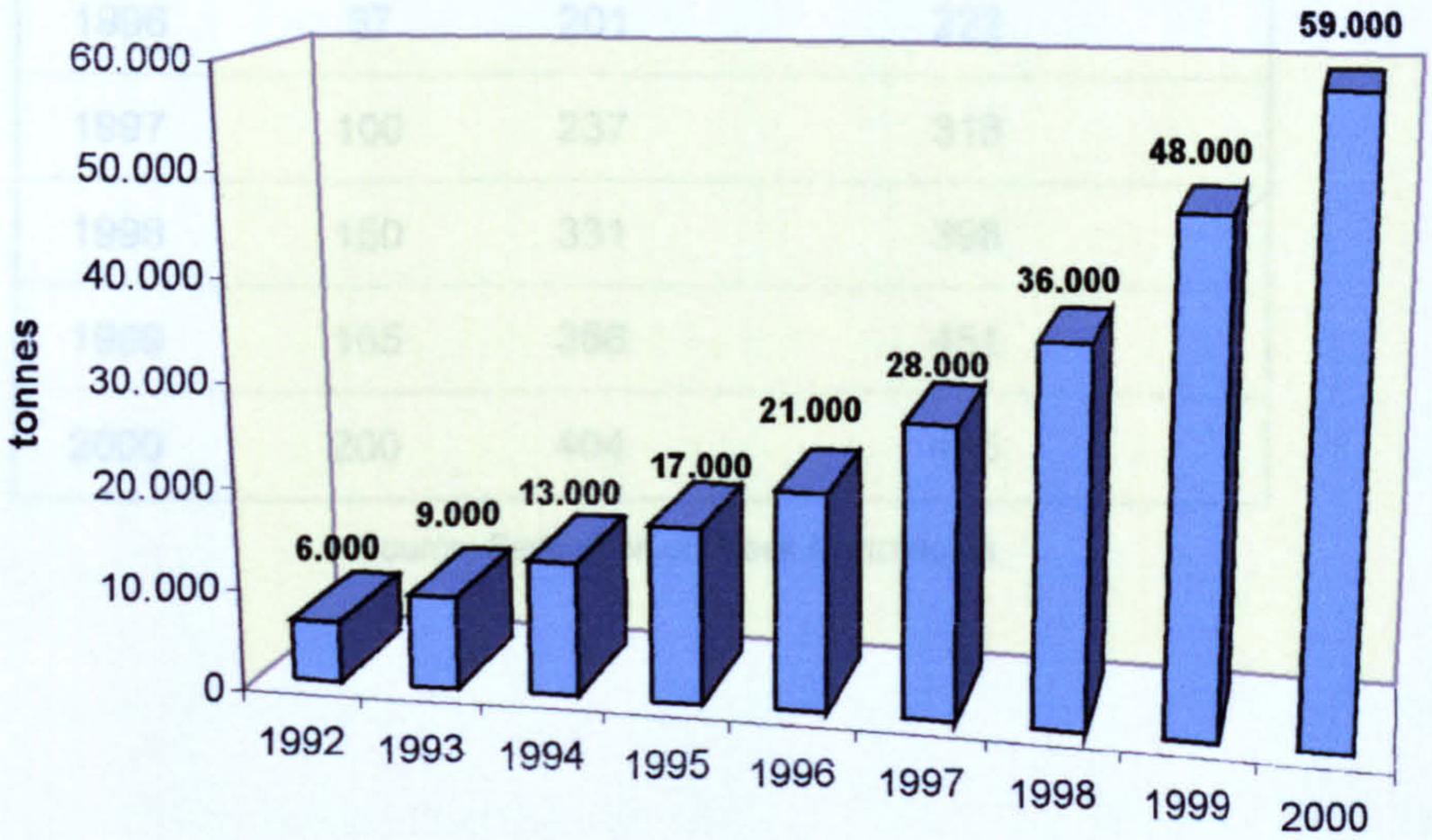


Figure 2: Total production of Sea bass and Sea bream in Greece (tons)



Source: Federation of Greek Maricultures

Table 1:Production of Sea bass and Sea bream (tons)

Year	*Greece*	*E.U*	*Mediterranean*
1992	6.000	12.480	14.650
1993	9.000	17.900	21.390
1994	13.000	24.100	35.710
1995	17.000	31.475	47.298
1996	21.000	37.706	55.404
1997	28.000	47.411	66.937
1998	36.000	60.188	79.052
1999	48.000	75.264	96.863
2000	59.000	92.414	112.114

Table 2: Production of Sea bass and Sea bream fry (millions)

Year	*Greece*	*E.U*	*Mediterranean*
1995	95	171	184
1996	57	201	222
1997	100	237	318
1998	150	331	398
1999	165	368	451
2000	200	404	445

*Source: Federation of Greek Maricultures

Table 3: Geographical distribution of fish farms in Greece

Evia	38	Kyklades	6
Fthiotida	27	Arkadia	4
Etoloakarnania	26	Xalkidiki	4
Attiki	25	Lesvos	4
Dodekanisa	22	Messinia	3
Chios	16	Lasithi	3
Korinthos	15	Samos	2
Argolida	15	Magnisia	2
Thesprotia	15	Kavala	2
Cefalonia	11	Lefkada	1
Fokida	10	Corfu	1
Prevesa	9	Pieria	1
Viotia	6	Rodopi	1
Total no. of Farms			***269***

*Source: Federation of Greek Maricultures

Figure 3: Production and value of Sea bass

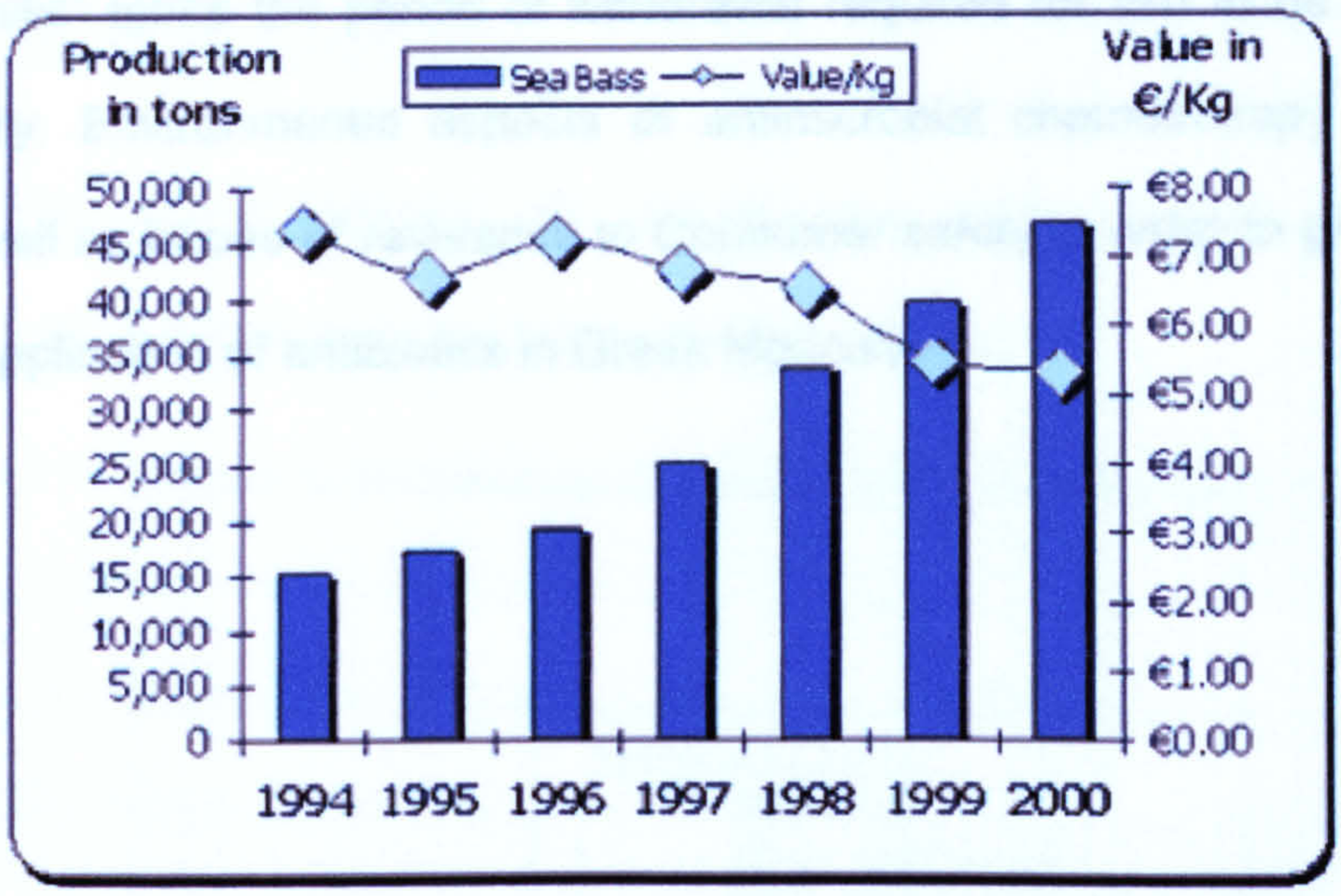
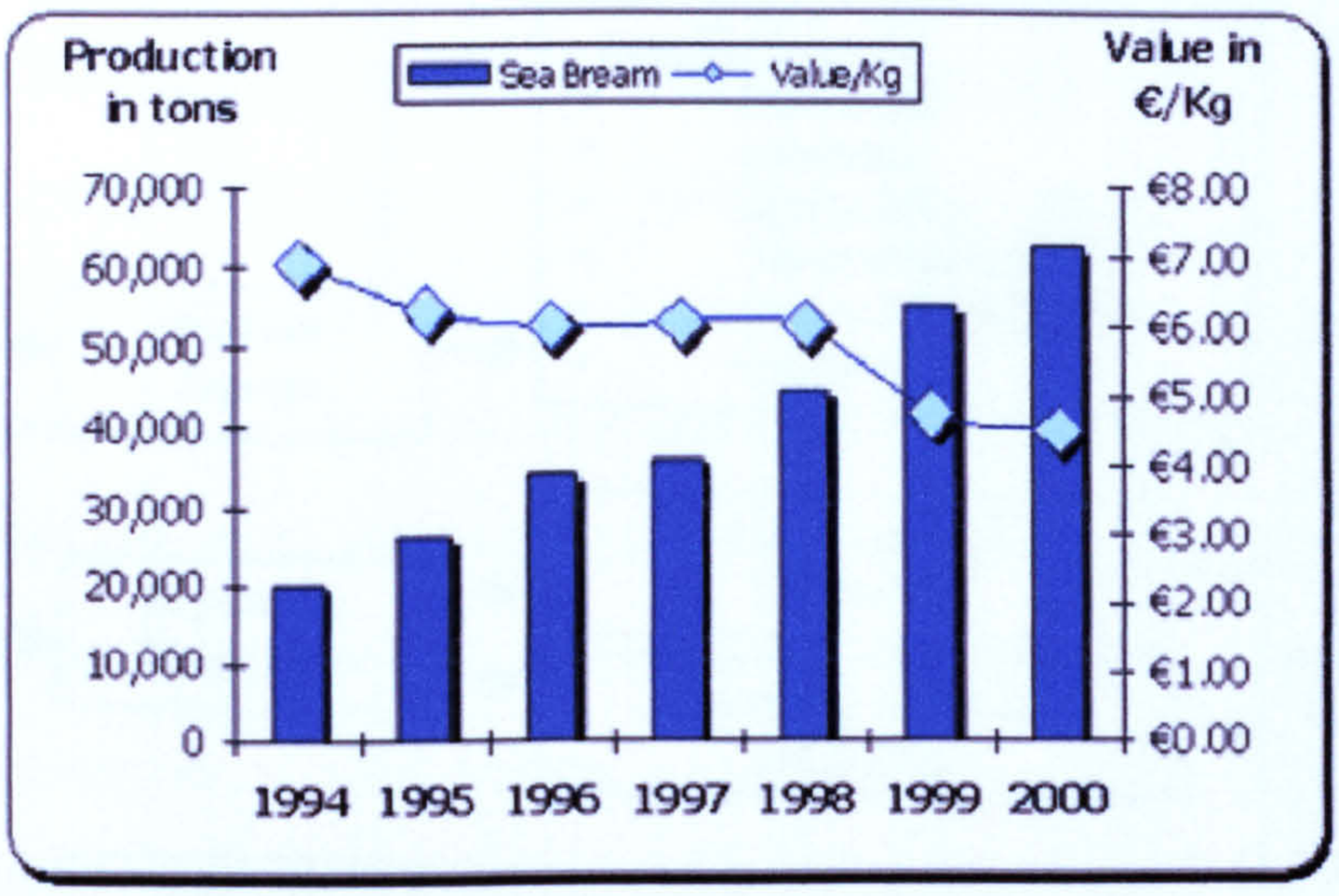


Figure 4: Production and value of Sea bream



*Source: Federation of European Aquaculture Producers

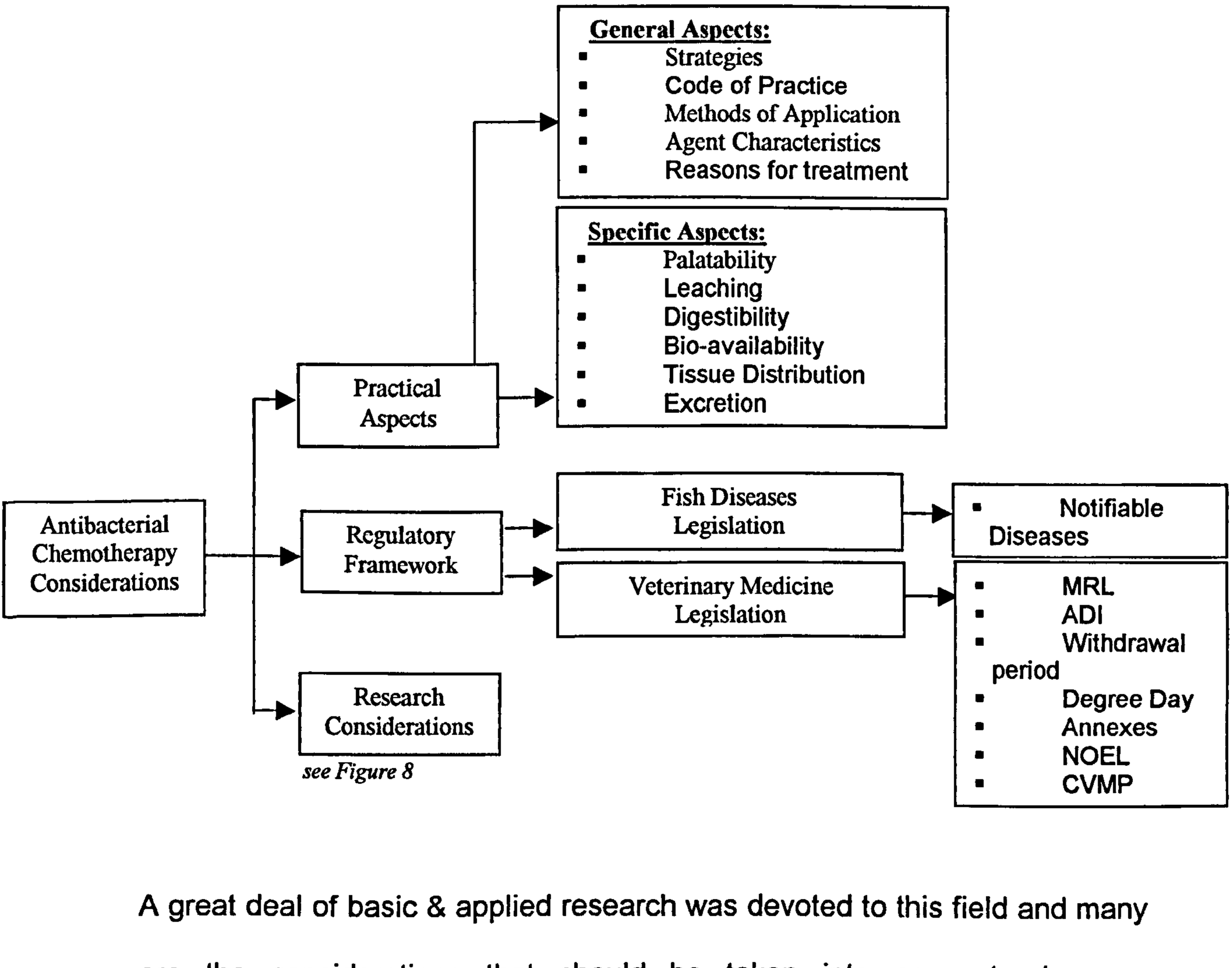
The Federation of Greek Maricultures, the leading producer association in Greece, founded in 1991 and since then coordinating the development of the sector, sponsored this research in order to provide practical views on antibiotic application in Greek Mariculture. Four years of research were devoted to detailed recording of bacterial fish pathogen frequency and resistance to the most commonly used antimicrobial agents in Greek mariculture. In addition this study included research on the kinetics of the two most widely used antimicrobial agents in Greek mariculture namely Oxytetracycline and Oxolinic acid. The aim was to investigate the clinical

efficacy of antimicrobial chemotherapy under "commercial" conditions and to try to define in "realistic" terms the period of withdrawal required for fish to be able to be marketed safely. Environmental aspects of antimicrobial chemotherapy were also described as well as issues of relevance to Consumer safety in order to give a global insight of the application of antibiotics in Greek Mariculture.

1.2 Antibacterial chemotherapy

Antimicrobial chemotherapy in aquaculture has raised considerable discussion and stimulated intense scientific interest.

Figure 5.: Antibacterial Chemotherapy Considerations



A great deal of basic & applied research was devoted to this field and many are the considerations that should be taken into account when an antimicrobial agent is applied in aquaculture. These considerations include Practical aspects, Regulatory issues and Research considerations (Figure 5).

1.2.1 Practical aspects

1.2.1.1 General practical aspects

Questions that are raised for the Practical aspects of the use of antimicrobial agents include, strategies for the control of bacterial infections in fish, codes of practice for the appropriate use of antimicrobial compounds in fish farms and methods of application of these agents. In order to control bacterial infections several strategies are employed. Eradication of the infection is not always possible or necessary. Decrease of bacterial numbers along with improvement of fish health status is necessary employing husbandry measures in order to "tip the balance" in favour of the fish when opportunistic bacterial pathogens cause mortalities. Reduction of stocking densities, handling and overfeeding reduces stress levels while avoidance of multiple hatchery origin of fry, testing of hatchery sources, broodstock screening, and separation of year classes are some of the prophylactic measures used to prevent the introduction of the pathogen. Adequate farm distances, fallowing of sites and reduction of cross contamination by equipment and personnel movements are on the other hand prophylactic measures to reduce the spread of bacterial pathogens. Simple suggestions for the appropriate use of drugs in aquaculture are presented in the following short and simple code of practice (Table 4):

Table 4.: Code of practice for the use of antimicrobial compounds in fish farms

Restriction of the compounds to authorised personnel, use under professional quittance	Restrict the use of some compounds to specific diseases
Use according to the instructions	Avoid the use of certain medically important drugs in fish
Proper storage and use before the expiry date	Rotate use of different antimicrobial compounds
Storage out of the way of children, inexperienced personnel, and laymen	Dispose of the unused compounds safely
Allow the recommended withdrawal periods	Ensure the quality of the medicated feed preparation
Organise a logbook for the drugs in stock with details for the treatments applied (cage no, quantity, consumption, problems.)	Ensure that the farm manager keeps a data logbook with mortalities, disease outbreaks, diagnosis and vet prescriptions and comments.
Avoid free release into the aquatic environment in order to avoid development of resistant microflora	Organise monitoring to ensure that the code of practice is maintained

The main methods of antimicrobial agent application in aquaculture along with basic comments on each method are presented below in Table 5. The selection of the antimicrobial agent is based on several necessary characteristics. The selected agent must be selective against the microorganism concerned, palatable and bio available to the fish. It should not cause toxic effects on the fish or exacerbate the disease but should be able to penetrate host's tissues and thereby reach the pathogen. It should break down easily and then be eliminated from the fish tissues and it should be metabolised into harmless compounds to fish and other marine organisms. Each antimicrobial agent should be safe for the consumers and even its residues should not form toxic or carcinogenic products during heating (cooking of contaminated flesh). In practical terms it should be stable under normal conditions of storage and use and should not contain harmful additives or impurities in the proprietary product, should be closely supervised during all stages of production and should be cost effective.

Table 5: Main methods of antimicrobial agent application in aquaculture

Methods of Application	**Comments**
Oral treatment (Medicated feed)	Palatable drug Minimal environmental risk
Bath	Soluble or adequately dispersed compound Lengthy exposure needed Disposal of dilute compound problematic
Dip	Soluble or adequately dispersed compound Brief immersion Environmental pollution considerations
Flush	Soluble or adequately dispersed compound Brief exposure Environmental pollution considerations
Injection	Feasibility only for large or valuable fish Anaesthesia may be required Slow process Negligible environmental risk
Local application	Feasible for treatment of ulcers on valuable pet fish

In epidemiological terms important drugs used in human medicine should be avoided in fish farming to protect the strategic advantage of their application. These compounds should not be used if plasmid mediated resistance is known to occur or if they have adverse effect on the user, the natural aquatic flora and fauna, or land animals which may come into contact with contaminated water.

The main reasons for treatment failures in clinical practice include possible selection of inappropriate antimicrobial agent (resistance), critical delay in diagnosis and treatment, inappropriate or variable dose rate (application, mixing) and uneven feeding response due to low temperature or hierarchy formation in the fish population. In addition palatability, stress factors, inappropriate management and inappropriate treatment strategy play significant roles in treatment failures.

Pathogen mechanisms to avoid the effect of the antimicrobial agents, reinfection due to mixing of year classes or populations with different epidemiological background, re-circulation of the effluents in hatcheries as well as cases where wild fish act as carriers or bacteria concealed in unusual sites like brain, necrotic tissues, macrophages or external surfaces make treatment very challenging and failure probable.

1.2.1.2 Specific practical aspects

The terminology of drug metabolism includes terms like palatability of the drug (pleasant in taste), leaching, digestibility (ability to be assimilated), absorption (ability to be absorbed in the gut epithelium), bioavailability (potential of achieving high concentration of "active" drug in the target tissues), excretion (expulsion of waste material as a result of metabolism) and depletion (reduction of drug residues in fish tissues). Palatability is affected by the antibiotic (low in OTC), by the method of drug administration (low at oil top coated feed) and by the species (sea bass: affected more than sea bream). Leaching is linked to the drug incorporation in the feed (high in oil top coated feed), the type of the drug (higher in OTC compared to OA) and it is proportionally increased with temperature. Digestibility is affected by the drug (lower in OTC compared to OA), it is increased proportionally for both antibiotics with increasing water temperature. Tissue distribution of the antibiotic in relation to the site of the infection is important. Localised infections like skin lesions as well as organised granulomas are regarded as difficult to treat because of the mechanical difficulties in accessing these tissues. Physicochemical microenvironment in the fish with factors like the concentration of Mg^{++} and Ca^{++}, protein binding as well as water temperature exert an important role on the *in vivo* efficiency of antimicrobial agents. The concentration of Mg^{++} and Ca^{++} in seawater reduce dramatically (>90%) the

biological activity of Oxytetracycline (Lunestad and Goksoyr, 1990) as well as in oxolinic acid and flumequine (Palmer et al., 1992; Smith et al., 1996). The concentration of these ions is higher in the hindgut of the marine fish by a factor 10 to 100 (Conte, 1969). Since tetracyclines are powerful chelating agents, their effectiveness can be reduced or eliminated by high concentrations of divalent and trivalent metal ions. Protein binding in the serum reduces the "biologically available" drug concentration against bacterial fish pathogens. Fish are poikilothermic and treatments are usually applied over a wide range of water and host temperatures. The metabolic rate of the fish and the kinetics of the antimicrobial agents are highly dependent on the temperature. Bjorklund et al., (1992) described the effect of temperature on the metabolism of oxolinic acid in rainbow trout. Drug Bioavailability depends on physiological species factors and physiochemical drug characteristics. Physiological factors that affect bioavailability include body weight / age / sex, physical state, sexual maturity, blood flow, liver and Kidney function, stomach emptying rate, motility, pH and enzyme variations in gastrointestinal tract as well as circadian / diurnal rhythm and stress levels. In addition factors like water temperature, nutrition, drug physiochemical characteristics / particle size, dose, type and amount of vehicle substances, manufacturing method, presence of a solvate or a hydrate and solubility characteristics vastly affect bioavailability. Oxolinic acid bioavailability was found to decrease with increasing dose rates, was enhanced by particle size reduction, while both OA and OTC bioavailability was markedly negatively affected by the health status of fish. Oxolinic acid was retained at a lower concentration and for shorter period in the fishes acclimated to seawater. Excretion was influenced by a series of factors like temperature, tissue, health status, maturity and environmental conditions.

1.2.2 Fish Defence mechanisms

Although Sea bass is known to be susceptible to stress and subsequent invasion from opportunistic as well as pathogenic bacteria, in recent years a series of studies demonstrated an array of immuno-competence mechanisms that include non-specific primary mechanisms like skin mucus (Soltani, 1998), antibacterial activity, phagocytosis (Esteban and Meseguer, 1997), macrophage activity (Angelidis et al., 1998), gut-associated lympoid organ activity (Abelli et al., 1997) and interferon-like activity (Pinto et al., 1993) as well as more specific mechanisms like IgM and B cells activity. (Bourmaud et al., 1992; Bakopoulos et al., 1997b; Coeurdacher et al., 1997; Dos Santos et al,1997).

1.2.3 Overview of the legislative framework

1.2.3.1 Fish Diseases

The European Commission has decided that after more than 10 years the Community's fish health legislation should be reviewed. DG SANCO, which now considers all veterinary issues, has invited representatives of the EU Aquaculture Advisory Committee to sit with representative state veterinary administrators to consider this. The first Community legislation concerning animal health in aquaculture production was adopted in 1991 (Council Directives 91/67/EEC & 98/45/EC). Today, detailed and harmonised legislation is in place covering animal health aspects of aquaculture production. The legislation includes conditions governing the placing on the market of aquaculture animals and products, measures for the control of certain fish diseases (Council Directive 93/53/EC & Commission Decision 2001/288/EC) and of certain diseases affecting bivalve molluscs (Council Directive 95/70/EEC & Commission Decision 2001/293/EC),

sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases (Commission Decisions 2001/183/EC) and of certain mollusc diseases (Commission Decision 94/306/EC) (Bjornerot, 2001).

For certain diseases listed in the legislation, several decisions for approval of farms and zones have been taken which protect and enhance the trade between disease free areas. In addition, decisions have been adopted concerning movements of different aquatic animal species and products within and into the Community (Commission Decisions 96/490/EC, 98/24/EC, 93/22/EEC, 1999/567/EC, 93/55/EEC, 93/169/EEC, 95/352/EC) and certain protective measures have been taken in respect of infectious salmon anaemia (ISA) in salmonids in Norway and Faroe Island (Commission Decisions 1999/766/EC, 2001/313/EC, 2000/574/EC, 2001/312/EC). Since 1998, when ISA first was detected within the Community, decisions have been taken to adapt the legislation to new situations, increase in knowledge and to give provisions to the Member States for combating the disease in a relevant manner (Council Directives 2000/27/EC, 93/53/EC). Recently, decisions were adopted on "withdrawal" measures to be applied in Scotland, England and Wales in case of outbreaks of ISA (Commission Decisions 2001/494/EC) and a Council Decision agreed to include ISA for compensation to the Member States in case of outbreaks within the Community (Council Directive 2001/572/EC). In addition to the regular legislation review, an animal health and zoosanitary review of the Community legislation concerning aquatic animals and products has been initiated by unit E2 (Health & Consumer Protection Directorate) of the European Commission. It has been concluded that a revision is needed to take into account the developments in aquaculture production, as well as the practical and scientific experiences gained during the last ten years. In the review process, the Member States, including well-reputed animal health experts within the aquatic field and the industry are

consulted. Although up until recently no diseases of Sea bass or Sea bream were listed in any of the EU lists of notifiable diseases, there is an interest on the review of the current legislation. Acting as FGM Fish Health Committee chairman and FEAP Fish Health Group Vice-chairman I participate twice a year in this review process as FEAP representative for the Mediterranean within the framework of the Advisory Committee of Fisheries and Aquaculture (ACFA) The main interest for Mediterranean Mariculture is to tackle issues like Marine zones allocation and conditions for the transport of fry in the future case of a notifiable disease adapting the current framework which is customised for salmonids. The Community aquatic animal health legislation should be simplified and made clearer and should be as consistent as possible with the International Aquatic Animal Health Code and the Diagnostic Manual for Aquatic Animal Diseases of the International Office for Epizooties (OIE) (Bjornerot, 2001) The fish farmer's point of view is that the objective of the legislation should be to facilitate free trade without the spread of disease. There has to be harmonisation with countries outside the Community with which member states trade, and the socio-economic objectives of aquaculture as a justifiable and sustainable method of food production must be maintained. These are well-established principles. Some restriction of trade is inevitable when diseases are controlled but this has to be as limited as possible. Controls must encompass the basic prevention of disease transmission, eradication and/or control programs, and also the trade and movement restrictions on fish, fish products, equipment, and possibly personnel. Where pertinent, e.g. in eradication programs, legislation will have to take into account any financial support be it from Member States, the Commission, and the insurance industry or from private funding. The legislation must also allow opportunities for international trading by allowing member states to demonstrate their disease free status. Official authentication by competent authorities is essential to ensure equivalence for exports and to facilitate trade (Aquamedia, 2002). The diseases to be regulated at

Community level need to be reviewed, as well as the susceptible species and vectors of concern. Future legislation should also consider the potential exchange of disease agents between farmed and wild aquatic animals and the regulations for fish should be separated from the ones for molluscs and crustaceans. Furthermore, it has been proposed that the legislation should be more flexible, giving provisions for urgent amendments in order to adapt the legislation to changed production and health conditions - including emerging diseases - and gained knowledge within the Member States and in other countries. Further proposals for major changes and issues to be carefully reviewed are: the requirements for National and Community reference laboratories; sampling and diagnostic methods; introduction of a risk based program including preventative measures, registration and monitoring of farms and other establishments; management routines for transport and waste; requirements and decentralized administrative procedures for approval of farms and zones, including criteria for zoning and movements of farmed animals and wild broodstock, control measures to be applied in different situations; and provisions for financial support and vaccination. There is also an urgent need to lay down harmonised animal health conditions for imports of aquatic animals and products from third countries (Bjornerot, 2001).

1.2.3.2 Veterinary Medicines

The importance of veterinary medicines in aquaculture and the increasing recognition of the problems of environmental interactions and impacts deriving from the use of such medicines led in 1992 to a conference on chemotherapy in aquaculture by the Office International des Epizooties (Michel and Alderman, 1992). Applicants for licenses for veterinary medicinal products are required to satisfy the Licensing Authority as to safety, quality and efficacy of their product. The term product includes not only active ingredients but also all other

components such as bulking agents, solvents etc. In the case of Veterinary medicinal products for use in aquaculture, any environmental impact must be assessed in order to satisfy the safety requirements of the license. The amount of information needed will depend on the proposed use, route of application and the expected extent of usage as well as on the nature of the product itself. Several EU directives regulate antimicrobial agent application in aquaculture. Five main terms have been designed in the light of these regulations, the Maximum Residue Level (MRL), the No Effect Level (NOEL), the Acceptable Daily Intake (ADI), the Withdrawal Period and the Degree Day concept.

Maximum residue Level (MRL) is the antimicrobial agent residue level, which if consumed, will not provide any risk to the health of the consumer. MRL is based on the "no effect" (NEL) of residue that is the highest dose level without visible or biochemical changes in pharmaco-toxicological testing. From the NEL, the acceptable daily intake (ADI) of the active substances by man is extrapolated. Acceptable Daily Intake (ADI) is calculated on an exaggerated daily food intake level (muscle 300g, liver 100g, kidney 50g, fat 50g, egg 100g, and milk 1500ml). MRL is calculated from the ADI with a large safety factor added (100-1000). The withdrawal period is the period which must elapse after the completion of a course of treatment of fish with a veterinary medicine before they may be slaughtered for human consumption. Withdrawal periods are affected by numerous factors that influence drug residence time in fish. The most important factors are water temperature, fish species, and fish tissue and drug characteristics. Withdrawal periods are formulated as a period of clearance while the level of drugs in edible tissues decrease below the MRL value for each antimicrobial agent. Four MRL Annexes were established by Regulation 2377/90 EEC and the fish related products are presented in a consolidated format (Table 6).

Degree Day is the concept established for the measurement of withdrawal periods in homoeothermic animals like fish, in which metabolism and drug excretion depends on the prevailing water temperature. It refers to the sum of the average daily water temperatures during the withdrawal period. For all new products a Maximum Residue Level (MRL) must be defined, that complies with EC regulations according to which all products will need an MRL by 1997 (Regulation 2377/90/EEC). The MRL pertains to a compound while a withdrawal period to a formulation or product. Different formulations of the same compound may require different withdrawal periods in order to achieve the same MRL. The evaluation of applications for a Community authorisation in the centralised procedure and in the residue data evaluation, is carried out by the Committee for Veterinary Medicinal Products (CVMP) at the European Agency for the Evaluation of Medicinal Products (EMEA). Both the Community marketing authorisations for veterinary medicinal products and the adoption of the results of residue evaluation are adopted in accordance with a standing committee procedure. Veterinary medicinal products subject to a national authorisation (Council Directive 81/851/EEC) must be authorised by the mutual recognition procedure, if they are to be placed on the market of more than one Member State. Each authorisation specifies the animal species in which it may be used, the therapeutic indications, the dosage and the withdrawal period for food-producing animals (time between the last dose and the slaughter and/or obtaining food products provided by treated animals). Authorisations are valid for 5 years and are renewed upon request and submission of all necessary information. A market surveillance of all market products is carried out by the marketing authorisation holder and by the competent authorities. For well-established antimicrobials, such as oxytetracycline and amoxycillin, the cost of adding farmed fish to the range of approved veterinary uses was not great particularly as these approvals took place before the increased vigour of licensing procedures was imposed, partly by the

needs of harmonisation within the European Union. These products are also licensed in most member states for use with aquaculture products, leading to a wide acceptability of the case for their safety and efficacy. Only the requirement to obtain the necessary data to support the establishment of a maximum residue limit (MRL) has so far affected their availability. Companies have made various attempts to develop several new compounds in the 1990s, one of which, sarafloxacin, would have been exclusively for fish use. Sarafloxacin was finally approved in 1998 after more than 1O years of effort. Other well-established antimicrobial agents have also been considered by various pharmaceutical companies for potential extension into the field of aquaculture, but none have so far reached the stage of receiving marketing authorisation. The reasons for this undoubtedly lie in the markedly increased regulatory costs imposed on the process of licensing veterinary medicines in the last 10 years. These are both in the terms of administrative costs and, more specifically, in the much greater requirements for demonstration of safety, to the environment, the operator and the consumer that are now required, particularly for newer molecules. Pharmaceutical companies are reluctant the invest in such products, so that, for example, oxolinic acid may well be lost for use with food fish species under the provisions of European Council Regulation 2377/90 if no MRL is set (Alderman and Hastings, 1998). An Unofficial Consolidated Updated up to 2002 version of the Annexes I to IV of Council Regulation n° 2377/ 90 is presented below in Table 6.

1.2.3.2.1 Oxolinic acid

4-Quinolones are broad spectrum compounds with bactericidal effect on a range of gram-negative and gram-positive bacteria (Stamm et al., 1986). The mode of action is by interference with the bacterial DNA gyrase inhibiting the supercoiling

of DNA. The 4-quinolones do not induce plasmid mediated drug resistance in bacteria, but resistance may be formed by mutation in a gene encoding a DNA gyrase subunit (Smith, 1990; Alderman 1998). The 4-quinolones can be combined with other classes of antimicrobial agents without deleterious effect on the antimicrobial activity and without any increased toxicity. A synergistic bactericidal effect has been observed for oxolinic acid-trimethoprim combinations due to a complementary blockage of bacterial DNA synthesis. Oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo [4,5g] quinolone-7-carboxylic acid) is widely successfully used to control furunculosis (Endo et al., 1973a; Austin et al., 1983) vibriosis (Endo et al., 1973b; Austin et al., 1981) and enteric redmouth disease (Rodgers and Austin, 1983). The LD_{50} of Oxolinic acid to rainbow trout *(Salmo gairdneri*) was higher than 5000mg /kg body weight /day in relation to the recommended dose in freshwater fish of 10mg/kg b.w /day (Austin et al., 1983). A dose rate of 10mg/kg b.w per day is recommended for routine use in freshwater while 30mg/kg b.w per day are required in the marine environment.

Table 6: Unofficial Consolidated version of the Annexes I to IV of Council Regulation n° 2377/ 90 - Updated up to 2002

Annex I (List of pharmacologically active substances for which maximum residue limits have been fixed)				
Anti- infectious agents				
Chemotheurapeutics				
Sulfonamides			100 µg/ kg Muscle**	All food producing species
Diamino pyrimidine derivatives	Trimethoprim		50 µg/ kg Muscle**	All food producing species except Equidae
Antibiotics				
Penicillins	Amoxicillin		50 µg/ kg Muscle**	All food producing species
	Ampicillin		50 µg/ kg Muscle**	All food producing species
Quinolones	Enrofloxacin	Sum of enrofloxacin and ciprofloxacin	100 µg/ kg Muscle**	All food producing species except bovine, ovine, caprine, porcine, rabbits and poultry
	Flumequine		600 µg/ kg Muscle**	Fin fish
	Sarafloxacin		30 µg/ kg Muscle**	Salmonidae
Macrolides	Erythromycin A		200 µg/ kg Muscle**	All food producing species
Florfenicol and related compounds	Florfenicol		1000 µg/ kg Muscle**	Fin fish
Tetracyclines	Chlortetracycline	Sum of parent drug and its 4-epimer	100 µg/ kg Muscle**	All food producing species
	Oxytetracycline	Sum of parent drug and its 4-epimer	100 µg/ kg Muscle**	All food producing species
Lincosamides	Lincomycin		100 µg/ kg Muscle**	All food producing species
Aminoglycosides	Neomycin B		500 µg/ kg Muscle**	All food producing species
	Spectinomycin		300 µg/ kg Muscle**	All food producing species except ovine

Antiparasitic agents (ANNEX I)				
Pyrethroids	Deltamethrin		10 µg/ kg Muscle**	Fin fish
Acyl urea derivatives	Diflubenzuron		1000 µg/ kg Muscle**	Salmonidae
	Teflubenzuron		500 µg/ kg Muscle**	Salmonidae
Annex II (List of substances not subject to maximum residue limits).				
Inorganic chemicals	Hydrogen peroxide			All food producing species
Organic compounds	Azamethiphos			Salmonidae
Substances generally recognised as safe	Formaldehyde			
Substances used in homeopathic veterinary medicinal products				
Substances used as food additives in foodstuffs for human consumption				
Substances of vegetable origin				
Annex III (List of pharmacologically active substances used in veterinary medicinal products for which provisional maximum residue limits have been fixed				
Anti- infectious agents				
Antibiotics				
Macrolides				
Cephalosporins				
Aminoglycosides				
Quinolones	Oxolinic acid		300 µg/ kg Muscle**	Fin fish
Provisional MRLs expires on 01/ 01/ 2003; Not for use in animals from which milk is produced for human consumption				
Agents acting against ectoparasites				
Pyrethroids	Cypermethrin		50 µg/ kg Muscle**	Salmonidae
Provisional MRL expires on 01/ 07/ 2003				

** For fin fish MRL relates to "muscle and skin in natural proportions"

Annex IV List of pharmacologically active substances for which no maximum levels can be fixed
Chloramphenicol
Dimetridazole
Metronidazole
Nitrofurans (including furazolidone)

(Source: EMEA Web site: www.emea.eu.int - MRL Frequently Asked Questions)

Oxolinic acid has relatively low solubility so that a micronised ultra-fine preparation has been developed to increase its bioavailability from 16 to 31%. In the EMEA decision EMEA/MRL/753/00-FINAL taken in July 2000, for Oxolinic acid like the other quinolones already recommended for inclusion in Annexes I and III of Council Regulation (EEC) No 2377/90, the parent compound was considered to be an appropriate marker residue for all the target species. A microbiological ADI of 2.5 µg/kg bw (i. e. 150 µg/person) was established for oxolinic acid, there was no information on the ratio of marker to total residue in the relevant tissues of any of the target species; taking into account the limited data available on the metabolism of oxolinic acid, and also the evidence that for those quinolones already included in the Annexes to Council Regulation (EEC) No 2377/90, the unmetabolised parent substance generally accounted for the major portion of the residues, a conservative marker to total ratio of 0.5 was used when calculating MRLs for oxolinic acid. The Committee for veterinary medicinal products (CVMP) recommended according to Article 4 of Council Regulation No 2377/90 as amended, a 2-year extension of the provisional MRL for oxolinic acid in accordance with the following table:

Table 7: Provisional MRL for Oxolinic acid

Pharmacologically active substances	Marker residue	Animal species	MRLs	Target tissues	Other provisions
Oxolinic acid	Oxolinic acid	Bovine	100 µg/kg	Muscle	Not for use in animals from which milk is produced for human consumption Provisional MRLs expire on 1.1.2003
			50 µg/kg	Fat	
			150 µg/kg	Liver	
			150 µg/kg	Kidney	
		Porcine	100 µg/kg	Muscle	Provisional MRLs expire on 1.1.2003
			50 µg/kg	Skin + fat	
			150 µg/kg	Liver	
			150 µg/kg	Kidney	
		Chicken	lOO µg/kg	Muscle	
			50 µg/kg	Skin + fat	
			150 µg/kg	Liver	
			150 µg/kg	Kidney	
			50 µg/kg	Eggs	
		Fin fish	300 µg/kg	Muscle and skin in natural proportions	

1.2.3.2.2 Oxytetracycline

Tetracyclines in nature are produced by *Streptomyces spp.* Their mechanism of action is twofold. They enter the bacterial cell either by passive diffusion through hydrophile pores in the outer cell membrane or by pumping the drug through inner cytoplasmic membrane via energy dependent transport mechanisms leading in both cases to the inhibition of protein synthesis through binding to 30S ribosomes. Tetracyclines are bacteriostatic and function by interfering with bacterial protein synthesis by reversibly binding to the bacterial 30S ribosomal subunit, preventing the binding of aminoacyl RNA to messenger RNA ribosome (Jawetz, 1987). They are broad-spectrum antibiotics effective against both Gram-positive and Gram-negative bacteria and some protozoans. They are used to control bacterial haemorrhagic septicaemia, columnaris, furunculosis, enteric redmouth, vibriosis, pasteurellosis and cold-water vibriosis. They may be incorporated in feed pellets at a dosage of 50-100 mg/kg body weight per day over a 10 day treatment period. In the EMEA decision (EMEA/MRL/023/95) for Oxytetracycline, Chlortetracycline, Tetracycline, the previously allocated MRLs were suggested to be considered as final for oxytetracycline, tetracycline and chlortetracycline and the Committee recommended the inclusion into Annex I of Council Regulation (EEC) No 2377/90 of these compounds for all food producing species (Table 8).

Table 8: Provisional MRL for Tetracycline, Oxytetracycline and Chlorotetracycline

Pharmacologically active substance(s)	Marker residue	Animal Species	MRLs	Target Tissues
Tetracycline	Sum of parent drug and its 4-epimer	All food producing species	600µg/Kg	Kidney
Oxytetracycline			300µg/Kg	Liver
Chlorotetracycline			100µg/Kg	Muscle
			100µg/Kg	Milk
			200µg/Kg	Eggs

In addition the Committee recommended that muscle and skin in natural proportions should be the target tissue for fish. The ADI level was set at 0-3µg/kg bw for these tetracycline compounds. Suitable and well-validated HPLC methods are available which can be used in the surveillance of residues of Oxytetracycline, Chlortetracycline and Tetracycline in tissues of cattle, sheep, pig, turkey, trout, carp and milk and eggs. The method takes into account the 4-epimers of Oxytetracycline, Chlortetracycline and Tetracycline. Residues of Oxytetracycline can be routinely monitored by a microbiological agar diffusion assay with detection limit of 100µg/kg for milk and meat, and 200µg/Kg for eggs. It appeared that the monitoring methods for residues in fat were not reliable and no MRL was therefore established.

Figure 6: OA Molecular form

Oxolinic acid

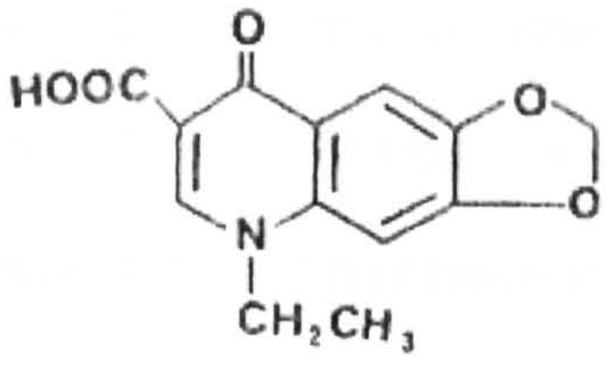
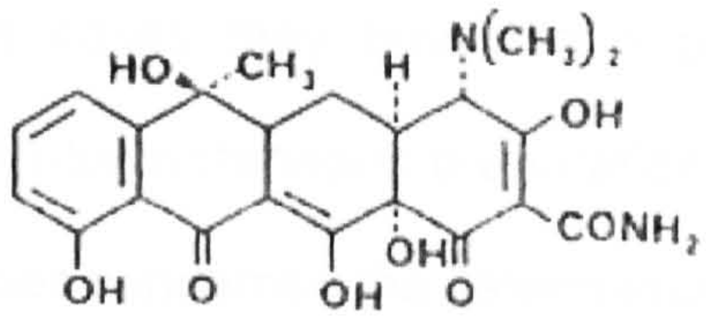


Figure 7: OTC Molecular Form

Oxytetracycline



(b)

Recommended dose rates and withdrawal periods are given below for products with a current marketing authorisation for use in aquaculture in the UK (Table 9).

Table 9: Recommended dose rates and withdrawal periods

Generic Name	Trade name	Fish Species	Dose (mg / Kg / day)	Withdrawal period (degree-days)
OTC	Aquatet	AS, RT	75	400
	Tetraplex	AS	75	400
	Tetraplex Forte	AS,RT, AC	75	AS 500, RT & AC 350
OA	Aqualinic	AS,RT,BT	10-30	500
	Aquinox	AS	10-30	500

(Alderman D. J. & T. S. Hastings ,1998)

Where no suitable licensed medicinal product is available, veterinarians may prescribe a product licensed for use in another animal species (here a guide minimum withdrawal period of 500 degree days is imposed) and in very limited cases may prescribe an unlicensed product for use on few animals. Such prescription is known as the "cascade principle". The limitation of the right to prescribe to only a few animals under the veterinarian's direct control effectively means that unlicensed medicines should not be used in aquaculture except perhaps on a few valuable broodstock animals. A veterinarian in extreme cases may prescribe a product licensed for use in man, or may prescribe an ex-temporaneous preparation. If no withdrawal period has been specified for the species concerned the veterinarian must ensure that a withdrawal period of at least 500 degree-days is observed. All instances of extra label use of veterinary medicines in aquaculture must be recorded and the records must be held available for inspection by the competent authorities for a period of at least three years.

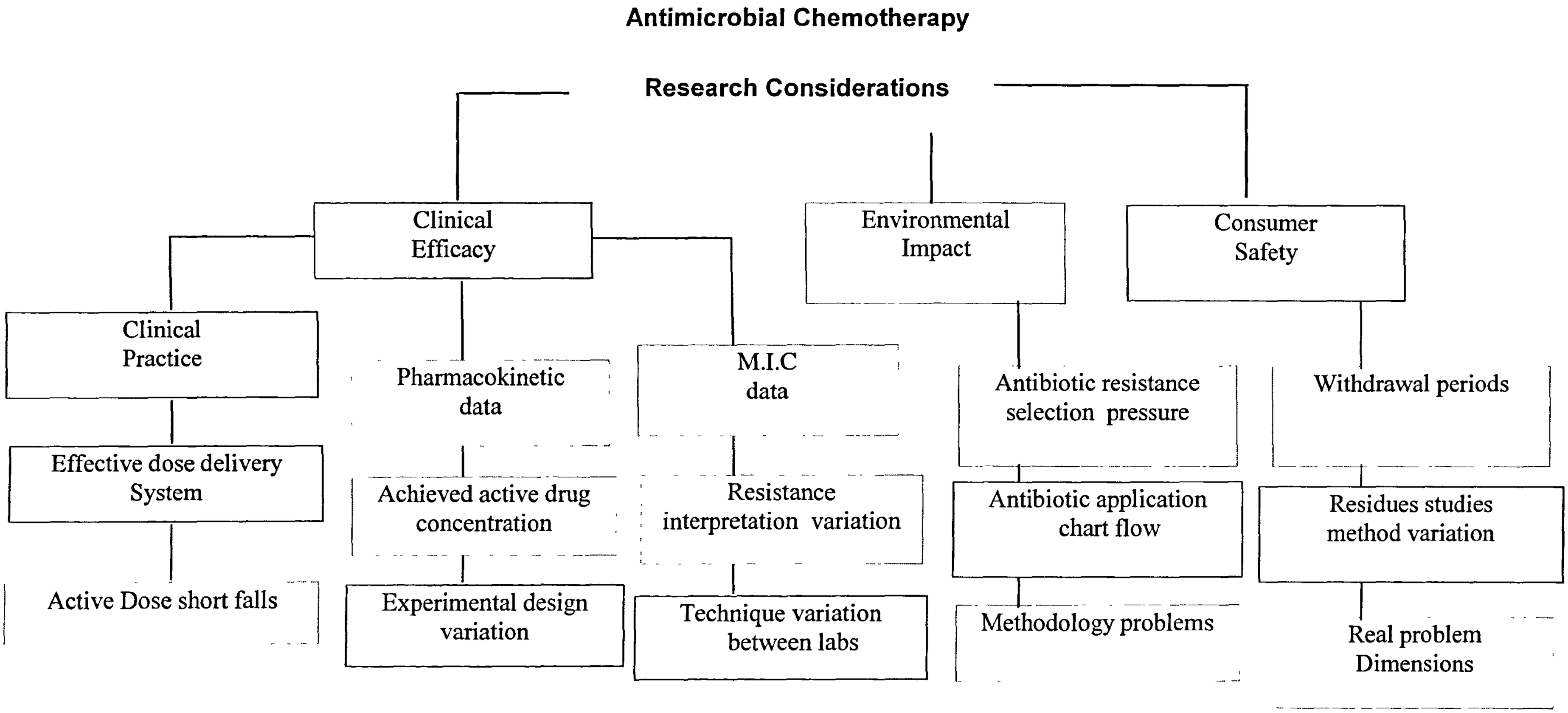
Unfortunately in comparison to other areas of veterinary medicine, the licensing of potential aquaculture chemotherapeutants has received limited attention, possibly due to the comparatively small size of the market and hence the reluctance of drug companies to commit the necessary resources for pursuing costly applications. Aquaculture medicines must be licensed to similar standards of quality, safety and

efficacy as human pharmaceutical products. However it should be underlined that the economic scale of the potential Aquaculture marketplace indicates that it may not be economically feasible to bring new products to the aquaculture market. Considerable effort has been devoted lately on consultation of the Commission in as an attempt to amend existing legislation on veterinary medicinal products. It is considered that the revision of the legislation is a big opportunity to present the specific nature and needs of Aquaculture industry to the legislator in order to ensure the sustainability of the sector, fish welfare, the production of high quality products and last but not least the public health. The European Aquaculture industry's aim is the gradual reduction of pharmaceutical levels applied but there are several key points that should be raised in order to ensure that the consumers as well as the industry's interests are better served. The new framework should induce flexibility for the licensing of aquaculture medicinal products. Although the foremost objective in the legislation is always consumer safety, the legislation should recognise the need for veterinary medicinal products in ensuring the health and welfare of animals. It should take into account animal health and welfare as well as public health, whereas previously the system has focussed exclusively on public health with little regard for the health and welfare of animals. The legislation should resolve the problems concerning the availability of medicinal products for aquaculture use and should in particular introduce a policy for 'orphan' medicinal products for veterinary use (with provisional MRLs – like oxolinic acid) analogous to that established for human medicinal products by Regulation (EC) No 141/2000, implemented through Regulation (EC) No 847/2000. This regulation should create the necessary mechanisms to ensure that all needs are covered by at least two therapeutic alternatives in European Aquaculture, with the objective of guaranteeing both competition and the diversity of available protection options and thereby preventing the emergence of resistance. The ten-year industrial-property protection period for veterinary medicinal products for fish should be extended to 15 years in order to enable the pharmaceutical industry to derive full benefit from such

products thus initiating renewed incentives to invest and achieve marketing authorisations for fish products. The time it takes to obtain registration and to develop the data required for the extension of a marketing authorisation to different species and to different diseases should be considerably shorter. Both centralised and decentralised procedure should be maintained providing the necessary flexibility in terms of faster launching of new products in the aquaculture market. Evaluation of the operation of the procedures for the granting of market authorisation has revealed the need to revise, in particular, the mutual recognition procedure in order to increase the scope for cooperation between Member States. Different levels of competence in different Member States as well as different level of vigilance often leave more options for treating or preventing fish diseases in some countries while leaving none in others. A simple example is that a few Member states refuse to act as reference Member states in the Mutual recognition procedure. A basic degree of harmonisation should be included in the enforcement of the new legislation in order to avoid the creation of indirect trade barriers. The large number of aquaculture species and relevant pathologies complicate the procedure of extension of use or species and emphasis should be made into making this process faster at a Member state level as well as at EU level. Systematic extrapolation of MRL values between species would make new drugs available faster for minor species. The 'cascade' principle needs to be modified. In the case of absence of authorised veterinary medicinal product for a condition affecting fish – a familiar case for veterinarians - in order to avoid causing unacceptable suffering to the fish population concerned, Member States shall, particularly, permit the administration of unauthorised medicines by a veterinarian or under his/her direct personal responsibility to a fish population on a particular fish farm. The reference to a small number of animals in the legislation is not applicable for aquaculture because fish are reared in cages or tanks in large numbers and these are treated as units in terms of epidemiology, prevention or treatment of fish diseases. The licensing and application of Fish vaccines should be encouraged in

order to support the European Aquaculture industry trend to limit the use of antimicrobials and to ensure high product quality and safety.

Figure 8: Antimicrobial Chemotherapy Research considerations



1.3 Research considerations

Research considerations on antimicrobial chemotherapy are revolved around three main areas of interest, those regarding the clinical efficacy, their environmental impact and their effect (if any) on consumer safety.

1.3.1 Clinical Efficacy

A range of factors affects the clinical efficacy of an antimicrobial agent in aquaculture. The ability to deliver the antimicrobial agent to the affected tissue of the diseased fish depends on the effective dose delivery system the application strategies and the shortfalls present in drug application in practice. The pharmaceutical industry's major research interest is more in the area of new chemical compounds, than in the development of new drug delivery systems like micro and nanocapsules, liposomes, osmotic pumps and pellet matrices. Factors that complicate the assessment of clinical efficacy of an antimicrobial agent in practice include the fish feeding response, the timing of the initiation of the treatment, the presence of unrelated stress factors, the economic and ethical reasons that proscribe the use of untreated control groups and the loss of appetite evident in diseased fish leading to the assumption that only the healthy fish eat, so while the clinician considers therapeutic any applied treatment this in a sense acts more like a prophylactic treatment. The pharmacokinetic properties of the agent for the specific fish species and at the prevailing water temperature affect the concentration of the agent in the infected fish. The third main parameter that dictates the clinical outcome of any treatment is the susceptibility of the bacterial pathogen to the obtained tissue drug concentration. Variations in the interpretation of the term of resistance to an antimicrobial agent exist among laboratories and in scientific papers regarding the evaluation of antimicrobial agent resistance expressed as M.I.C values or zones of inhibition. Classification of bacterial pathogens according to their clinical relevance divides a bacterial population into three groups, namely: a) Resistant, b) Intermediate and c)

Sensitive or susceptible bacterial pathogen to a certain antimicrobial agent. Establishment of critical breakpoint values Minimum Inhibitory Concentration (M.I.C) or areas of zones of inhibition are possible only when Bimodal or Polymodal distributions of bacterial pathogens exist in relation to their sensitivity to a certain antimicrobial agent. Minimum Inhibitory concentration (M.I.C) of a bacterial strain to a certain antimicrobial agent is the concentration of this agent that is required in order to inhibit the growth of this strain. The disadvantage in this type of extrapolation is that they have in them no consideration of the pharmacokinetic data. Problems of drug resistance revolve around two main axons. The first is the development of resistance to the few effective and legally available antibacterial drugs (Aoki and Kitao, 1981; Aoki et al., 1981; Austin, 1985; Aoki, 1988; Richards, 1992; Richards et al., 1992). The second is the expressed anxiety that the use of antimicrobials in the aquatic environment may lead to transmission of plasmid-mediated resistance, producing resistant populations of human microbial pathogens. Whilst the possibility of such an event can't be ignored, so far it appears to remain something which perhaps may be demonstrated under lab conditions but which has not so far been found to occur under natural conditions. Bacteria acquire resistance mechanisms to antimicrobial agents by mutation, transduction, transformation and conjugation. Mutation is important for 4-quinolones. It refers to a stable genetic change, which is often presented naturally; it is not a result of exposure to the agent and persists in the absence of drug "selection" leaving only resistant microorganisms while virulence may change similarly. Transduction is important for antimicrobial agents like Erythromycin, Oxytetracycline and Chloramphenicol. It is based on the ability of bacteriophages to carry bacterial DNA in their protein core being able sometimes to pass bacterial extra chromosomal DNA (plasmids) on to newly infected bacterial cells. This mechanism was proven to exist for plasmids encoding penicillinase production. Transformation is an uncommon and probably not very important mechanism. It refers to incorporation of free DNA present in the aquatic environment

in bacterial cells. Conjugation is an extremely important mechanism for the spread of resistance (often multiple drug) especially common in Gram-negative bacteria. It was first described in Japan in 1959. It involves the passage of genes from cell to cell by direct contact through a "sex pilus" or "bridge". Transferable genetic material has two different DNA sequences. The "R" factor or "R Determinant Plasmid" that encodes the actual resistance while the "Resistance Transfer Factor" or "RTF" or" Transfer Factor Plasmid" that encodes for sex factor which is the transfer apparatus (sex pilus). Each of the above can be transferred individually but both must be present for successful transfer of antibiotic resistance. Under natural conditions the efficiency of transfer is low but antibiotics can exert a selective pressure that allows the emergence of resistant bacterial strains. Resistance to tetracyclines is usually plasmid mediated and transferable. It is often inducible and appears to be associated with the ability to prevent accumulation of the antibiotic in the cytoplasm. The frequency rises with extended use and has become pronounced in some farms. Where the development of resistance to tetracyclines rises from a change of bacterial cell membrane permeability, this can result in cross-resistance to other drugs including 4-quinolones. Plasmid -encoded drug resistance of fish pathogens was described in several studies (Aoki and Kitao, 1981, 1985; Austin, 1985).

Bacterial strains develop special mechanisms to prevent drug antimicrobial effect by the production of enzymes to inactivate the drug at bacterial cell surface, impermeability of cell membranes in order to prevent influx of the drug, production of local pH changes in order to deactivate some of the antibiotics that are organic acids and whose action is pH dependent and production of a local anaerobic environment that affects the transport mechanisms of some drugs that are energy dependent. The in vivo antibiotic resistance is also a relative term. It depends on the method of administration of the antibacterial agent, tissue distribution of the agent in relation to the site of the infection and physicochemical microenvironment in the fish.

Techniques that are usually applied for in vitro sensitivity testing belong either to Disc diffusion method or Dilution methods. Disc Diffusion methods are usually modifications of the Kirby-Bauer method. The method is based on the measurement of zones of inhibition around antibiotic disks on solid culture media. These zones of inhibition depend on several factors like the sensitivity of the microorganism, the amount of the agent used, the diffusion rate of the agent, the growth rate of the organism, the conditions, the medium composition, pH and volume. Incubation temperature, inoculum production method and size and method of inoculation also affect the sensitivity of the diffusion methods. Standardised methods are based either on detailed internal comparative standards or on rigorous specification. For many but not all the antimicrobial agents there is linear relationship between diameter of zone of inhibition produced using a standard method and the log of the M.I.C of the agent against the organism. However no regression lines have been produced for fish pathogens. Dilution methods use either solid or liquid media. These methods have several disadvantages. They are time consuming and questioning exists on their value for the detection of Quinolone M.I.C. However generally they are perceived as the correct approach, they easily can be adapted to determine Minimum bactericidal concentrations (M.B.Cs), their use reduces the logistical problems, can provide direct extrapolation of numerical data and they are preferred for slow growing bacteria. In vitro sensitivity test variations are widely accepted to increase the inability of data correlation between laboratories involved in fish diseases diagnosis. The main obstacle in obtaining realistic and applied laboratory data originates from the in vitro test inability to simulate in vivo conditions. The importance of inter- and intra-laboratory reproducibility that can be achieved using a particular in vitro method may be more important than the relevance of its results to the in vivo situation (Smith et al., 1994). Laboratory test variations refer to the laboratory media formulation and to technique protocols. Laboratory media formulation depends on factors like pH, nutrient conditions, ionic concentrations (phosphate, iron) while technique protocols

differ in factors like the centrifugation of the inocula and temperature used. Temperature affects the results of *in vitro* tests like M.I.C of oxytetracycline and oxolinic acid (Giles et al., 1991; Inglis et al., 1991; Martinsen et al., 1992). By convention a concentration of an antibiotic three times the MIC value in serum is considered sufficient to kill or inhibit the bacteria at the site of infection. Inability to simulate real life situations depends on many factors like the host-defence mechanisms. Phenomena and mechanisms like the immunomodulating effect of antibiotics, the non specific immune response and its effect on the pathogen as well as the effect of sub-inhibitory concentrations on the pathogen are some of the factors that prohibit the simulation of the in vivo situation at least based on the existing experimental designs and available techniques.

This is the first study in the Mediterranean where quantitative antibiogram and MIC data are linked to achieved antibiotic concentrations in sea bass and sea bream at two prevailing temperatures following applied treatment protocols.

1.3.2 Environmental impact – Residues in Aquatic Environment

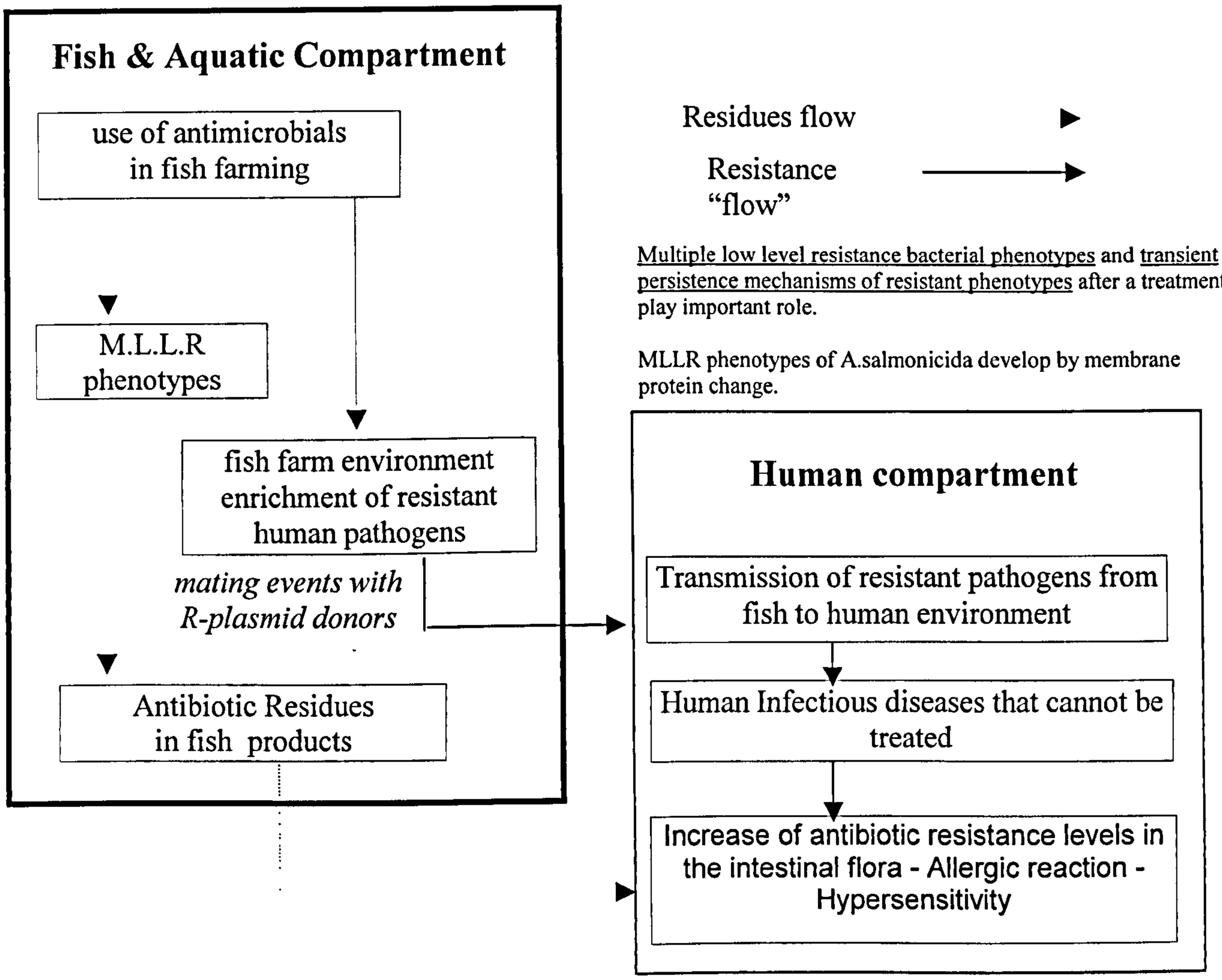
When drugs and chemicals are applied in Mariculture it is inevitable that some of that material will enter the environment (Alderman et al., 1994). The proportion will depend on several factors, most important of which is the route of application. Compounds applied as oral therapeutants will enter the environment by three routes. The most direct will be on uneaten medicated feed where it has been estimated that an average of 30% of feed is not eaten by the farmed fish but falls through the sea cage to the seabed beneath. This waste feed may then be consumed by feral fish, invertebrates or pelagic filter organisms, and may or may not be broken down microbiologically, depending on the state of the below-cage environment and the drug concerned. The second route will be in the form of un-metabolised drug in the faeces and excretions from the kidney, liver or gill of the farmed fish. Faeces will fall

to the sea bottom and enter the detrital chain as with waste feed. The third and final route will be the excretion of a metabolically altered drug by the fish. Wild fish feeding on medicated feed fragments may be caught by human consumers or filter feeding organisms (mussels) may be predated by birds and possibly enter the human food chain indirectly. Drug withdrawal periods set to ensure that this elimination process is complete were displaced later by the concept of Maximum Residue Limit (MRL) in order to ensure that no unacceptable residues enter the human food chain via farmed fish meat. Nearly all studies show high levels of resistance. The prevalence of resistance reflects frequency of the use of the agent and R-plasmid encoded resistance is common in fish pathogens. The definition of resistant bacteria in the aquatic environment depends on three major considerations. The agent concentration used as breakpoint, the method employed and the sensitivity, specificity of the methodology and the relevance to chemical concentration and bioavailability of the antimicrobials.

1.3.3 Consumer safety

The considerations on the potential risks are usually based on the following diagrammatically presented pattern:

Figure 9: Considerations on the potential risks for consumer safety



The survival of a resistant strain in the environment is based on persistence mechanisms: This type of mechanism becomes apparent only in the presence of the antimicrobial and may lead to the generation of bacterial strains of intermediate resistance. The resistance may result from genotypic or phenotypic changes in the cell (e.g. through changes in membrane proteins or the permeability of cell membranes). These phenotypes are detected during and shortly after therapy and could cause

problems in surveys of the frequency of resistance. Bryan (1989) suggested that in the case of Quinolones persistence was probably the dominant mechanism of clinical resistance. *A.salmonicida* strains in this study became resistant to Oxolinic acid and have been isolated years after the last use of Quinolones. R-plasmids have been reported to lose resistance genes under low nutrient conditions (Godwin and Slater, 1979; Griffiths et al., 1990) and scepticism exists on the survival of the plasmids and stability in the adjacent aquatic environment. Several studies exist in the literature on R-plasmid transfer in the lab (Nakajima et al., 1983; Ervik et al., 1994a; Sandaa et al., 1994), in simulated marine environment (Coughter and Stewart, 1989; Angles et al., 1993; Goodman et al., 1993), in marine environment (Saye and Miller, 1989), in the sediment under sea cages (Santaa et al., 1992), and in freshwater farms (Brazil et al., 1986). These studies suggested low frequency of transfer in nature although movement of R-plasmid bacteria (not human pathogens) to the human environment - transfer of R-plasmids to human pathogens was proven possible.

Questions refer not only to the existence of human therapy risks associated with fish farming but also on the magnitude of such risks. In a simplified version the basic question is: "Can fish act as vectors of human diseases?" Inglis et al. (1993) reported a list of human pathogens that can be associated with fish farming. The bacteria amongst these pathogens are associated with the fish and the aquatic environment, are related to pollution of the marine environment and most of these are isolated during processing, marketing and preparation of fish. The danger of these infections, which most of the time develop as self-limited gastroenteritis, depends on climatic condition (appropriate conservation) and sometimes-cultural conditions (consumption of raw fish). There are 20 groups of "microbes" isolated from fish that have been pathogenic to humans. The bacteria amongst these pathogens can cause intestinal infections entering the human host via the mouth as well as septicaemias or localised infections entering via the skin. In the case of *Salmonella spp.* and *Campylobacter jejuni* it is

proven that food products and /or infected humans act as vectors. Resistant *Salmonella spp.* have been isolated from fish. *V.parahaemolyticus* has been isolated from raw and undercooked fish but only 1% of fish pathogens are Kanagawa positive while this test is positive in 96% of the human pahogenic strains. *Aeromonas hydrophila* affects only immunocompetent patients while controversial information exists on the pathogenicity of *Plesiomonas shigelloides* and circumstantial evidence only relating to *Edwardsiella tarda*. Infections via the skin refer to bacterial pathogens like *Erysipelothrix rhusiopathiae* (swine pathogen), *A.hydrophila, Leptospira interrogans* the causative agent of Weil's disease and *Pseudomonas spp.* which is pathogenic for hospitalised patients, *Mycobacterium marinum* which is widely distributed in nature swimming pools, freshwater & marine aquaria and *Mycobacterium fortuitum* that has been isolated from aquarium fish as well as human patients after open heart surgery, venous stripping and renal homograft recipience. The review of the available literature indicates that the health risk from the potential risk of transmission of antibiotic resistance from fish pathogens to potential human pathogens (Table 10) is minimal and extremely difficult to prove *in vivo* (Alderman and Hastings, 1998; Smith et al., 1994).

Table 10: Bacteria of significance as human pathogens isolated from fish and their immediate environment and preferred antimicrobial agents for the treatment they cause

[illegible]	[illegible]	[illegible]
Pathogens primarily entering the host via the mouth		
Salmonella spp.	Food poisoning	Ampicillin,amoxicillin, trimethoprim-sulphamethoxazole
Vibrio parahaemolyticus	Food poisoning	
Campylobacter jejuni	Gastroenteritis	Erythromycin
Aeromonas hydrophila	Diarrhoea	Ciprofloxacin, norfloxacin, trimethoprim-sulphamethoxazole
Aeromonas hydrophila	Septicaemia	Cephalosporins
Plesiomonas shigelloides	Gastroenteritis	trimethoprim-sulphamethoxazole, tetracycline, ciprofloxacin
Edwardsiella tarda	Diarrhoea	Ampicillin
Pathogens primarily entering the host via the skin		
Pseudomonas aeruginosa	Wound infection	Aminoglycoside + antipseudomonad penicillin
Pseudomonas fluorescens	Wound infection	
Mycobacterium fortuitum	Mycobacteriosis	Amicacin + cefoxitin
Mycobacterium marinum	Mycobacteriosis	Rifampicin + ethmbutol, trimethoprim-sulphamethoxazole
Erysipelothrix rhusiopathiae	Erysipeloid	Penicillin
Leptospira interrogans	Leptospirosis	Penicillin G, ampicillin

a. After Austin and Austin (1987)

Modified from Smith et al (1994)

b. After Bartlett (1992)

The methods used for the detection of antimicrobials include Biological methods (Bioassays) that measure the biologically active agent based on standard addition

curves and High Performance Liquid Chromatography methods (H.P.L.C) that measure the chemical concentration of the agent. However conflicting data arise from single dose studies where different protocols, different methods and assumptions are used for the description of the short-term kinetics of an agent and the extrapolation of the real life situation through mathematical models. Mathematical modelling is used to define resemblance and common ground between the studies, without questioning of the approach and its relevance to clinical practice. Only a few studies exist that simulate in a more realistic and less optimistic way the actual practice of antimicrobial agent application in aquaculture far from the "near perfect" study profile of the highly controlled studies. This is the first study where Box Plot Analysis gives the «full» picture of the clinical relevance of antibiotic application presenting pharmacokinetic data of the population.

Materials & Methods

2.1 BACTERIOLOGY

2.1.1 Bacteriological Survey

During a period of two years (Sept 1994-Dec 1996), samples from five cultured species in the Mediterranean region [Sea bass (*Dicentrarchus labrax)*, Sea bream (*Sparus aurata)*, Common dentex (*Dentex dentex*), Grey mullet (*Mugil cephalus*) and Sharpsnout bream (*Puntazzo puntazzo*)] were taken. Fish originated from 24 farms in 9 geographical regions of Greece.

Figure 10: Geographical regions of Greece – Origin of Bacterial Strains

Samples were taken using aseptic technique with a sterile loop from kidney of diseased fish that were not undergoing treatment. Sampling of diseased moribund fish was performed personally either on site at the farm or in the lab of Fish Diseases of N.C.M.R, Athens, when samples of moribund fish were send to the lab by fish farmers or fish veterinarians in the field. In a limited number of cases bacterial strains already isolated in regional labs were sent and were simply subcultured in the NCMR lab.

Following initial growth and in some cases enrichment, distinct colonies were isolated and were subcultured separately in order to identify all bacterial strains involved in an outbreak. Tryptone Soy Agar (TSA), Tryptone Citrate Bile Salt Agar (TCBS) and Tryptone Soy Broth (TSB) (all supplemented with 1.5% NaCl) were used for the initial isolation. Resulting bacterial strains were maintained at 4°C, at -20°C and at -70°C. Basic tests were performed for preliminary classification of all bacterial isolates, namely, Gram strain, motility test ("hanging drop" method), oxidase test, Oxidative versus fermentative metabolism in Hugh-Leifson glucose medium supplemented with 1.5% NaCl (Hugh-Leifson 1953) and sensitivity to vibriostat 0/129 (2,4-diamino-6,7-diisopropylpterydine) and Novobiocin using disks impregnated with 10 and 150 µg 0/129 (oxoid) and disks of 5 µg Novobiocin (Pasteur Diagnostics). The Gram stain is a fundamental differential stain in microbiology. Gram positive are the bacterial that are not decolourised by ethanol or acetone and they are stained blue, while Gram negative are the bacteria that are decolourised by ethanol or acetone and are stained red. This different colour is due to differences on the cell wall and membrane. The Gram stain can be applied either on pure bacterial colonies or on direct tissue smears (as in the case of Bacterial Kidney Disease). Some bacterial strains are capable of independent directional movement by means of polar flagella, which is different from the non-directional random Brown movement. The Oxidase test demonstrates the ability of a bacterial strain to produce oxidative enzymes that contribute in the process of electron transfer as "electron donors". When the Redox dye tetramethyl-p-phenylenediamine is used as electron receptor this is reduced producing deep blue colour. This test is used to demonstrate the aerobic (oxidative) and anaerobic (fermentative) metabolism of glucose by the bacterial strains examined. O-F agar (Hugh-Leifson 1953) supplemented with 1.5% NaCl includes Bromothymol blue a pH indicator which turns from blue to yellow when acid is produced during the metabolism of Glucose.

Table 11: O-F agar result analysis

Open tube	Closed tube	Result
Green	Green	No reaction
Blue (top layer)	Green	Alkaline reaction
Yellow	Green	Oxidation
Yellow	Yellow	Fermentation

Reagent 0/129 (2,4-Diamino-6,7-di-iso-propyl pteridine phosphate) is bacteriostatic for *Vibrio* species and it is used for the differentiation of Aeromonads and Vibrios. Sensitivity to Vibriostat 0/129 and to Novobiocin is determined by using disks impregnated with 10 and 150 μg 0/129 (Oxoid) and disks of 5 μg Novobiocin (Pasteur Diagnostics).

Table 12: 0/129 result analysis

Disk 150μg	Disk 10μg	Result
No zone of inhibition	No zone of inhibition	Resistance
Zone of inhibition	No zone of inhibition	Intermediate sensitivity
Zone of inhibition	Zone of inhibition	Sensitivity

2.1.1.1 Quantitative Antibiogram

A Quantitative Antibiogram is used in order to provide comparable results on the zones of inhibition of a certain bacterial pathogen to a range of antimicrobial agents. The Antibiogram is performed only in pure cultures. Sensitivity to an antimicrobial agent is demonstrated by the formation of zones of inhibition of the bacterial growth around the disk impregnated with the antimicrobial agent. If the target is to measure resistance to Potentiated sulphonamides agar must not contain inhibitors like lysed blood or thymidine. The most appropriate agars for antibiogram use are Mueller-Hinton agar and Isosensitest agar. Of the two above Mueller-Hinton was selected because it supported better the growth of more fastidious organisms like *Photobacterium damsela subsp. piscicida*. The methods used for inoculum standardisation in the Quantitative antibiogram are presented in Figure 11. In this study a number of colonies were picked up, a suspension was made and cfus/ml were adjusted by spectophotometer (wave

length: 480 – OD:0.2). A predetermined volume of 500μl of the adjusted suspension was placed on the petri dish and was spread evenly on the surface. The problem of the above process was that although for Vibrio anguilarum the final volume referred approximately to 10^3 cells, this process was employed for all bacterial isolates with different properties and growth potential. However due to the large number of bacteria, a guide based on the study for Vibrio anguillarum was considered the standard operating procedure in this study. OD values for every isolate are presented in Annex 1. Antimicrobial agent disks employed in this study had the following concentration of antibiotic:

Table 13: Antimicrobial agent - Quantitative Antibiogram

Antimicrobial agent	**Abbreviation**	**Concentration (μg)**
Oxytetracycline	OTC	30
Oxolinic acid	OA	2
Co-trimoxazole	SXT	25
Furazolidone	FR	50
Amoxicillin	AML	10

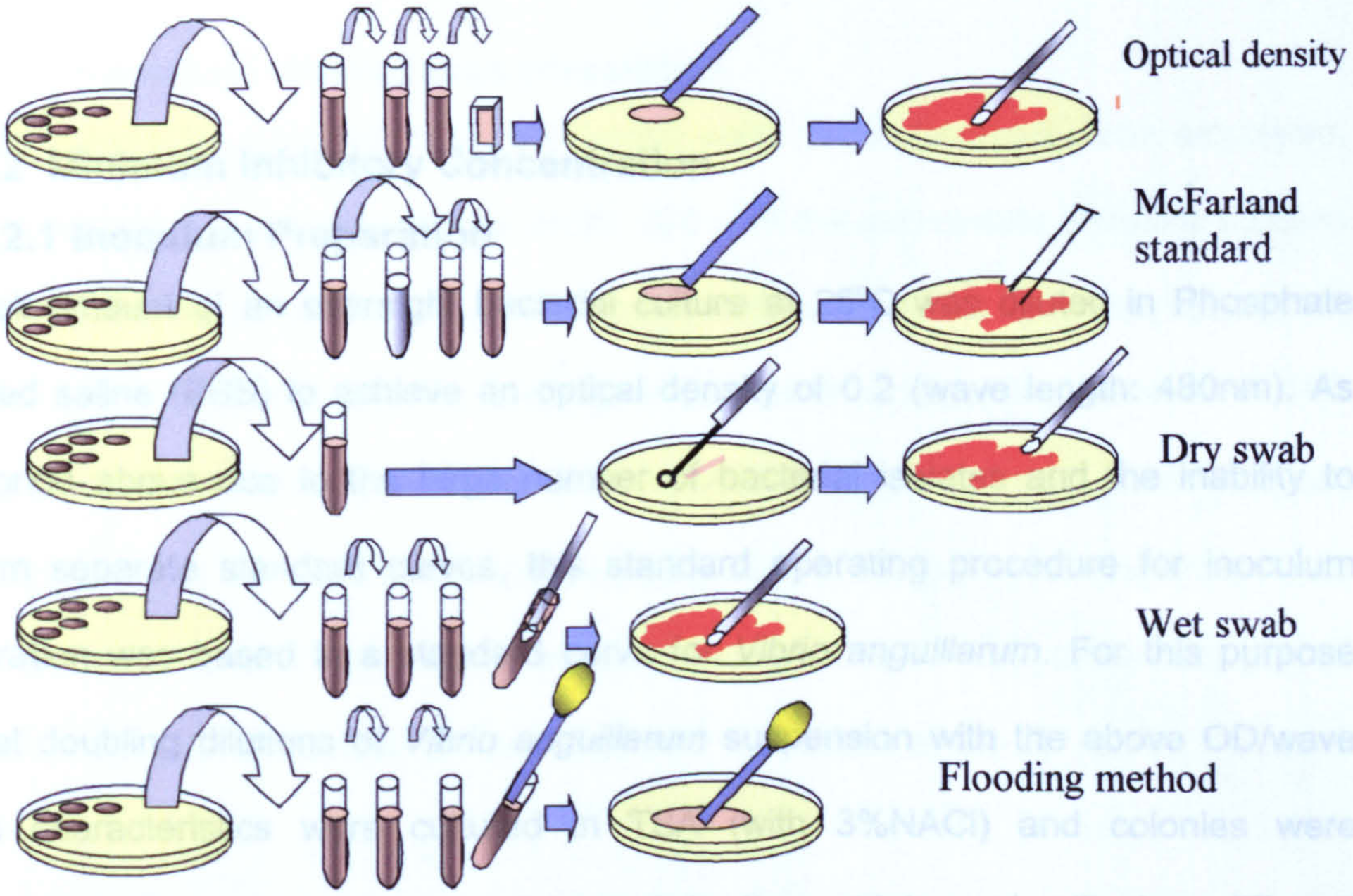


Figure 11: Inoculation Protocols for disk diffusion method (from Christofilogiannis 2001)

Figure 11: presents the two quantitative methods (Optical density / McFarland standard) and three more semiquantitative methods of inoculation in MIC determination with disk diffusion method.

Optical density: a number of colonies are picked up and a suspension is made and cfus/ml are adjusted by spectophotometer. A predetermined volume of the adjusted suspension is placed on the petri dish and is spread evenly on the surface. This method was employed for Quantitative Antibiogram evaluation in this study.

McFarland standard: a number of colonies are picked up and a suspension is made and cfus/ml are adjusted by visual comparison to a known McFarland standard. A predetermined volume of the adjusted suspension is placed on the petri dish and is spread evenly on the surface.

Dry swab: a number of colonies are picked up and an approximate suspension is made in a known volume. A loop full volume of the suspension is spread over all the plate using a loop.

Wet swab: a number of colonies are picked up and an approximate suspension is made in a known volume. Dilutions are performed if necessary. A sterile cotton swab is submersed in the solution and is then used for spreading a quantity on the agar surface.

Flooding method: a number of colonies are picked up and an approximate suspension is made in a known volume. Dilutions are performed if necessary. A known volume of the suspension is removed using a volumetric tube and then is spread on the agar surface. After a tilting circular movement excess liquid is removed and plates are left to dry.

2.1.1.2 Minimum Inhibitory Concentration

2.1.1.2.1 Inoculum Preparation

A small amount of an overnight bacterial culture at 25°C was diluted in Phosphate buffered saline (PBS) to achieve an optical density of 0.2 (wave length: 480nm). As mentioned above due to the large number of bacterial isolates and the inability to perform separate standard curves, this standard operating procedure for inoculum preparation was based in a standard curve for *Vibrio anguillarum*. For this purpose several doubling dilutions of *Vibrio anguillarum* suspension with the above OD/wave length characteristics were cultured in TSA (with 3%NACl) and colonies were enumerated. The procedure was repeated in three trials and with two different operators. This procedure indicated that a *Vibrio anguillarum* suspension in the above conditions equates to 10^6 cells/ml, so that a further dilution of 1:100 produced the final working MIC inoculation solution equating to 10^4 cells/ml.

2.1.1.2.2 Antibiotic Solution

An aqueous solution of Oxytetracycline (OTC) of 160 µg\ml was produced from dry powder, and the solution was filter sterilised through a 0.22 µm filter. Serial two fold dilutions of the antibiotic were made giving concentrations that ranged from 80 to 0.15625 ppm of OTC. Oxolinic acid as other 4-quinolone antimicrobials is not easily dissolved in distilled water. Oxolinic acid was therefore first suspended in distilled water, fully dissolved by the dropwise addition of 0.1M NaOH and readjusted to pH: 9.3 by drop-wise addition of 0.1M hydrochloric acid to a nominal concentration of 1280 µg\ml. The solution was filter sterilised using disposable 0.22µm filters and serial two-fold dilutions were made to a final dilution of 0.15625 ppm of OA.

2.1.1.2.3 Procedure - 96-well plate preparation

A 96 well microtitre plate with round-bottomed wells (Nunc Denmark) was employed. 100µl of Double-strength Isosensitest broth, 100 µl of the appropriate antibiotic solution and 5µl of bacterial culture were added to each well. Isosensitest agar was selected in this test due to lower interference and higher degree of repeatability achieved in MIC values. Two rows of two fold antibiotic dilutions were employed for each bacterial strain (4 bacterial strains for each 96 well plate). Inoculation of the bacterial suspension was performed from the low to the higher antibiotic dilution. The 96-well plates were placed in plastic bags (to avoid loss of humidity) and incubated at 22-25° C for 24 hours (Figure 12).

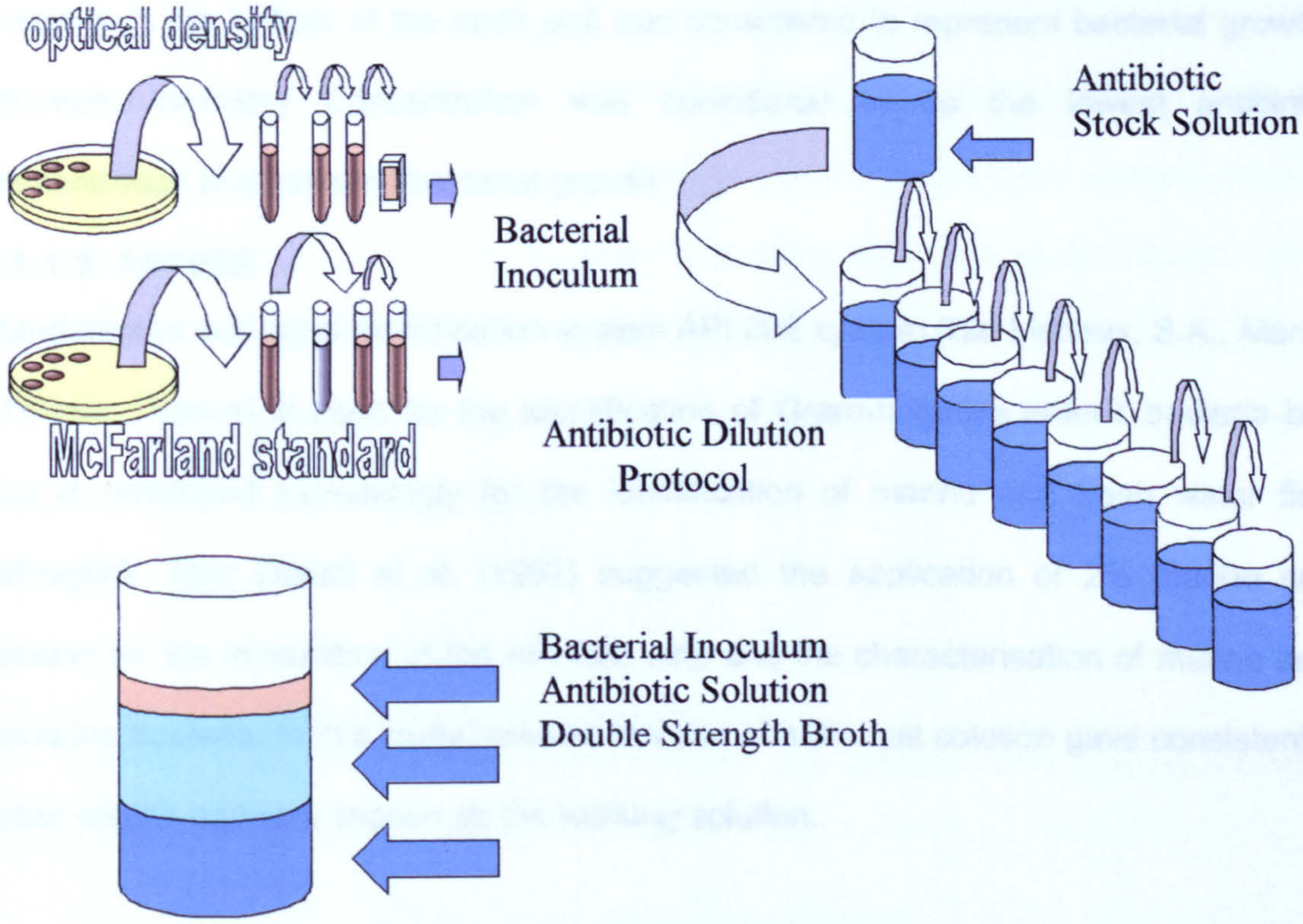


Figure 12: Inoculation Protocol for Broth Microdilution method (from Christofilogiannis 2001)

Figure 12 presents the two quantitative methods (Optical density / McFarland standard) of inoculation in MIC determination with broth microdilution method.

Optical density: a number of colonies are picked up and a suspension is made and cfus/ml are adjusted by spectrophotometer. A predetermined volume of the adjusted suspension is placed in a double series of wells in a 96 well plate where equal volumes of double strength broth as well as known antibiotic concentration exist. This method was employed for MIC evaluation in this study.
McFarland standard: a number of colonies are picked up and a suspension is made and cfus/ml are adjusted by visual comparison to a known McFarland standard. A predetermined volume of the adjusted suspension is placed in a double series of wells in a 96 well plate where equal volumes of double strength broth as well as known antibiotic concentration exist.

2.1.1.2.4 Interpretation of the results

Turbidity in the bottom of the each well was considered to represent bacterial growth. Minimum Inhibitory Concentration was considered to be the lowest antibiotic concentration that inhibited bacterial growth.

2.1.1.3 API 20E

Standardised and rapid identification system API 20E system (Bio Merieux. S.A., Marcy - l'Etoile, France) is used for the identification of Gram-negative enteric bacteria but also is employed increasingly for the identification of marine and fresh water fish pathogens. Mac Donell et al. (1982) suggested the application of 2% marine salt solution for the inoculation of the API 20E strip and the characterisation of marine and estuarine bacteria. In this study however the use of a 3% salt solution gave consistently better results and was chosen as the working solution.

2.2 KINETICS – RESIDUE WORK

The aim of the pharmacokinetics and residue work was to investigate the kinetics of the two most widely used antimicrobials in Mediterranean mariculture, namely Oxolinic acid and Oxytetracycline, in two species of cultured fish, Sea bass (*Dicentrarchus labrax*) and Sea bream (*Sparus aurata*) kept at two water temperatures (high/ "summer" and low/"winter" temperatures). In this context four experiments were designed for each antimicrobial agent.

2.2.1 Oxolinic acid kinetics and residue work

The following experiments were performed for the Oxolinic acid pharmacokinetic investigation.

Table 14: Oxolinic Acid Experiments

Experiment	Fish Species	Conditions
I	Sea bream (65g)	22 ± 1 °C
II	Sea bream (200g)	17 ± 1 °C
III	Sea bass (250g)	26 ± 1 °C
IV	Sea bass (200g)	18 ± 1 °C

2.2.2 Oxytetracycline kinetics and residue work

The following experiments were performed for the Oxytetracycline pharmacokinetic investigation.

Table 15: Oxytetracycline Experiments

Experiment	Fish Species	Conditions
V	Sea bream (230g)	24 ± 1 °C
VI	Sea bream (200g)	18 ± 1 °C
VII	Sea bass (200g)	24 ± 1 °C
VIII	Sea bass (220g)	14 ± 1 °C

Experiments I,V,VI,VII and VIII were performed in the Aquarium of the National Centre for Marine Research in Athens, Greece while Experiments II, III and IV were performed in Kalloni Poros LTD fish farm in Taktikoupoli Troizinias near Poros Island in the Saronikos Gulf. Temperature was carefully controlled in aquarium experiments and specific periods with minimal temperature fluctuations were selected when experiments were performed in the fish farm. Temperature was recorded twice every day at a depth of 1 meter in the fish farm and in the bottom of all tanks in the aquarium studies.

2.2.3 HPLC system – Oxolinic Acid

The system used comprised of a pump [Waters™ 510 HPLC pump], an autosampler [Waters™ 717 plus], a column [H&P Deactivated Hypersil BDS C18 100mm/4mm] and a fluorescence detector [WATERS 470 Fluorescence Detector]. The system was centrally controlled by a Pentium computer and Waters ™ SAT/IN modules using chromatography software [Millennium™ Chromatography Manager Waters® 1994]. Flow rate was adjusted to 0.5ml/minute and pressure upper limit to 2500 psi. Fluorescence detector emission wavelength was adjusted to 369 nm and excitation

wavelength to 327 nm. Oxolinic acid retention time was 5 minutes. Mean recovery of the method was estimated at 77%. Detection limit was 30ppb of Oxolinic acid.

2.2.4 HPLC system – Oxytetracycline

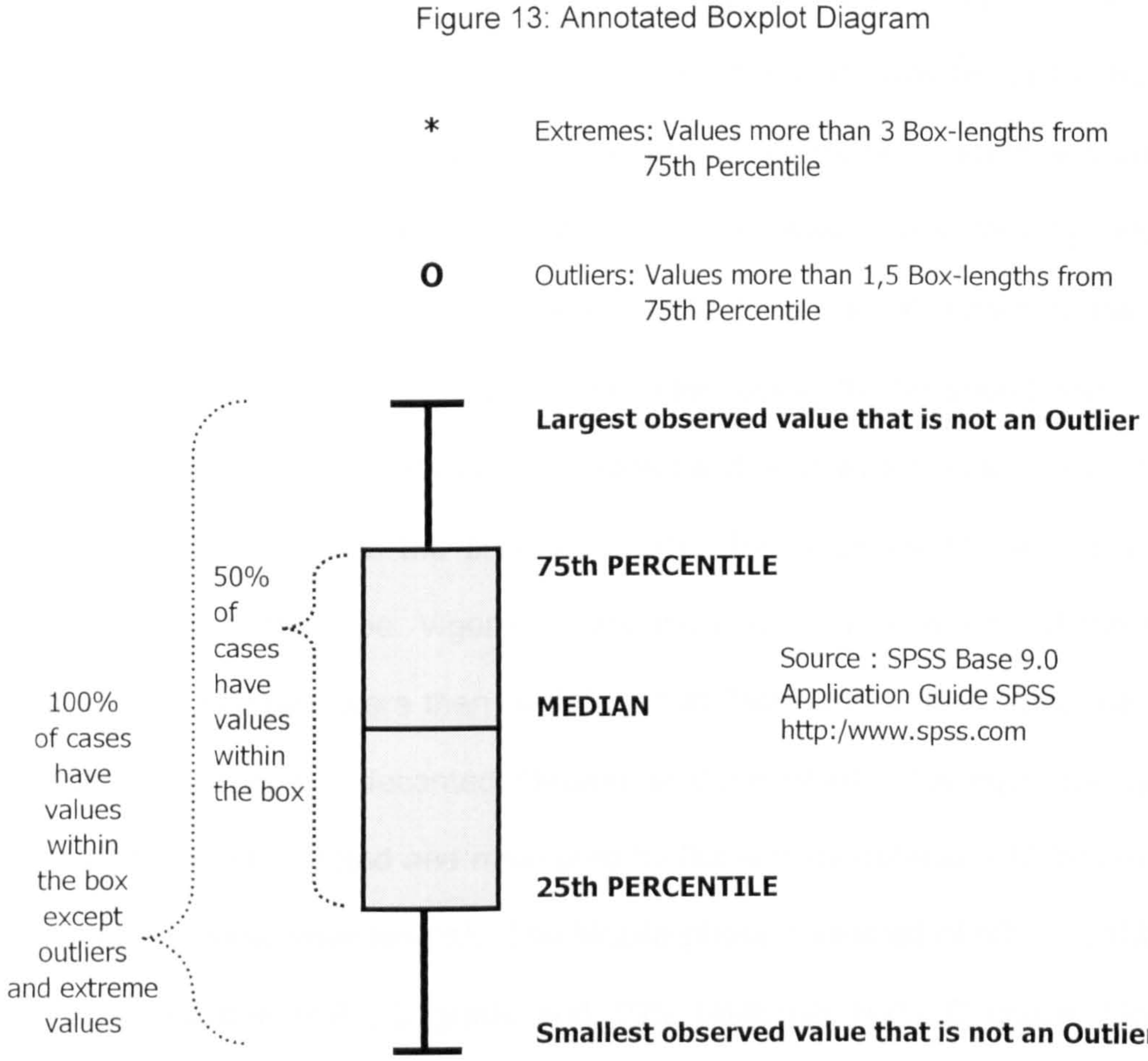
The system used comprised of a pump [Waters™ 510 HPLC pump], an autosampler [Waters™ 717 plus], a column [H&P Deactivated Hypersil BDS C18 100mm/4mm] and a UV detector [Waters™ 484 Tunable Absorbance Detector]. The system was centrally controlled by a Pentium computer and Waters ™ SAT/IN modules using chromatography software [Millennium ™ Chromatography Manager Waters® 1994]. Flow rate was adjusted to 0.5ml/minute and pressure upper limit to 2500 psi. Tunable Absorbance detector wavelength was adjusted to 360nm. Oxytetracycline retention time was 8 minutes. Mean recovery of the method was estimated at 70%. Detection limit was 20ppb of Oxytetracycline

2.2.5 Data Analysis & Presentation

There are two ways for the presentation of H.P.L.C data. The first includes the presentation of the minimum, mean and maximum concentration achieved each day of the experiment, while the other includes statistical analysis and presentation of the distribution of O.A concentration in the population sampled at each sampling time. In the first type of presentation the blue line represented the maximum concentration achieved at each sampling time, the green line, the mean concentration and the red line the minimum concentration achieved in the muscle of Sea bream and Sea bass. The detailed statistical analysis of the H.P.L.C data with Box-Plot Analysis (SPSS® Base, SPSS Inc. Chicago, Illinois, USA) gave a more realistic approach on the O.A concentrations achieved in the tissues of the fish sampled. *Boxplots* are summary plots based on the median, quartiles, outlier and extreme values. Boxplots were formed from

"boxes", which contain the 50% of values falling between the 25th and 75th percentiles, and the "whiskers", lines that extend from the box to the highest and lowest values, excluding outliers. A horizontal line inside the box indicates the median. Boxplots also convey information about spread and skewness. SPSS boxplot output also identifies outliers (o) and extreme values (*). Outliers (o) are considered the values that are more than 1.5 box lengths from the 25^{th} or the 75^{th} percentile, while Extremes (*) are considered the values that are more than 3 box lengths from the 25^{th} or the 75^{th} percentile.

Figure 13: Annotated Boxplot Diagram

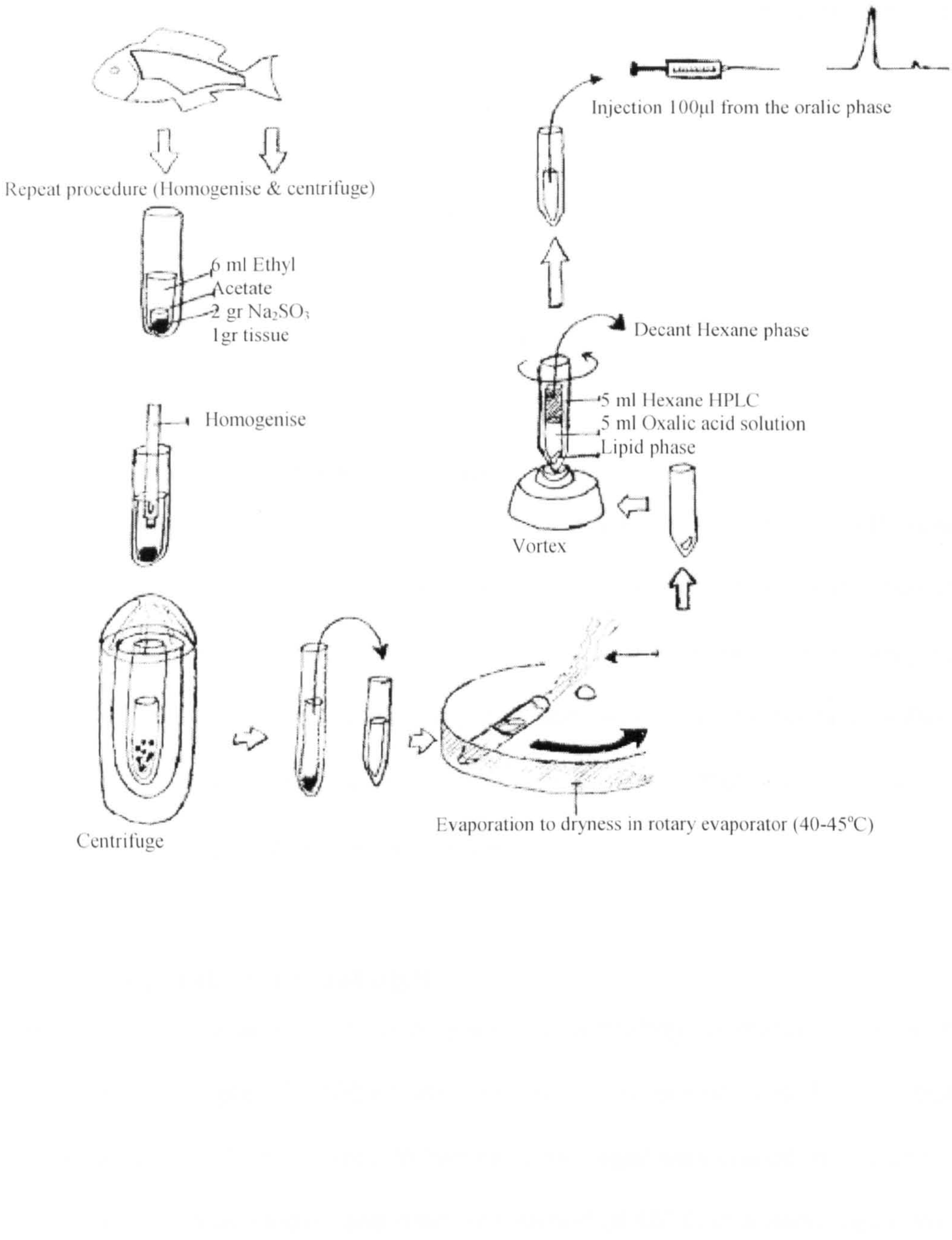


2.3 OXOLINIC ACID KINETICS

2.3.1 Method of HPLC Analysis – Oxolinic Acid

Two grams of muscle and 0.5 grams of liver samples were placed in glass tubes with an equal quantity of Sodium sulphate (Na_2SO_4) and 12 ml of Ethyl acetate (H.P.L.C. Grade). Samples were homogenised using an Ultra turrax homogenator, while the tube was kept in ice-cold water (around 2°C) in order to avoid temperature increase resulting in decay and loss of the antimicrobial agent. Samples were centrifuged at 1500rpm for 2min and the supernatant was removed and placed into special tubes that fit in a Speed Vac system where samples were evaporated under vacuum. This step lasted for 50min. In the meantime 12ml of Ethyl acetate were placed in the original tubes and all the above steps were repeated for a second time. Care was taken for the samples to be "near" dry in order to be able to recover the antimicrobial agent at a later stage. In the next step, the remaining combined extract was redissolved by adding 5ml of Hexane H.P.L.C. and vortexing for 45sec (dissolving the fat content of the extract) and the liquid was removed and placed in other tubes. In the speed Vac tubes 5ml of aqueous 0.01M oxalic acid were also added and after vortexing for 30 sec the aqueous phase was placed with the previous phase. The aqueous phase (heavier) was the lower phase in the tube. Vigorous vortexing lead to good mixing of the two phases. Tubes with samples were then centrifuged at 2800rpm for 10min and the supernatant (organic solvent) was decanted. Oxolinic acid remained in the aqueous layer. 100µl of this phase were injected and measured by fluorescent detector (327nm excitation and 369nm emission wave length). The Mobile phase consisted of 60% 0.01M oxalic acid, 30% Acetonitrile H.P.L.C grade and 10% Methanol H.P.L.C grade. Flow rate was adjusted to 1ml /min. Retention time was 5 minutes. Flumequine 200ppb final solution was used as internal standard. The method was standardised with several standard dilution curves (R-squared=99.93 percent, slope 95.83, intercept: 405.86) and spiked tissue curves (R square: 99.59%). Mean recovery of the method was found to be around 77%.

Figure 14: OA residues detection by HPLC



2.3.2 Bioassay–Detection of Oxolinic acid residues

Yersinia ruckeri (FDL 39/81) was used as the indicator organism for the detection of oxolinic acid residues. The method used was a modification of that described by Barker (1994) and included:

a. Preparation of test bacterium

b. Preparation of test agar

c. Preparation of antimicrobial agent

d. Preparation of the muscle/serum samples

2.3.2.1 Preparation of test bacterium

A 250 µl Ehrlenmeyer flask containing 100ml Tryptone Soy broth (TSB) (Oxoid) was inoculated with Y. ruckeri from an overnight plate. The flask was then incubated overnight on a rotary shaker at 100rpm at 30° C. Cells were subsequently harvested, centrifuged at 4° C for 20 min at 1000g and washed in fresh phosphate-buffered saline (PBSA). They were then re-centrifuged, re-suspended in PBSA and adjusted to a final concentration of 10^7 cells/ml by optical density.

2.3.2.2 Preparation of test agar

Isosensitest test agar (Oxoid) was prepared according to manufacturer's instruction and adjusted to pH: 6. 100ml aliquots were dispensed into 500-ml bottles and autoclaved at 121° C for 15 min. When required, agar was placed in a microwave oven and heated until fully molten and then maintained at 45° C in a water bath. Immediately before pouring, 0.1ml of the bacterial working concentration of 10^4 cells/ml (Optical Density) Agar was poured into pre-levelled bioassay trays (Nunc, Rosklide, Denmark) to give a depth of 1.5-2mm. When set and cooled, 36 evenly spaced wells (Latin square), 5mm in diameter, were punched into the agar.

2.3.2.3 Preparation of the antimicrobial agent

Oxolinic acid (Sigma) as other 4-quinolone antimicrobials is not easily dissolved in distilled water. Oxolinic acid was therefore first suspended in distilled water to a nominal concentration of 1024 µg/ml, fully dissolved by the dropwise addition of 0.1M NaOH and readjusted to pH: 9.3 by drop-wise addition of 0.1M hydrochloric acid. Using this stock solution further serial two fold dilutions were made in distilled water to a final, theoretical concentration of 0.008µg/ml.

2.3.2.4 Preparation of muscle samples

One-gram samples of antibiotic-free Sea bream and Sea bass muscle were homogenised in an equal volume of appropriate drug dilution. These homogenates were then centrifuged (1000g for 20 min) and 3 x 50µl aliquots of supernatant were added to 3 random wells per assay tray using an electronic pipette. Assay trays were performed in triplicate and following overnight incubation at 30°C, zones of inhibition were measured using a digital calliper.

2.3.3 Oxolinic Acid Kinetics in Sea Bream (*Sparus aurata*)

2.3.3.1 High Water Temperature

2.3.3.1.1 Experimental Design

One hundred and twenty Gilthead bream (*Sparus aurata*) of average weight 65g were kept a semi-closed circulation system comprised of 15 fibreglass rectangular tanks (160lt volume) in the aquarium of N.C.M.R. The NCMR aquarium pumping system provides a degree of recirculation but a 20% of system water volume is exchanged per hour. Water temperature was kept at 22 ± 1°C and fish were left for a 10-day adaptation period before the experiment.

2.3.3.1.2 Medicated Feed Preparation

Fish were fed on a 2 % per body weight ratio and a dose rate of 30mg O.A/Kg b.w/day was used for a period of 10 days. Due to pilot trials where obvious palatability problems were encountered and leaching was suspected, it was decided that in all the experiments the antibiotics would be incorporated in the diet. The NCMR Fish Nutrition lab has extensive experience in feed formulation and manufacturing and based on initial observations a parallel study was initiated to elaborate on palatability and leaching of medicated diets for sea bass and sea bream. However in order to avoid deviation problems commercial extruded feed was decided to be transformed into powder and the antibiotic was initially incorporated in a “premix” form and then was included in the total quantity of the diet. A small quantity of distilled water was added to increase humidity and make pellet preparation easier. Feed was then lyophilised, pellet size was adjusted by use of a crumbler and the feed was kept at 4°C.

2.3.3.1.3 Sampling

Fish were sampled on the 0,3,10,11,12,14,16,20,23 and 25th day of the experiment. In this manner 3 samplings took place during the treatment period and sampling was intense in the first days after treatment in order to evaluate the maximum levels of O.A obtained in the muscle and liver as well as the speed of O.A depletion in the tissues. Each time 9 fish were sampled. Fish in all cases were killed, by a single severing cut of the spinal cord, weighed and quickly all the skeletal muscle and liver were removed and kept at -20°C until analysed by H.P.L.C. This method of slaughter was selected in order to avoid possible interference of the anaesthetic with the HPLC analysis.

2.3.3.2 Low Water Temperature

2.3.3.2.1 Experimental Design

Experiment II investigated the metabolism of Oxolinic acid in sea bream kept at water temperature 17 $\pm$ 1°C. In spring 1995, one hundred and forty sea bream (*Sparus aurata*) of average weight 212g $\pm$ 23.6g (m.w $\pm$ s.d) were kept in a specially designed net (3x2x6 m dimensions) in Kalloni Ltd. Water temperature was fairly stable throughout all the experimental period at 17 $\pm$ 1.4°C and fish were left for a 3-week adaptation period just before the experiment. Sea bream were treated orally with Oxolinic acid at a dose rate of 30mg/kg body weight / day for a period of 10 days.

2.3.3.2.2 Medicated Feed Preparation

Fish were fed on a 0.3 % per body weight ratio and a dose rate of 30mg O.A/Kg b.w/day was used for a period of 10 days. Medicated diet was prepared using the same method mentioned above with incorporation of the antibiotic in the diet.

2.3.3.2.3 Sampling

Samples of muscle and liver were taken at 0, 1st, 3rd, 7th, 10th, 11th (morning and afternoon), 12th, 13th, 14th, 16th, 18th, 20th, 25th, 30th and 35th day of the experiment. In

this manner 4 samplings took place during the treatment period and sampling was intense in the first days after treatment (11th to 14th) in order to evaluate the maximum levels of O.A obtained in the muscle and liver as well as the speed of O.A depletion in the tissues. Each time an average of 9 fish was sampled. Fish were killed, by a single severing cut of the spinal cord, weighed and quickly all the skeletal muscle and liver were removed and kept at -20°C until analysed by H.P.L.C.

2.3.4 Oxolinic Acid Kinetics in Sea Bass (*Dicentrarchus labrax*)

2.3.4.1 High Water Temperature

2.3.4.1.1 Experimental Design

Experiment III investigated the metabolism of Oxolinic acid in sea bass kept at water temperature 26 ± 1°C. In summer 1995, one hundred and twenty adult Sea bass (*Dicentrarchus labrax*) of average weight 252g ± 24.8g (m.w ± s.d) were kept in a specially designed net (3x2x6 m dimensions) in Kalloni Ltd. Water temperature was fairly stable throughout all the experimental period at 26 ± 1.8°C and fish were left for a 3-week day adaptation period just before the experiment. Sea bass were treated orally with Oxolinic acid at a dose rate of 30mg/kg body weight / day for a period of 10 days.

2.3.4.1.2 Medicated Feed Preparation

Fish were fed on a 1% per body weight ratio and a dose rate of 30mg O.A/Kg b.w/day was used for a period of 10 days. Medicated diet was prepared using the same method mentioned above with incorporation of the antibiotic in the diet.

2.3.4.1.3 Sampling

Samples of muscle and liver were taken at 0, 1st, 3rd, 7th, 10th, 11th (morning and afternoon), 12th, 13th, 14th, 16th day of the experiment. In this manner 4 samplings took place during the treatment period and sampling was intense in the first days after treatment (11th to 14th) in order to evaluate the maximum levels of O.A obtained in the

muscle and liver as well as the speed of O.A depletion in the tissues. Each time an average of 9 fish was sampled. Fish were killed, weighed and quickly all the skeletal muscle and liver were removed and kept at -20°C until analysed by H.P.L.C.

2.3.4.2 Low Water Temperature

2.3.4.2.1 Experimental Design

Experiment IV investigated the metabolism of Oxolinic acid in sea bass kept at water temperature 18 ± 1°C. In spring 1995, one hundred and eighty adult Sea bass (*Dicentrarchus labrax*) of average weight 215g ± 32g (m.w ± s.d) were kept in a specially designed net (3x2x6 m dimensions) in Kalloni Ltd. Water temperature was fairly stable throughout all the experimental period at 18 ± 1.4°C and fish were left for a 3-week day adaptation period just before the experiment. Sea bass were treated orally with Oxolinic acid at a dose rate of 30mg/kg body weight / day for a period of 10 days.

2.3.4.2.2 Medicated Feed Preparation

Fish were fed on a 0.2 % per body weight ratio and a dose rate of 30mg O.A/Kg b.w/day was used for a period of 10 days. Medicated diet was prepared using the same method mentioned above with incorporation of the antibiotic in the diet.

2.3.4.2.3 Sampling

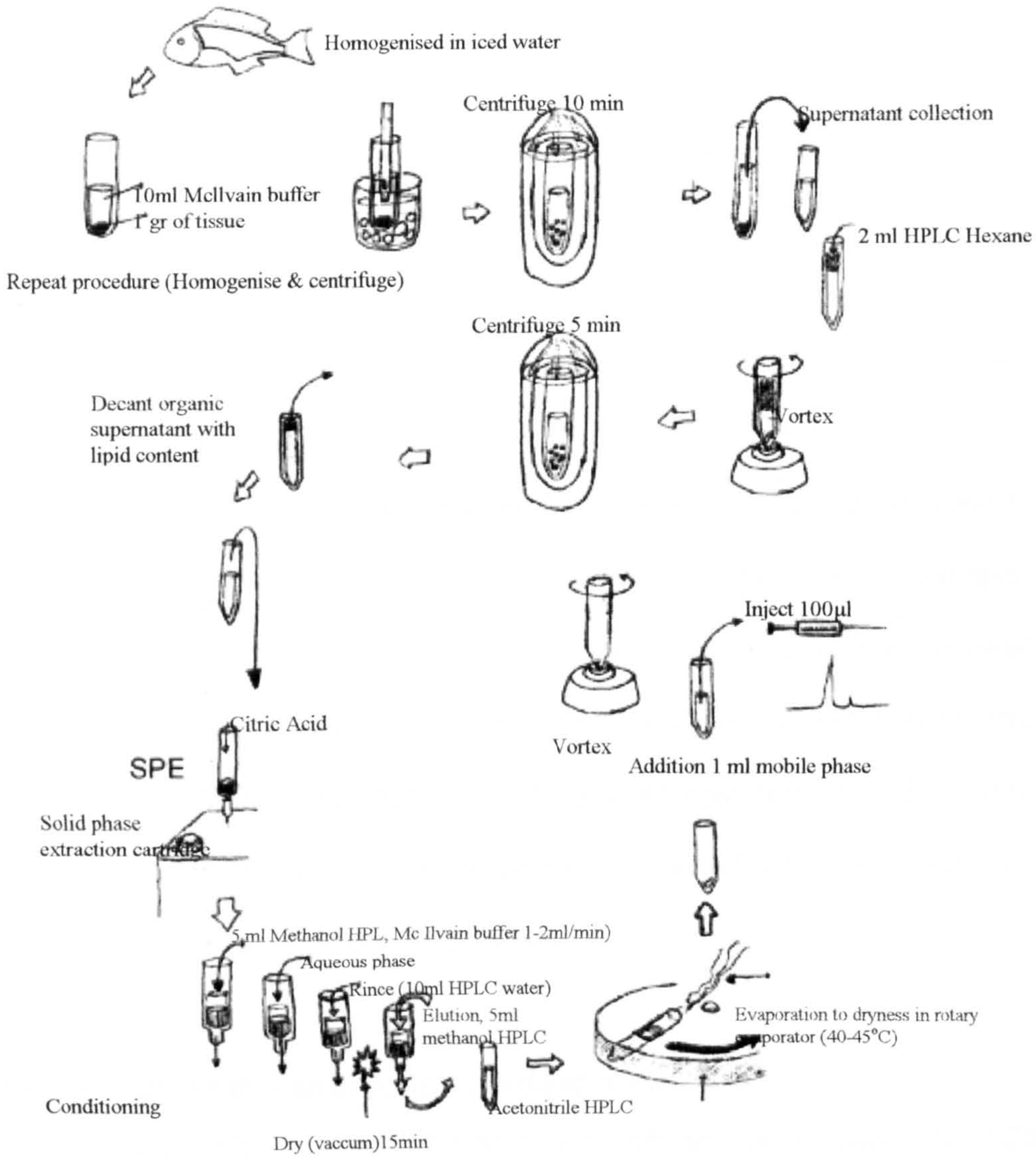
Samples of muscle and liver were taken at 0, 1st, 3rd, 7th, 10th, 11th (morning and afternoon), 12th, 13th, 14th, 16th, 18th, 20th, 25th, 30th and 35th day of the experiment. In this manner 4 samplings took place during the treatment period and sampling was intense in the first days after treatment (11th to 14th) in order to evaluate the maximum levels of O.A obtained in the muscle and liver as well as the speed of O.A depletion in the tissues. Each time an average of 9 fish was sampled. Fish were killed, weighed and quickly all the skeletal muscle and liver were removed and kept at -20°C until analysed by H.P.L.C.

2.4 OXYTETRACYCLINE KINETICS

2.4.1 Method of HPLC Analysis – Oxytetracycline

The detection of OTC in tissues was performed according to Tyrpenou (1995). One gram of muscle, liver or skin tissue were homogenised [Ultra Turrax T-25, IKA Labortechnik] with 10 ml McIlvain modified buffer (0.06 M citric acid, 0.02 M $K_2HPO_4.2H_20$) at low temperature (4°C). The sample was then centrifuged for 10 minutes at 15000 rpm [Sorvall ® SUPERSPEED RC2-B Automatic Refrigerated Centrifuge] and the supernatant was removed. The procedure was repeated twice and combined supernatants were passed through the Solid Phase Extraction column (SPE) [Bond Elute C18 500mg (IST, Isolute®)] using a Waters↓ Sep-Pak® Vacuum Manifold. Each SPE column, prior sample application, was conditioned with 5ml methanol HPLC grade [Lab-Scan Analytical Sciences], then with 5ml McIlvain modified buffer. In the combined supernatants from liver samples 2ml of Hexane HPLC grade (Lab-Scan) were added, vortexed thoroughly and centrifuged for 5 minutes at 15000 rpm. The organic layer (upper layer) with all the lipid content of the sample was decanted and the aquatic phase was passed through the SPE column. After sample application, 10ml of water HPLC grade were passed through the SPE column in order to rinse it and then the column was vacuum dried for 15 minutes. Elution was performed with 10ml of Methanol/Acetonitrile (50:50) HPLC grade [Lab-Scan] and the eluent was evaporated to dryness in Rotary Flask Evaporator [Bucchi] connected with a Refrigerated Bath [RB-12A Techne] with temperature control [Tempette® TE-8D Techne]. Oxytetracyline residue was then re-suspended to 0.5 ml of mobile phase (0.01M oxalic acid/ Acetonitrile 85:15). Injection volume was 100µl.

Figure 15: OTC residues detection by HPLC



2.4.2 Bioassay - Detection of oxytetracycline residues

A commercial spore suspension of Bacillus cereus (Difco) was employed as the indicator organism.

2.4.2.1 Preparation of Bioassay trays

Isosensitest agar was prepared according to manufacturer's instructions and adjusted to pH: 5.6 by the dropwise addition of 0.1M hydrochloric acid. 100 ml aliquots were dispensed into 500ml bottles and autoclaved at 121° C for 15 minutes. When required, agar was placed in a microwave oven, heated until fully molten and then maintained at 50^{0}C in a water bath. Before pouring, each bottle was allowed to stand at room temperature for 5 minutes. Fifty microlitres of the spore suspension was added to the agar and the bottle was gently rolled to give a final bacterial concentration of 10^3 cfu's /ml agar. The seeded agar was then slowly poured into pre-levelled sterile plastic bioassay trays (Nunc) to give an agar depth of 1.5 - 2 mm. When set and cooled at 4^{0}C for 30 minutes, 36 evenly spaced wells were punched into the agar using a cork borer 6.5mm in diameter.

2.4.2.2 Preparation of the antibiotic standard solutions

Oxytetracycline was dissolved in distilled water to a nominal concentration of 1024 µg/ml. Serial two-fold dilutions were made in distilled water to a final theoretical concentration of 0.03125 µg/ml. Solutions that ranged from 4µg/ml to 0.03125 µg/ml were used while the rest were discarded. Fifty microlitre aliquots were placed into each of 3 wells randomly positioned on the bioassay tray. The remaining wells were filled with 50 µl of distilled water and used as negative controls. Trays were then incubated for 18 hours at 30^{0}C. Zones of inhibition were measured to one decimal place using a digital calliper.

2.4.3 Oxytetracycline Kinetics in Sea Bream (*Sparus aurata*)

2.4.3.1 High Water Temperature

2.4.3.1.1 Experimental Design

The OTC kinetics experiment in sea bream at high temperature was performed in the aquarium of the NCMR in January 1997. Water was heated to achieve constant temperature of 24 ± 1 o C. One hundred and twenty sea bream of mean weight 180 ±23g (m.w ± s.d) were divided in three groups and placed in rectangular tanks of 2 m3 each and were left to acclimatised for two weeks.

2.4.3.1.2 Medicated feed Preparation

Fish were fed OTC medicated diet at a feeding rate of 1% b.w per day. OTC dose rate was 75 mg / kg b.w / day for 10 days and the antibiotic was incorporated in the diet as mentioned above because in initial experiments low palatability was evident when OTC was superficially coated.

2.4.3.1.3 Sampling

Seven fish were sampled per sampling time at 0, 1st, 3rd, 5th, 7th, 10th, 11th, 12th, 13th, 14th, 17th, 21st, 23rd and 25th day from alternate tanks in order to avoid stressing the fish and interfere with their feeding response.

2.4.3.2 Low water temperature

2.4.3.2.1 Experimental Design

The OTC kinetics experiment in sea bream at low temperature was performed in the aquarium of the NCMR in March 1997. Water temperature during the experimental period had an average of 18 $\pm$1.7 ° C. One hundred twenty sea bream of mean weight 200 $\pm$ 28mg (m.w $\pm$ s.d) were divided into three groups and placed in cylindroconical tanks of 2 m^3 each and were left to acclimate for two weeks.

2.4.3.2.2 Medicated feed preparation

Fish were fed OTC medicated diet at a feeding rate of 0.3% b.w per day for 10 days. OTC dose rate was 75 mg / kg b.w / day and the antibiotic was incorporated in the diet, as mentioned above because in initial experiments low palatability was evident when OTC was superficially coated.

2.4.3.2.3 Fish Sampling

An average of eight fish were sampled per sampling time at 0,1st, 3rd, 5th, 7th, 10th, 11th, 13th, 15th, 18th, 20th, 25th, 30th and 40th day from alternate tanks in order to avoid stressing the fish and interfere with their feeding response.

2.4.4 Oxytetracycline Kinetics in Sea Bass (*Dicentrarchus labrax*)

2.4.4.1 High Water Temperature

2.4.4.1.1 Experimental Design

OTC kinetics experiment in sea bream at low temperature was performed in the aquarium of the NCMR in January 1997. Water temperature during the experimental period had an average of 24 ± 1.8 ° C. One hundred twenty sea bass of mean weight 200 ± 15g (m.w ± s.d) were divided into three groups and placed in cylindroconical tanks of 2 m^3 each and were left to acclimatise for two weeks.

2.4.4.1.2 Medicated feed preparation

Fish were fed OTC medicated diet at a feeding rate of 1 % b.w per day. OTC dose rate was 75mg/kg b.w/day and the antibiotic was incorporated into the diet because in initial experiments low palatability was evident when OTC was superficially coated.

2.4.4.1.3 Sampling

An average of eight fish were sampled per sampling time at 0, 1st, 3rd, 5th, 7th, 10th, 11th, 12th, 13th, 14th, 17th, 21st, 23rd and 25th day from alternate tanks in order to avoid stressing the fish and interfere with their feeding response.

2.4.4.2 Low Water Temperature

2.4.4.2.1 Experimental Design

In March 1997, in the aquarium of NCMR an oxytetracycline kinetics experiment was performed in sea bass at low temperature (14° C). One hundred and twenty fish of average weight 180 g were placed in three cylindroconical tanks of 2 m^3 and left for a two-week adaptation period.

2.4.4.2.2 Medicated feed preparation

Fish were fed OTC medicated diet at a feeding rate of 0.3 % per body weight and an OTC dose rate of 75mg/kg b.w/day. Medicated diet was prepared using the same method mentioned above with incorporation of the antibiotic in the diet.

2.4.4.2.3 Sampling

An average of seven fish were sampled on the 0, 5^{th}, 7^{th}, 10^{th}, 11^{th}, 13^{th}, 15^{th}, 18^{th}, 20^{th}, 23^{rd}, 26^{th}, 31^{st}, 35^{th} and 39^{th} day of the experiment in order to monitor efficiently the OTC concentration increase as well as the relatively slow depletion process.

3 RESULTS

3.1 Bacteriology

The presentation of bacteriological data includes 15 different sections of data:

3.1.1 Biochemical profile - quantitative antibiogram & MIC data

All the data for each strain were presented in Appendix I in a card format which includes information concerning the fish species which the strain was isolated from, basic data such as Gram stain, Oxidase test, Motility test, Colony form and colouration, Growth on TCBS agar, Oxidative-Fermentative metabolism as well as API20E biochemical profile, zones of inhibition in a quantitative antibiogram and the M.I.C values for Oxytetracycline and Oxolinic acid.

3.1.2 Summary statistics - bacterial strain origin & geographical distribution

Five different species of farmed fish were sampled. Bacterial strain % incidence isolated from each species was briefly described.

- sea bass contributed 86.13% of the strains
- sea bream 16.83%
- sharpsnout bream 4.95%
- common dentex 1.89%
- grey mullet 0.99%

■ Fish samples originated from moribund fish examined on site or send to the lab by fish veterinarians or farmers from nine different geographical regions in Greece.

- Saronikos gulf with 25% of the farms contributed 44.55% of the strains
- South Evoikos, 16.67% of the farms contributed 16.83% of the strains

- Twenty-four different fish farms were sampled in this study. Samples mainly originated from 3 farms (namely farm 8, 21 and 22) where every disease outbreak was monitored.

Table 16: Summary statistics: Origin of Bacterial Strains and Geographical distribution of fish farms mentioned in the study.

Bacterial Strain - summary statistics			
	Origin code	No strains	Gulf
1	RP	4	Patraikos
2	Argo	3	Argolikos
3	SF	3	Ionian sea
4	NE	1	North Evoikos
5	Deep	1	North Evoikos
6	Ast	3	Ionian sea
7	Ovr	7	Saronikos
8	Kal	12	Saronikos
9	Pl	4	Saronikos
10	Agr	2	Saronikos
11	Petr	2	Saronikos
12	Chios	1	North Aegian
13	Nous	8	Ionian sea
14	Fido	4	South Evoikos
15	Prap	5	North Evoikos
16	Eyr	1	Argolikos
17	Ocean	3	North Evoikos
18	Zant	4	Korinthiakos
19	Ler	1	South Aegian
20	Myt	1	North Aegian
21	Makr	9	South Evoikos
22	Kant	18	Saronikos
23	Vas	1	South Evoikos
24	AqH	3	South Evoikos
		101	

Geographical region strain			
	Gulf	No Sites	No Strains
1	Patraikos	1	4
2	North Aegian	2	2
3	Saronikos	6	45
4	Argolikos	2	4
5	Korinthiakos	1	4
6	South Evoikos	4	17
7	South Aegian	1	1
8	North Evoikos	4	10
9	Ionian sea	3	14
		24	101

The aim was to include bacterial pathogenic bacteria that caused mortalities in the field from fish farms throughout Greece. Bacterial isolation was coupled with parasitological analysis and histopathological analysis but these data are not included here because they are beyond the scope of this study.

3.1.3 API 20E biochemical profiles: Number of strains - % incidence

Table 17, demonstrates the biochemical profile of the bacterial strains isolated from diseased sea bass. 93.1 % of these strains are Gram- while 6.9% are Gram + cocci. Among the 87 strains isolated, 21.85% exhibited 12 biochemical profiles from which species identification was not possible. The rest (78.15%) exhibited 14 biochemical profiles and were identified as

- Vibrio anguillarum 1 phenon 4b 33.3%
- V. alginolyticus 8.05%
- V. anguillarum 1 phenon 2 6.9%
- V.parahaemolyticus 2.3%
- V.fluvialis 1.15%
- V.vulnificus 1.15%
- Pseudomonas fluorescens 1.15%
- Flavobacterium meningosepticum 2.30%

- Aeromonas salmonicida 1.15%
- Photobacterium damsela subsp. piscicida 2.30%

Gram+ cocci (6 strains) constituted 6.9% of the bacterial strains isolated. These were isolated from 4 different fish farms in 3 different geographical regions. *V.anguillarum1* phenon 4b (Grizez et al., 1991) was the dominant bacterial strain being isolated from diseased sea bass in 12 fish farms (33.33%).

Table 17: Bacterial Strains isolated from Sea Bass (*Dicentrarchus labrax*)

A/A	Sea Bass Api20E	Code number	No
1	0003004	Agr1, Agr2	2
2	0000004	Kant4	1
3	1003004	Kant1, Kant 17, Makr1	3
4	1007004	Kant 2, RP1 , Flavobacerium menigosepticum	2
5	1047524	Petr3, Ovr5, Prap1, Ler1, Kal2, Pl2 ,V.anguillarum 1 phenon 2	6
6	1047724	Ovr2	1
7	2000104	Ovr3 A.salmonicida	1
8	2200004	Ast2, Pseudomonas fluorescens 81%	1
9	2004004	Nous7, Nous8, Photobacterium damsela subsp. piscicida	2
10	3047524	Petr2, Pl3,4, Ovr6,7, Makr 2,4, 5,6,8,10 Kant8,9,12,14,15, Prap 3,4,5, Sf2,3, NE 1, Deep1, Myt1, Chios1,SFI1, Kal10,11,12 V.anguillarum 1 phenon 4b	29
11	3003004	Kant3	1
12	3042524	Makr3	1
13	3044125	Ast1, probably (V.fluvialis)	1
14	4046105	Prap2, V.parahaemolyticus	1
15	4046505	Ovr4	1
16	4047520	Ovr1, V.alginolyticus	1
17	4047125	Ocean3, V.alginolyticus	1
18	4114735	Nous4, V.parahaemolyticus (ALO test: - + + & SAC -)	1
19	4146505	Nous 6	1
20	4146525	Nous2, Nous3, Kant5, Kal 8, V.alginolyticus	4
21	4144525	Ocean1, Kant7,19, SFI1	4
22	4174525	Kant16	1
23	4366525	Kant6	1
24	4347525	Nous1, V.alginolyticus	1
25	5046105	Ocean 2, V. vulnificus	1
26	6144525	Nous 5	1
27	Gram+ cocci	Pl1, Eyr1, Argo2, Kant10, Kant11, Kant13	6
28	Gram+ cocci	Argo1	1
			87

Table 18: Bacterial Strains isolated from Sea Bream (*Sparus aurata*), Sharpsnout bream (*Puntazzo puntazzo*), Common dentex (*Dentex dentex*) and Grey mullet (*Mugil cephalus*)

A/A	Sea Bream Api 20E	Code number	No
1	0004000	Zant4	1
2	0004125	Zant3 ,*V.ordalii*	1
3	0040777	Fido2	1
4	0002000	Ast3	1
5	1004124	Zant2, Pasteurella spp.	1
6	2005024	Vasni 1 , *Photobacterium damsela subsp. piscicida.*	1
7	4146525	AqH1,*V.alginolyticus*	1
8	4154525	Fido1,*V.alginolyticus* (ALO test: - + +)	1
9	5144565	Fido 3	1
10	5446025	Aq.H3	1
11	5046305	Fido4	1
12	6005004	Makr7, Kal4 ,*Listonella damsela* 99.5%	2
13	6544164	Aq.H2	1
14	7005100	Zant1	1
15	Gram+cocci	Argo3, Kal1	2
			17

A/A	Punt.Puntazzo Api 20E	Code number	No
1	4144525	Kal6 ,probably *V.alginolyticus* (ALO test: - + +)	1
2	3047524	Kal3, Kal 9, *V.anguillarum* 1	2
3	4154525	Kal7	1
4	6005004	Kal5 , *Listonella damsela* 99.5%	1
			5

A/A	Dentex. Dentex Api	Code number	No
1	Gram+cocci	RP2, RP4	2

A/A	Mugli cephalus Api	Code number	No
1	Gram+cocci	RP3	1

- Table 18, demonstrates the biochemical profiles reported for bacterial strains isolated from sea bream, sharpsnout bream, common dentex and grey mullet.
- In sea bream where 16.83% (17) of the bacterial strains were identified among the 95 biochemical profiles (API 20E) only 6 allowed reasonable identification. 88.24% of the strains were Gram- and 11.97% were Gram + cocci.
  - V.ordalli 5.88%
  - V.alginolyticus 11.76%
  - Photobacterium damsela subsp. piscicida. 11.76%
  - Listonella damsela 11.76%
- In sharpnout bream 80% of the strains were identified as
  - V.alginolyticus 20%
  - V.anguillarum1 phenon 4b 40%
  - Listonella damsela 20%

while in common dentex and grey mullet 100% of the bacterial strains were Gram+ cocci.

3.1.4 API biochemical profiles: frequency and % incidence statistical analysis

Table 19: Frequency of different biochemical profiles

Api 20E Profile	Frequency	Api 20E Profile	Frequenc
1003004	3	4146525	5
1004124	1	4154525	2
1007004	2	4174525	1
1047524	6	4347525	1
1047724	1	4366525	1
2000104	1	5046105	1
2004004	2	5046305	1
2005024	1	5144565	1
2200004	1	5446025	1
3003004	1	6005004	3
3040125	1	6114525	1
3042524	1	6544164	1
3047524	30	7005100	1
4046105	1	Gram- cocci	1
4046505	1	Gram+ cocci	11
4047125	1	0044777	1
4047520	1	0002000	1
4114735	1	0003004	2
4144525	5	0004125	1
4146505	1	0004000	1
		0000004	1
		TOTAL	**101**

Table 19, demonstrates all the API 20E biochemical profiles reported in this study along with their frequency. *Vibrio anguillarum1* phenon 4b was the dominant isolate while Gram+ cocci were very common in all fish species (10.9%).

3.1.5 API identifiable biochemical profiles: number of strains and % incidence

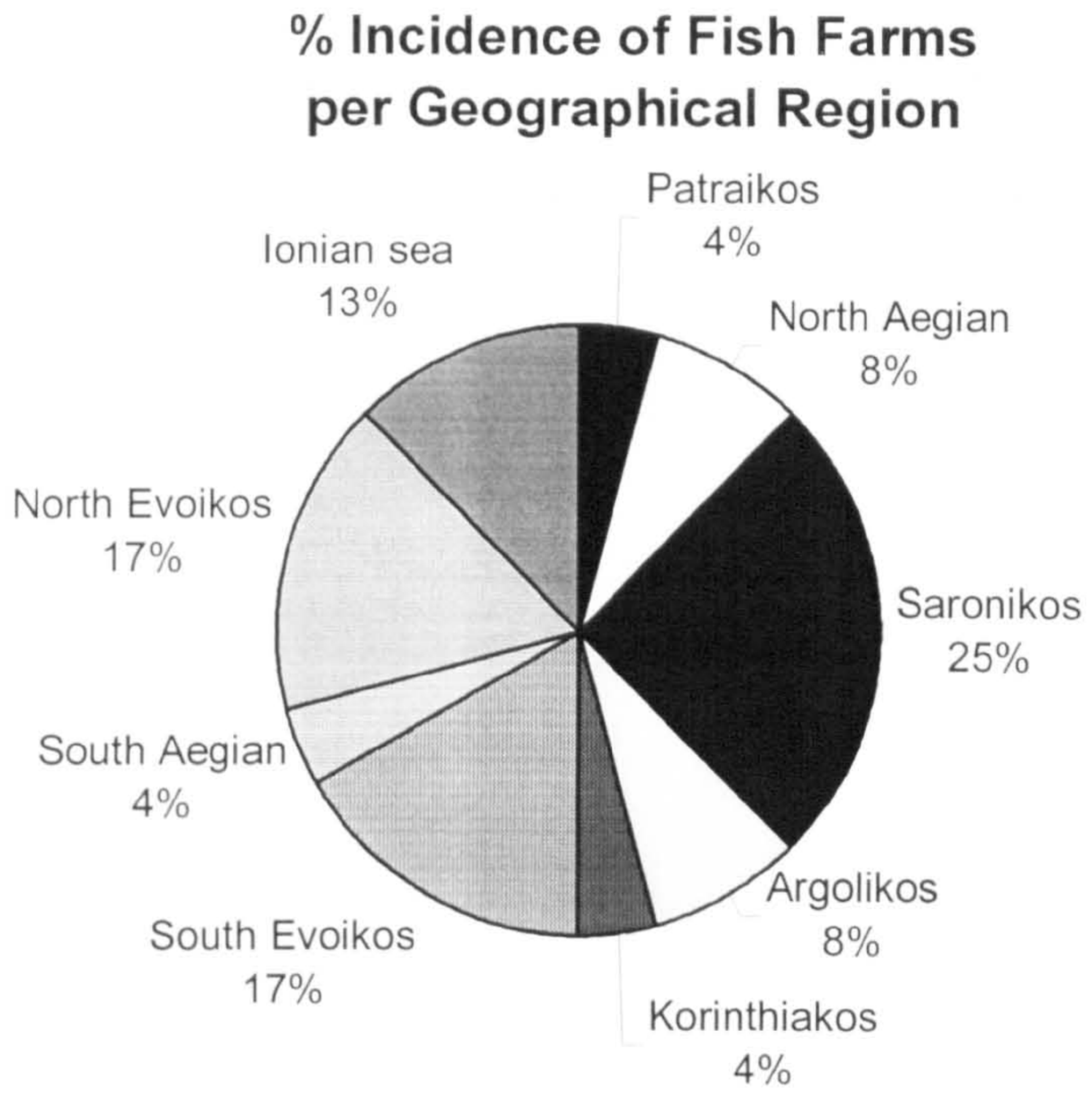
Table 20, presents different bacterial strains identified by API 20E. The identification of the strain, their API 20E profiles, their code name along with their origin and % incidence are presented.

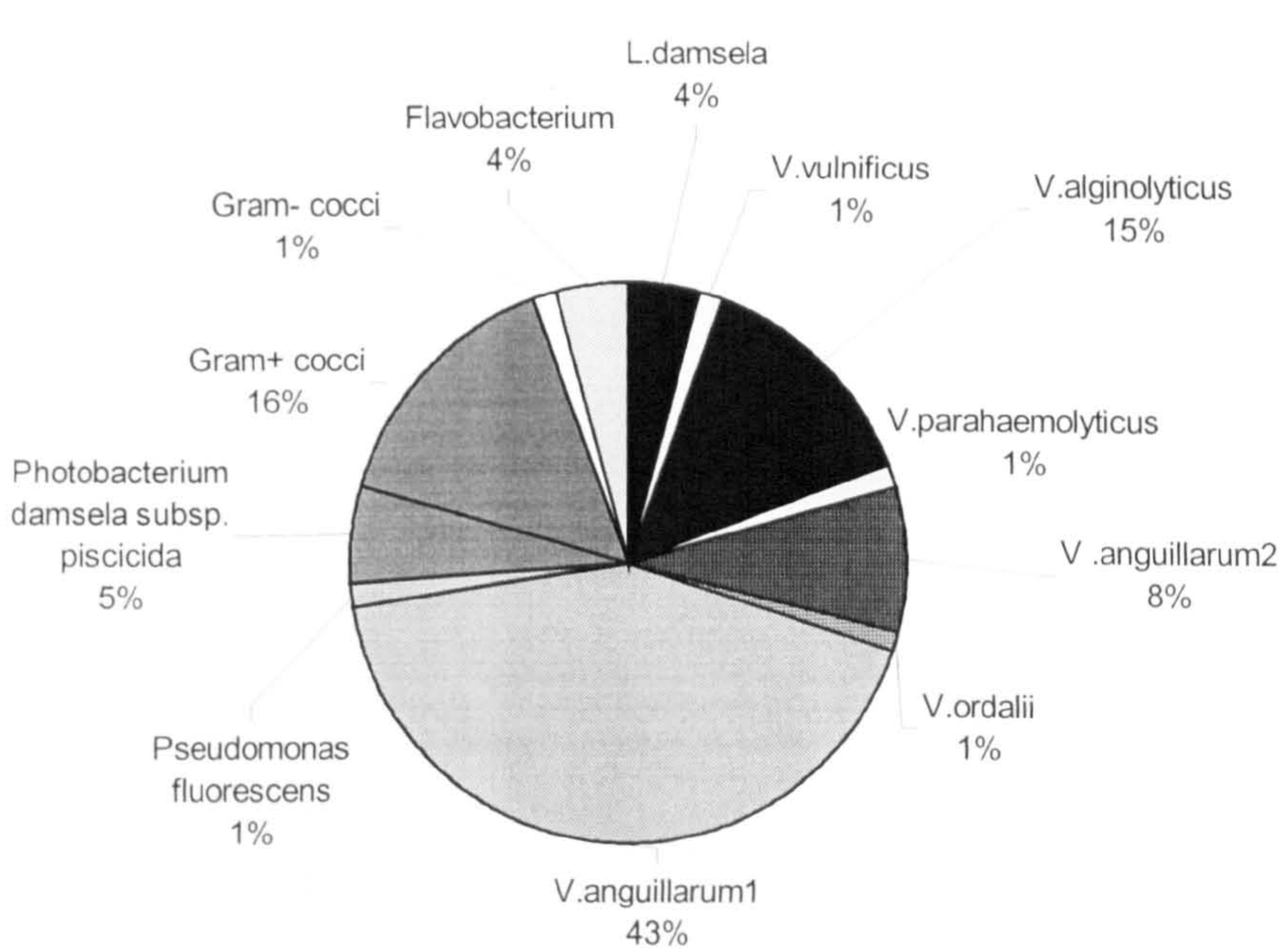
Table 20: Biochemical profiles of identified bacterial strains

A/A	Bacterium	Api20E	Code	No
1	L.damsela	6005004	Kal4 Makr7 Kal5	3
2	V.vulnificus	5046105	Ocean2	1
3	V.alginolyticus	4047520	Ovr1	1
	V.alginolyticus	4347525	Nous1	1
	V.alginolyticus	4146525	Nous2,3 Kant5 Kal8, AqH1	5
	V.alginolyticus	4047125	Ocean3	1
	V.alginolyticus	4144525	Kal6	1
	V.alginolyticus	4154525	Fido1	1
4	V.anguillarum 1	3047524	Petr2, Pl3,4, Ovr6,7, Makr 2,4, 5,6,8,10 Kant8,9,12,14,15, Prap 3,4,5, Sf2,3, NE 1, Deep1, Myt1, Chios1,SFI1, Kal10,11,12 Kal3, Kal9	31
5	V .anguillarum 1*	1047524	Petr3 Ovr5 Prap1 Ler1 Kal2 Pl2	6
6	V.parahaemolyticus	4046105	Prap2	1
7	V.ordalii	0004125	Zant3	1
8	Pseud.fluorescens	2200004	Ast2	1
9	Photobacterium damsela subsp. piscicida	1004124	Zant2	1
	Photobacterium damsela subsp. piscicida	2005024	Vasni1	1
	Photobacterium damsela subsp. piscicida	2004004	Nous7, Nous8	2
10	Gram+ cocci		Pl1, Eyr1, Argo1, Kant10, Kant11, Kant13, Argo3, Kal 1, RP2, RP4, RP3	11
11	Gram- cocci		Argo2	1
12	Flavobacterium spp	1002004	Kant1, 17, Makr1	3
				73

3.1.6 Bacterial strains – farms: % incidence per geographical region

Figure 16: Graphical Presentation, Bacterial Strains /Geographical distribution





This percentage refers to the number of isolates among the identified bacterial strains in this study giving an indication of the clinical relevance of these bacterial pathogens.

3.1.7 Bacterial Strains: Antibiogram data Summary

The presentation of the Quantitative Antibiogram data in the Table 21 is based on the following condition: The bacterial isolates were considered resistant to certain

antimicrobials when the zone of inhibition to the antimicrobial ranged between diameter of the 6 and 15 mm, of intermediate resistance exhibited when zones of inhibition varied between 15 and 25 mm and susceptible when zones of inhibition shared a diameter greater than 25 mm.

Table 21: Presentation of the Quantitative Antibiogram data for bacterial isolates

Farm	Strain	Fish Species	API20E profile	OTC	OA	SXT	FR	O129	P	NV	AMP
1	Rp1	Bass	1007004	I	S	S	S	I	S		
	Rp2	Dentex	Gram+ cocci	S	R	S	I	R	I		
	Rp3	Mugil	Gram+ cocci	S	R	S	S	R	S		
	Rp4	Dentex	Gram+ cocci	S	R	S	S	R	I		
2	Argo1	Bass	Gram- cocci	S	R	S	R	I	S		
	Argo2	Bass	Gram +	S	R	S	R	S	R		
	Argo3	Bream	Gram+ cocci	S	R	S	I	R	I		
3	SF1	Bass	4144525	S	S	S	S		R		
	SF2	Bass	3047524	R	S	S	S	S	R		
	SF3	Bass	3047524	S	S	S	I	S	R		
4	NE1	Bass	3047524	S	S	S	S	S	R		
5	Deep1	Bass	3047524	S	S	S	I	S	R		
6	Ast1	Bass	3040125	S	S	S	S	I		S	
	Ast2	Bass	2200004	S	S	I	S	S	I		
	Ast3	Bream	0002000	S	R	S	I	R	S		
7	Ovr1	Bass	4047520	S	I	I	I	S	R		
	Ovr2	Bass	1047724	S	R	R	R	R	R		
	Ovr3	Bass	2000104	I	R	I	R	R	R		
	Ovr4	Bass	4046505	I	I	I	I	R	R		
	Ovr5	Bass	1047524	S	I	I	I	R	R		
	Ovr6	Bass	3047524	S	S	S	S	S	R		
	Ovr7	Bass	3047524	S	I	I	R	R			
8	Kal1	Bream	Gram+ cocci	S	R	S	S	R	S		
	Kal2	Bass	1047524	S	S	S		I			
	Kal3	Puntazzo	3047524	S	R	I	I	R	R	R	
	Kal4	Bream	6005004	S	S	I	S	R	R	R	R
	Kal5	Puntazzo	6005004	S	I	S	I	S			
	Kal6	Puntazzo	4144525	S	I	I	I	R			
	Kal7	Puntazzo	4154525	S	R	I	I	R	R	R	R
	Kal8	Bass	4146525	S		S	S	R	R	R	R
	Kal9	Puntazzo	3047524	S	S	S	I	I	R	R	R
	Kal10	Bass	3047524	S	S	R	S	S	R	S	R
	Kal11	Bass	3047524	S	S	S	S	S		S	
	Kal12	Bass	3047524	S	S	S	S	S	R	I	R
9	Pl1	Bass	Gram +	S	R	I	R	R	R		
	Pl2	Bass	1047524	S	S	S	S				
	Pl3	Bass	3047524	S	S	S	S	I			
	Pl4	Bass	3047524	S	S	S	S	S	R		

Farm	Strain	Fish Species	API20E profile	OTC	OA	SXT	FR	O129	P	NV	AMP
10	Agr1	Bass	0003004	S	R	S	I	R	S		
	Agr2	Bass	0003004	S	R	S	I	R	S		
11	Petr2	Bass	3047524	S	S	S	S	S	R		
	Petr3	Bass	1047524	S	S	S	S		R		
12	Chios1	Bass	3047524	S	S	S	S	S	R		
13	Nous1	Bass	4347525	S	I	S	I	R		I	
	Nous2	Bass	4146525	S	S	S	S	R		R	
	Nous3	Bass	4146525	S	I	S	I	R		R	
	Nous4	Bass	4114735	S	S	S	I	R			
	Nous5	Bass	6114525								
	Nous6	Bass	4146505	S	I	I	I	R		R	R
	Nous7	Bass	2004004			R					
	Nous8	Bass	2004004	R	R	S	S	R		R	S
14	Fido1	Bream	4154525	S	I	I	I	I	R		
	Fido2	Bream	0044777	S	I	S	I	R	R		
	Fido3	Bream	5144565	S	I	S	I	I	R		
	Fido4	Bream	5046305	R	I	R	I	R	R		
15	Prap1	Bass	1047524	S	S	S	S	S	R		
	Prap2	Bass	4046105	S	I	I	I	R	R		
	Prap3	Bass	3047524	S	S	S	S	R	R		
	Prap4	Bass	3047524	S	S	S	S	I		I	
	Prap5	Bass	3047524	S	S	S	S	I		I	R
16	Eyr1	Bass	Gram+ cocci	S	R	S	S	R	S		
17	Ocean1	Bass	4144525	I	R	I	I	R		R	R
	Ocean2	Bass	5046105	S	I	S	I	R		R	R
	Ocean3	Bass	4047125	S	R	I	R	R		R	
18	Zant1	Bream	7005100	S	S	I	I	R			
	Zant2	Bream	1004124	S	I	S	I	I		R	R
	Zant3	Bream	0004125	S	S	S	S	S	R	I	I
	Zant4	Bream	0004000	R	R	R	R	R		R	R
19	Ler1	Bass	1047524	S	S	S	S	S	R		
20	Myt1	Bass	3047524	S	S	S	I	R	R	R	R
24	AqH1	Bream	4146525	I	R	I	R	R		R	R
	AqH2	Bream	6544164	R	S	R	I	R	R	I	R
	AqH3	Bream	5446025	I	I	I	I	R	R	R	I
21	Makr1	Bass	1003004	I	S	S	S	I	S		
	Makr2	Bass	3047524	S	S	S	S	S	R		
	Makr3	Bass	3042524	S	S	S	S	R	R		

Farm	Strain	Fish Species	API20E profile	OTC	OA	SXT	FR	O129	P	NV	AMP
	Makr4	Bass	3047524	I	S	S	S	I	S		
	Makr5	Bass	3047524	S	S	S	S	S	R	I	
	Makr6	Bass	3047524	S	S	S	S	S	R	I	
	Makr7	Bream	6005004	S	S	S	I	I	R		
	Makr8	Bass	3047524	S	S	S	S	S		R	
	Makr10	Bass	3047524	S	S	S	S	S		I	
22	Kant1	Bass	1003004	I	I	S	S	R	S		
	Kant2	Bass	1007004	I	S	S	S	I			
	Kant3	Bass	3003004	I	R	S	R	R	S		
	Kant4	Bass	0000004	S	S	S	R	R			
	Kant5	Bass	4146525	S	I	S	I	R		R	
	Kant6	Bass	4366525	S	R	S	S	R	R		
	Kant7	Bass	4144525	S	I	S	I	I	R		
	Kant8	Bass	3047524	S	S	S	S	S	R		
	Kant9	Bass	3047524	S	S	S	S	S	R		
	Kant10	Bass	Gram+	I	R	R	R		I		
	Kant11	Bass	Gram+	S	R	S	R	R			
	Kant12	Bass	3047524	S	S	S	S	S			
	Kant13	Bass	Gram+	S	S	S	I	S	R		
	Kant14	Bass	3047524	S	S	S		S			
	Kant15	Bass	3047524	S	S	S	S	S	R		
	Kant16	Bass	4174525	I	R	I	R	R	R		
	Kant17	Bass	1003004	I	I	S	S		I	I	R
	Kant 19	Bass	4144525	S	S	S	S	S	S	S	I
23	Vasni1	Bream	2005024	S	S	S	S	R	I	S	S

3.1.8 Resistant bacterial strains: API 20E profiles

Table 22: Presentation of resistant biochemical profiles of bacterial isolates

Oxolinic Acid	Oxytetracycline	Potentiated Sulphonamides	Furazolidone
1047724	2004004	1047724	1047724
2000104	5046305	5046305	200104
2004004	6544164	6544164	3003004
3003004	0004000	0004000	4047125
4047125			
4366525			
Gram- cocci			
Gram+ cocci			
0002000			
0003004			
0004000			

The API 20E profiles that exhibited resistance to each of the four most commonly used antimicrobials in aquaculture. V.anguillarum 1 phenon 2 exhibited resistance to OA, Pot. Sulphonamides and Furazolidone while Gram+ and Gram- cocci were resistant only to oxolinic acid.

3.1.9 Bacterial Strains: Statistical analysis-Antibiotic sensitivity % & frequency

Table 23, presented the Antibiotic Sensitivity % incidence distribution of the bacterial strains for Oxytetracycline, Oxolinic acid, Potentiated Sulphonamides, Furazolidone, Penicillin, Novobiocin and Ampicillin.

Table 23: Antibiotic Sensitivity frequencies

Oxolinic Acid	**Frequency**	**Ampicillin**	**Frequency**
Susceptible	51	Susceptible	2
Intermediate	21	Intermediate	3
Resistant	26	Resistant	16
Missing	3	Missing	80
	101		*101*
Penicilin	**Frequency**	**Novobiocin**	**Frequency**
Susceptible	13	Susceptible	5
Intermediate	7	Intermediate	10
Resistant	48	Resistant	19
Missing	33	Missing	67
	101		*101*
P. Sulphonamides	**Frequency**	**Furazolidone**	**Frequency**
Susceptible	73	Susceptible	48
Intermediate	20	Intermediate	35
Resistant	7	Resistant	14
Missing	1	Missing	4
	101		*101*
Oxytetracycline	**Frequency**		
Susceptible	80		
Intermediate	14		
Resistant	5		
	101		

3.1.10 *V.anguillarum* 1 API 20E-3047524: Antibiotic sensitivity % & frequency

Table 24: Statistical Analysis of Antibiotic Sensitivity
Frequencies and Percentages of *V.anguillarum* 1 API20E:3047525

	OA		**OTC**		**SXT**		**FR**		**P**	
	Fr	%	Fr	%	Fr	%	Fr	%	Fr	%
Susceptible	28	93.3	28	93.3	27	90.0	23	79.3	13	19.1
Intermediate	1	3.3	1	3.3	2	6.7	5	17.2	7	10.3
Resistant	1	3.3	1	3.3	1	3.3	1	3.4	48	70.6
(missing)	-	-	-	-	-	-	1	-	33	-

Table 24 shows the susceptibility profile of the dominant bacterial isolate (*Vibrio anguillarum* 1 phenon 4b) to five antimicrobial agents. It is clear that this strain exhibited susceptibility to Oxytetracycline, Oxolinic acid, Potentiated Sulphonamides and Furazolidone while showing resistance to Penicillin.

3.1.11 Statistical Analysis (cross tabulation) of antibiogram data in each farm

- In 4 *(16.6%)* farms (Farms 2,10,16,17) only resistant strains to oxolinic acid were isolated while in 9 (37.5%) farms (3,4,5,11,12,,19,20,22,24) only oxolinic acid susceptible strains were isolated.
- Regarding oxytetracycline 14 *(58.3%)* farms demonstrated only susceptible bacterial isolates while in only 1 (4.16%) farm 21 intermediate and 8 resistant strains were isolated. Potentatiated sulfonamides gave similar result.
- In 13 fish farms *(54.1%)* (2,4,5,6,8,9,10,11,12,15,16,19,20,24) only susceptible strains were isolated and in 1 farm (21) *(4.16%)* intermediate and resistant strains were dominant. Furazolidone resistance only analysis indicated that only susceptible strains were reported in 5 farms *(20.8%)* (4,11,12,16,19) while, in 3 farms *(12.5%)* (2,17,21) only resistant strains were isolated.

Concluding the analysis of these data referring to antibiotic resistance profiles to 4 antibiotics of bacteria isolated in each farm, least resistance was reported in farms 4,11,12,and 19 (susceptible strains to 4 antibiotics) while in farms 3,16,20 and 24 strains susceptible to 3 antibiotics were reported. On the other hand, farm 21 provided strains resistant to 3 antibiotics, farms 2 & 17 to 2 antibiotics. Only in farm 21 it is possible to comment that the incidence of only one strain that is resistant to 3 antibiotics could indicate inappropriate use of antibiotics in the farm. In the majority of the farms bacterial strains exhibited all the range from sensitivity to intermediate resistance, as well as resistance to antimicrobial agents tested.

Table 25: Statistical Analysis (Cross tabulation) of Antibiogram data in each fish farm

	Oxolinic Acid	Oxytetracycline	Potentiated Sulphonamides	Furazolidone
Farms with ONLY Susceptible Bacterial strains (%)	3,4,5,11,12, 19,20,22,24 (37.5%)	2,4,5,6,8,9,10, 11,12,15,16,19, 20,24 (58.3%)	1,2,3,4,5,10, 11,12,16,19,20, 22,24 (54.16%)	4,11,12,16,19 (20.83%)
Farms with only Resistant or Intermediate and resistant strains (%)	2,10,16,17 (16.6%)	21 (4.1%)	21 (4.1%)	2,17,19 (12.5%)

3.1.12 Statistical Analysis (% & frequency) of multiresistant bacterial strains

Table 26: Statistical Analysis Frequency of multi-resistant bacterial strains

Value	Frequency	Value	Frequency
1111	5	0200	5
1212	2	0201	5
1222	3	0202	3
2121	1	0211	2
2222	1	0212	2
0000	1	0222	1
0001	31	1000	4
0002	7	1100	2
0010	1	1202	1
0011	2	1202	1
0020	1	1211	1
0101	9	2000	1
0111	6	2021	1
0112	1	2200	101

The parameter Value on the above table refers to a four-digit figure. Number 2 is used to demonstrate Resistance to antibiotics, 1 refers to strains of intermediate resistance and 0 is used to demonstrate susceptibility to antimicrobials. In the four digit numbers the first refers to resistance to Oxytetracycline, the second to Oxolinic acid, the third to Potentiated Sulphonamides and the last to Furazolidone. Profile 0000 for example

means susceptibility to all four antimicrobials, 1111 intermediate resistance to all and 2222 resistance to all antimicrobials. Resistance to four antimicrobial agents was exhibited by 1% of the strains, intermediate resistance (1111 profile) was present in 2% and susceptibility to all four was present in 30.7% of the bacterial strains. Sixty nine point three percent (69.3%) of the strains exhibited intermediate resistance and / or resistance to at least one antimicrobial agent. Twelve percent (12%) of the strains were resistant to two antimicrobial agents. Finally in 74% of the strains same pattern of resistance was evident for both Oxolinic acid and Furazolidone.

3.1.13 Statistical analysis of % incidence of MIC values to OA and OTC

Table 27: Statistical Analysis of % Incidence of MIC values to Oxolinic acid and Oxytetracycline

Value	OA	OTC
M.I.C (µg/ml)	Frequency	Frequency
0.075	2	1
0.15	23	6
0.3	20	38
0.625	18	25
1.25	6	11
2.5	2	4
5	6	2
10	3	2
20	0	2
40	3	2
80	7	1
160	0	1

Figure 17: Presentation of the frequency M.I.C value incidence of all bacterial isolates to Oxolinic acid and Oxytetracycline

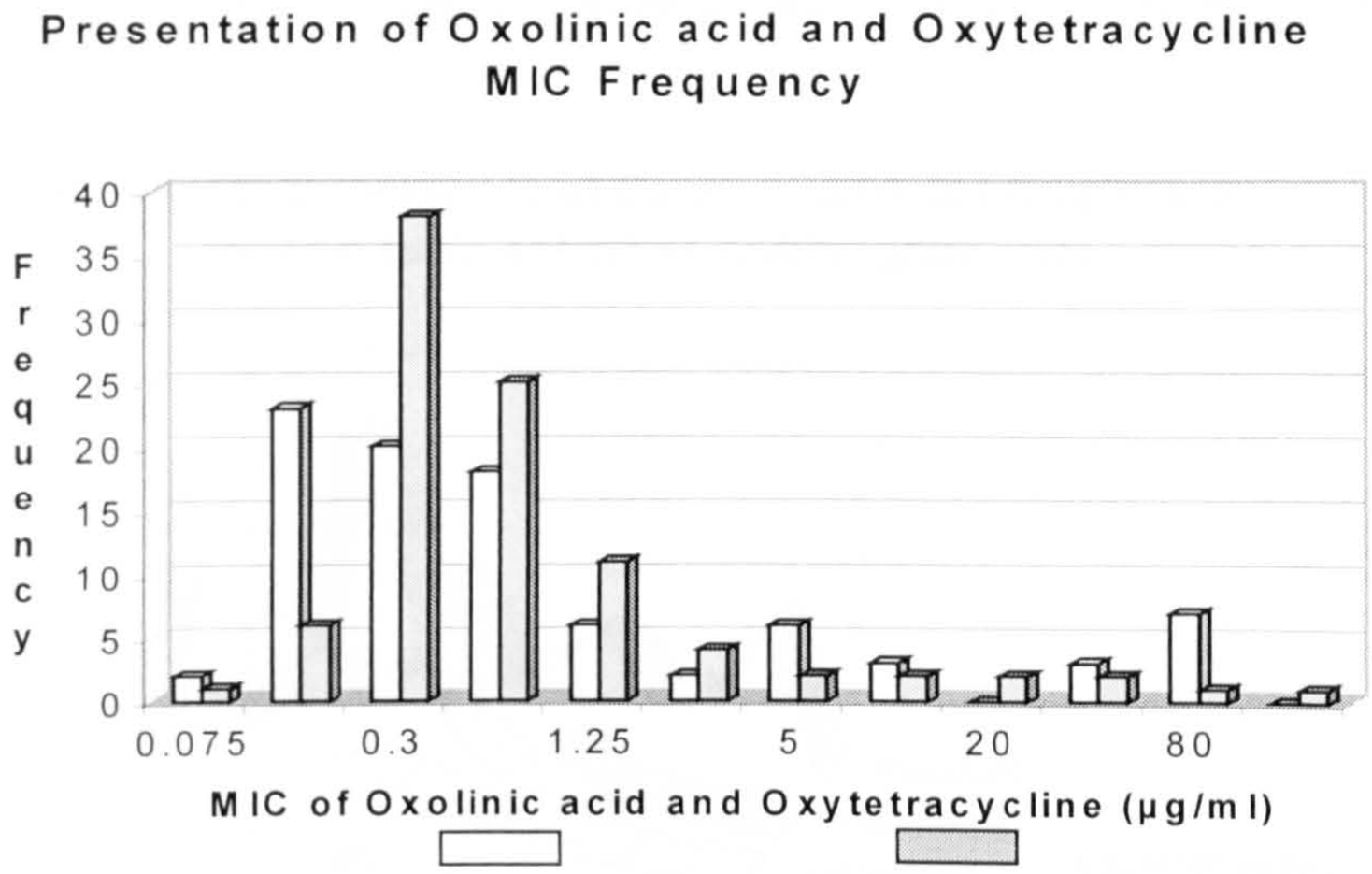


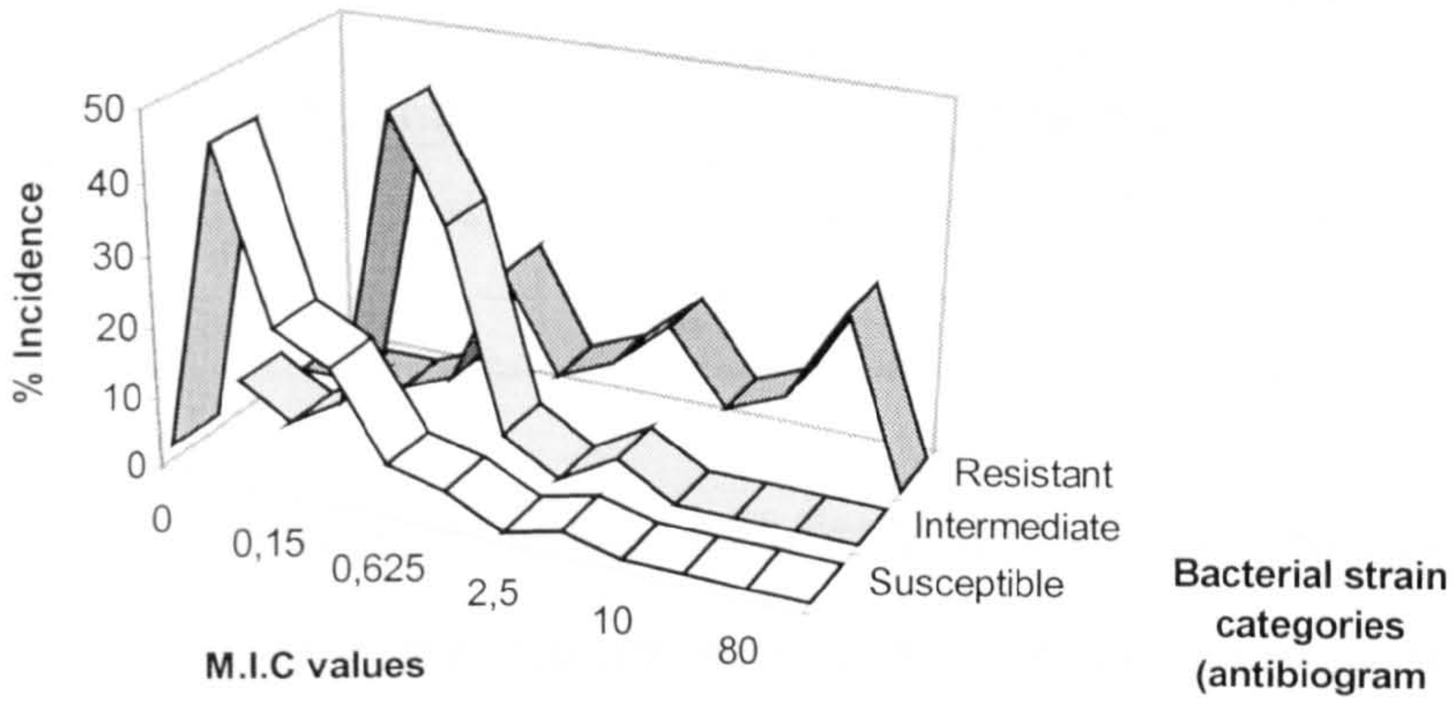
Table 27, and Figure 17 demonstrate the statistical analysis and the graphical presentation of M.I.C value (ppm) distribution for all bacterial isolates regarding Oxolinic acid and Oxytetracycline.

Table 28: Statistical Analysis of Oxolinic acid and Oxytetracycline MIC values in relation to Quantitative Antibiogram data

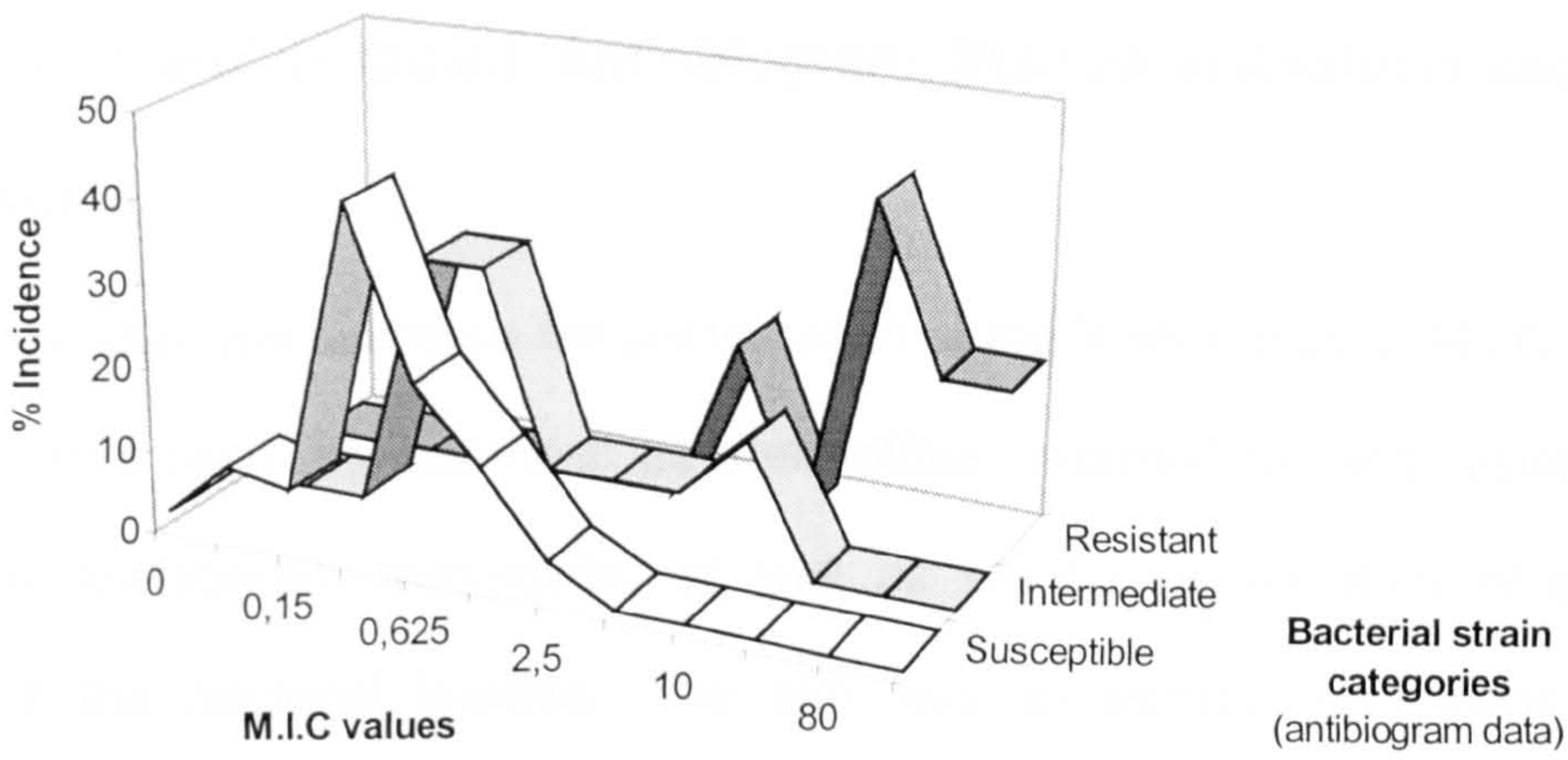
	Oxytetracycline			Oxolinic Acid		
mic	**Susceptible**	**Intermediate**	**Resistant**	**Susceptible**	**Intermediate**	**Resistant**
0	1.4	0	0	2	4.8	0
0.075	8.5	0	0	46	0	0
0.15	7	0	0	22	4.8	0
0.3	42.3	30.8	0	18	47.6	3.4
0.625	22.5	30.8	0	6	33.3	20.7
1.25	14.1	7.7	0	4	4.8	6.9
2.5	4.2	7.7	0	0	0	10.3
5	0	7.7	20	2	4.8	17.2
10	0	15.4	0	0	0	6.9
40	0	0	40	0	0	10.3
80	0	0	20	0	0	24.1
160	0	0	20	0	0	0

Figure 18 & Figure 19: Representation of the correlation between Quantitative Antibiogram data and M.I.C value of the bacterial isolates to Oxolinic acid and Oxytetracycline

Figure 18: % Incidence of Oxolinic acid M.I.C values in relation to Quantitative Antibiogram Data



Fiure 19: % Incidence of Oxytetracycline M.I.C values in relation to Quantitative Antibiogram Data



3.1.14 Statistical Analysis of OA/OTC MIC vs Quantitative Antibiogram Data

The statistical analysis of the correlation between Quantitative Antibiogram data and M.I.C values for Oxolinic acid and Oxytetracycline was used in order to establish M.I.C breakpoints in relation to the categories obtained from the Quantitative Antibiogram. The condition in order to establish M.I.C breakpoints in this study was the following: For each of the categories described in the Quantitative Antibiogram, M.I.C breakpoint was considered the M.I.C concentration under which at least 75% of the bacterial isolates of

this category belonged in. Employment of this condition made possible to consider the following M.I.C breakpoints for Oxolinic acid and Oxytetracycline.

Table 29: M.I.C breakpoints for Oxolinic acid and Oxytetracycline.

	Oxolinic acid		**Oxytetracycline**	
	M.I.C breakpoints	**% Incidence**	**M.I.C breakpoints**	**% Incidence**
Susceptible	< 0.3	88 %	< 0.625	81.7 %
Intermediate	0.15 - 1.25	85.7 %	0.15 - 2.5	77 %
Resistant	> 1.25	75.7 %	> 2.5	100 %

Strains of intermediate resistance considered belonging in a grey area and regarding the extrapolation of % incidence the presence of strains of intermediate resistance with M.I.C that ranged from 0 to 1.25 (Oxolinic acid) was taken under consideration.

3.1.15 OA/OTC MIC vs Quant. Antiobiogram: Statistical Analysis each fish species

Statistical analysis was based on the presentation of the % incidence of M.I.C values for strains that had been characterised as susceptible, intermediate and resistant using Quantitative Antibiogram techniques, as well as on the presentation of the actual numbers of the bacterial isolates. The aim was to deduct correlations between Antibiogram data and M.I.C values for each antibiotic and possibly for each fish species. The interpretation of the actual values seemed to be more effective tool in order to estimate M.I.C breakpoints.

As M.I.C breakpoint for each antibiotic and each bacterial strain group (susceptible / of intermediate resistance / resistant) was defined the Minimum inhibitory concentration, which inhibited the growth of at least 80% of, the bacterial isolates of this group.

3.1.15.1 Sea Bass

In sea bass as it is described in Table 29, Oxolinic acid M.I.C breakpoints were:

Susceptible: < 0.3 ppm

Intermediate: 0.3 - 0.625 ppm

Resistant: > 0.625 ppm

while the Oxytetracycline M.I.C breakpoints were considered the following:

Susceptible: < 0.625 ppm

Intermediate: 0.625 - 5 ppm and

Resistant: > 5 ppm.

Table 30: Correlation of Oxolinic acid and Oxytetracycline M.I.C data and Antibiogram data for Sea bass

Sea bass - Frequency %						
	Oxolinic Acid			**Oxytetracycline**		
MIC	*Susceptible*	*Intermediate*	*Resistant*	*Susceptible*	*Intermediate*	*Resistant*
0.075	100	0	0	100	0	0
0.15	91.7	8.3	0	100	0	0
0.3	57.1	35.7	7.1	86.2	13.8	0
0.625	11.1	44.4	44.4	82.4	17.6	0
1.25	50	25	25	87.5	12.5	0
2.5	0	0	100	66.7	33.3	0
5	0	33.3	66.7	0	100	0
10	33.3	0	66.7	0	100	0
40	0	0	100	100	0	0
80	0	0	100	0	0	100
160				0	0	100
Number of Strains						
	Oxolinic Acid			**Oxytetracycline**		
MIC	*Susceptible*	*Intermediate*	*Resistant*	*Susceptible*	*Intermediate*	*Resistant*
0.075	19	0	0	6	0	0
0.15	11	1	0	3	0	0
0.3	8	5	1	25	4	0
0.625	1	4	4	14	3	0
1.25	2	1	1	7	1	0
2.5	0	0	1	2	1	0
5	0	1	3	0	1	0
10	1	0	2	0	3	0
40	0	0	3	1	0	0
80	0	0	3	0	0	1
160				0	0	1

Figures 20, 21, 22, 23: Correlation of Oxolinic acid and Oxytetracyline M.I.C data and Antibiogram data for Sea bass

Figure 20: Sea bass: % Incidence of Oxolinic and M.I.C values in relation to Quantitative Antibiogram Data

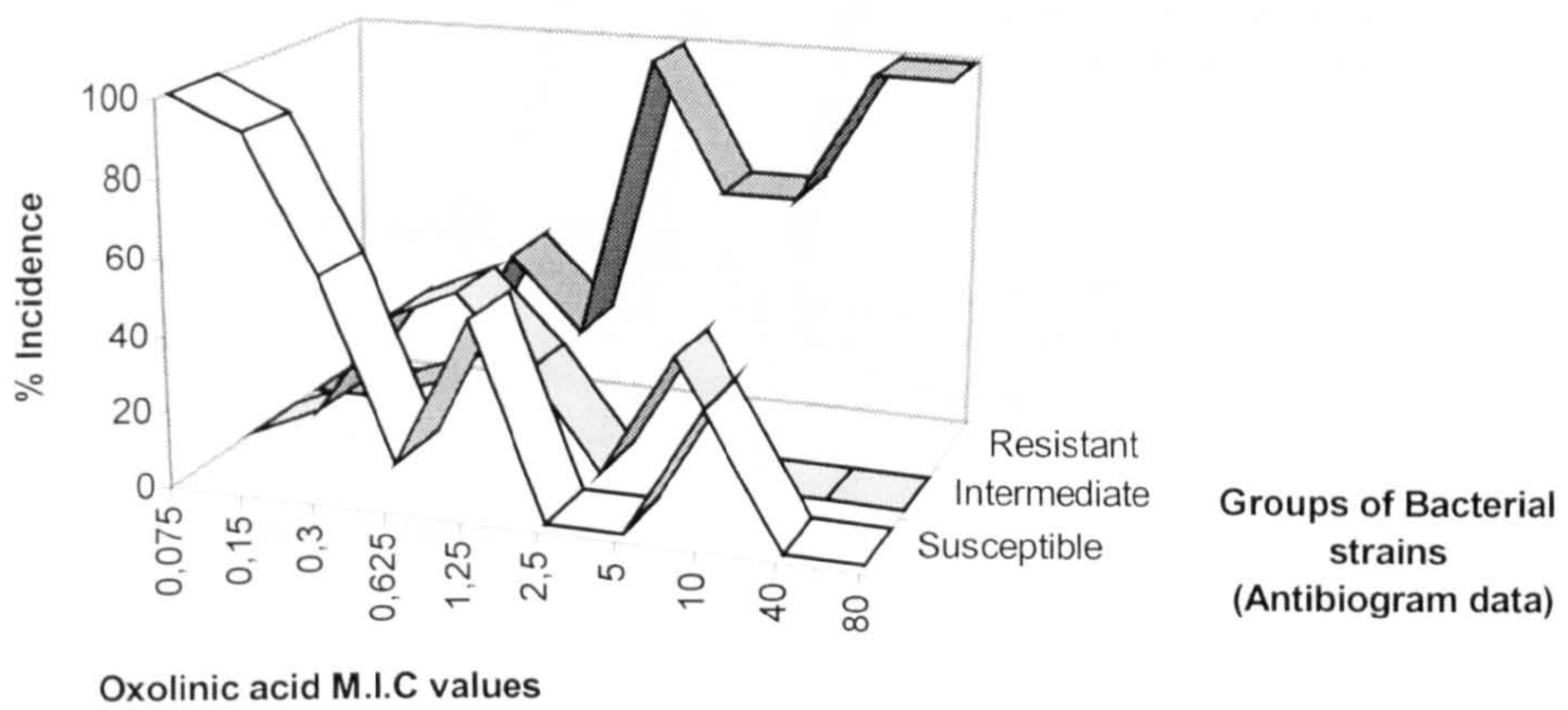


Figure 21: Sea bass : Correlation of Oxolinic acid M.I.C data and Quantitative Antibiogram Data

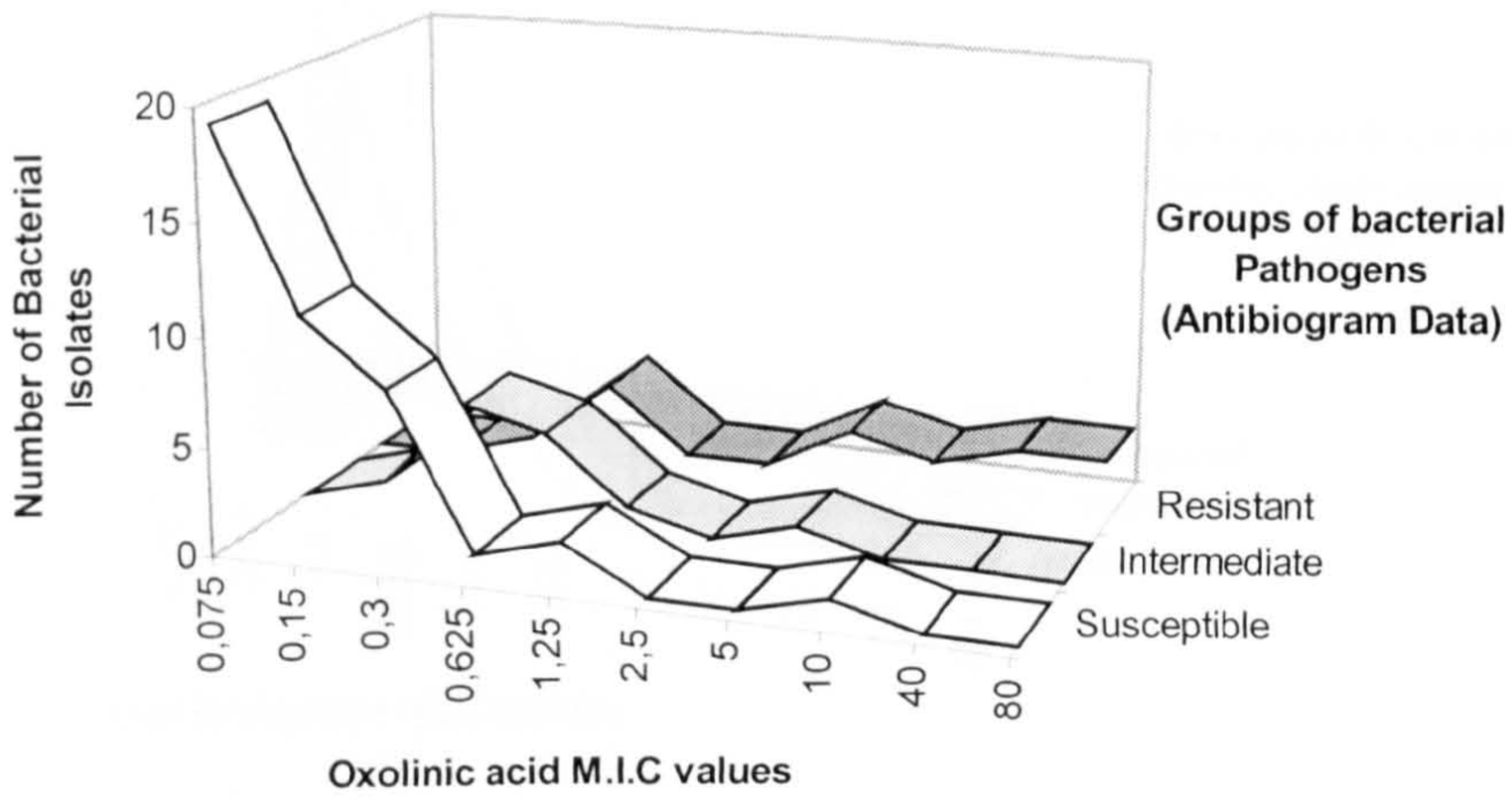


Figure 22: Sea bass: % Incidence of Oxytetracycline M.I.C values in relation to Quantitative Antibiogram Data

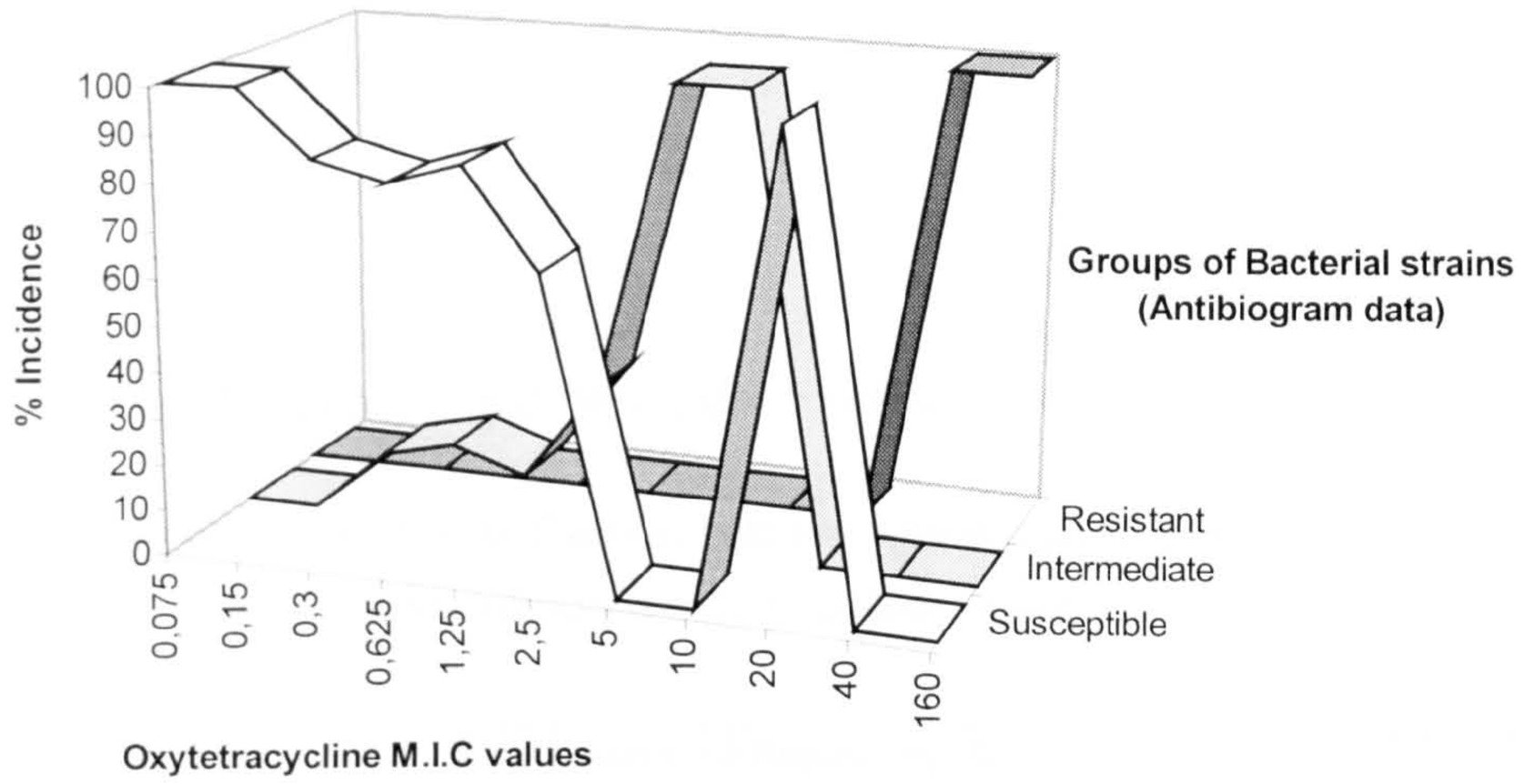
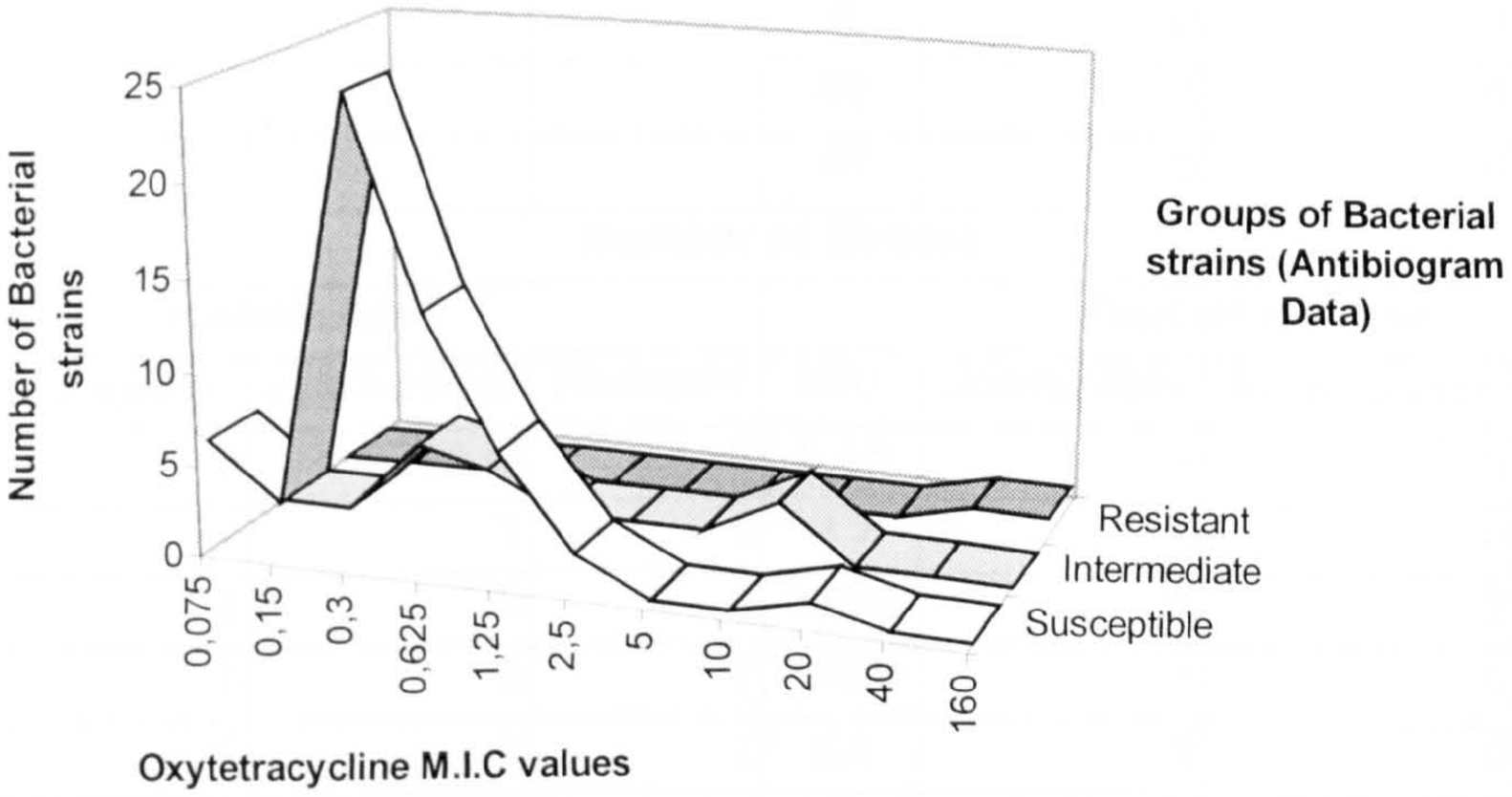


Figure 23: Sea bass: Correlation of Oxytetracycline M.I.C data and Quantitative Antibiogram Data



3.1.15.2 Sea Bream

In sea bream O.A M.I.C as well as OTC M.I.C breakpoint values were:

Susceptible: < 0.625 ppm

Intermediate: -

Resistant: > 0.625 ppm

In this species M.I.C breakpoint was very clearly defined.

Table 31: Correlation of Oxolinic acid and Oxytetracycline M.I.C data and Antibiogram data for Sea bream

Sea Bream - Frequency %							
Oxolinic Acid				**Oxytetracycline**			
MIC	*Susceptible*	*Intermediate*	*Resistant*	**MIC**	*Susceptible*	*Intermediate*	*Resistant*
0.075	100	0	0	**0.15**	100	0	0
0.3	25	75	0	**0.3**	100	0	0
0.625	33.3	50	16.7	**0.625**	60	40	0
5	0	0	100	**1.250**	100	0	0
80	0	0	100	**2.5**	100	0	0
				5	0	0	100
				40	0	0	100
				80	0	0	100
Number of Strains							
Oxolinic Acid				**Oxytetracycline**			
MIC	*Susceptible*	*Intermediate*	*Resistant*	**MIC**	*Susceptible*	*Intermediate*	*Resistant*
0.075	3	0	0	**0.15**	1	0	0
0.3	1	3	0	**0.3**	6	0	0
0.625	2	3	1	**0.625**	3	2	0
5	0	0	2	**1.250**	1	0	0
80	0	0	2	**2.5**	1	0	0
				5	0	0	1
				40	0	0	1
				80	0	0	1

Figures 24, 25, 26, 27: Correlation of Oxolinic acid and Oxytetracyline M.I.C data and Antibiogram data for Sea bream

Figure 24: Sea bream: % Incidence of Oxolinic acid M.I.C values in relation to Quantitative Antibiogram data

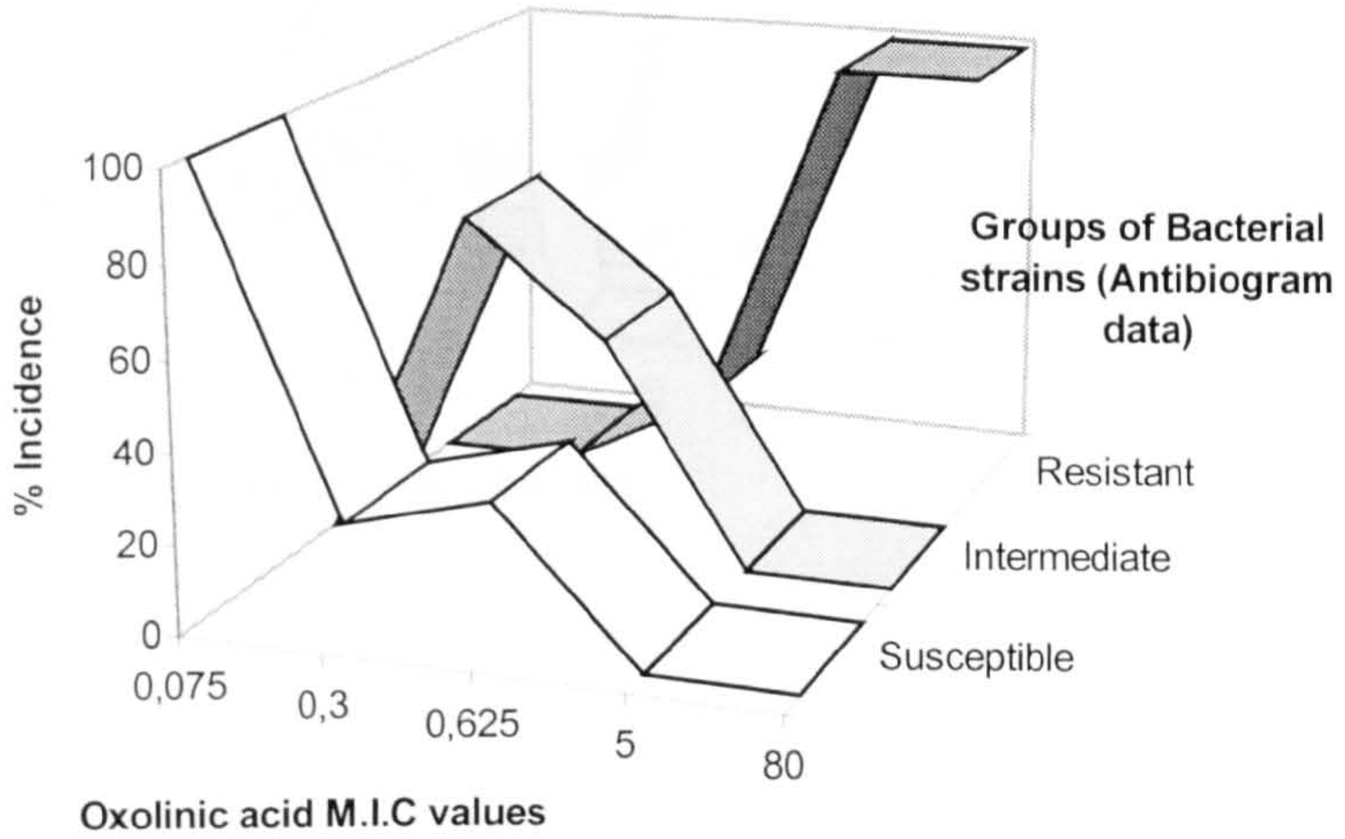


Figure 25: Sea bream: Correlation of Oxolinic acid M.I.C values and Quantitative Antibiogram data

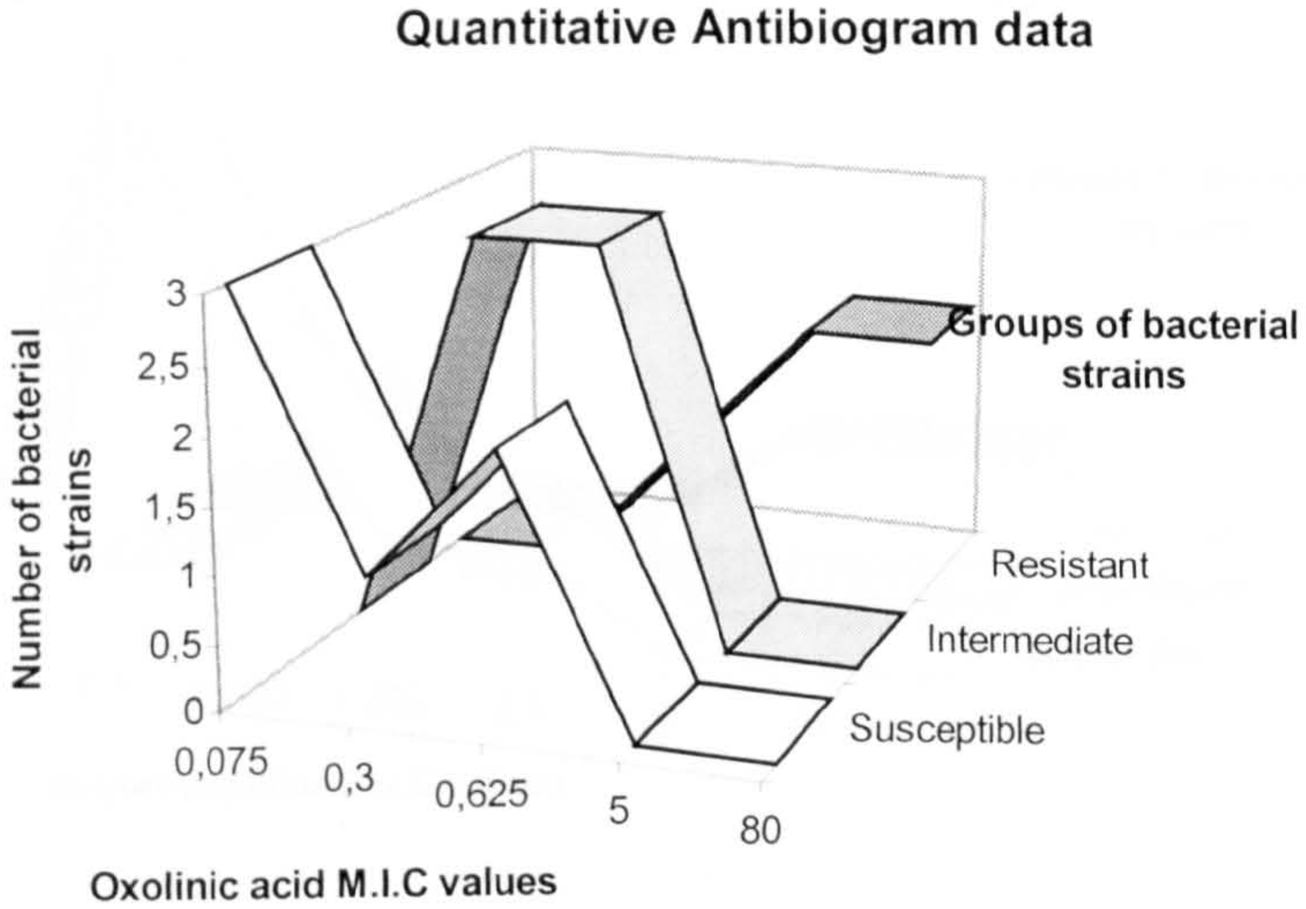


Figure 26: Sea bream: % Incidence of Oxytetracycline M.I.C values in relation to Quantitative Antibiogram data

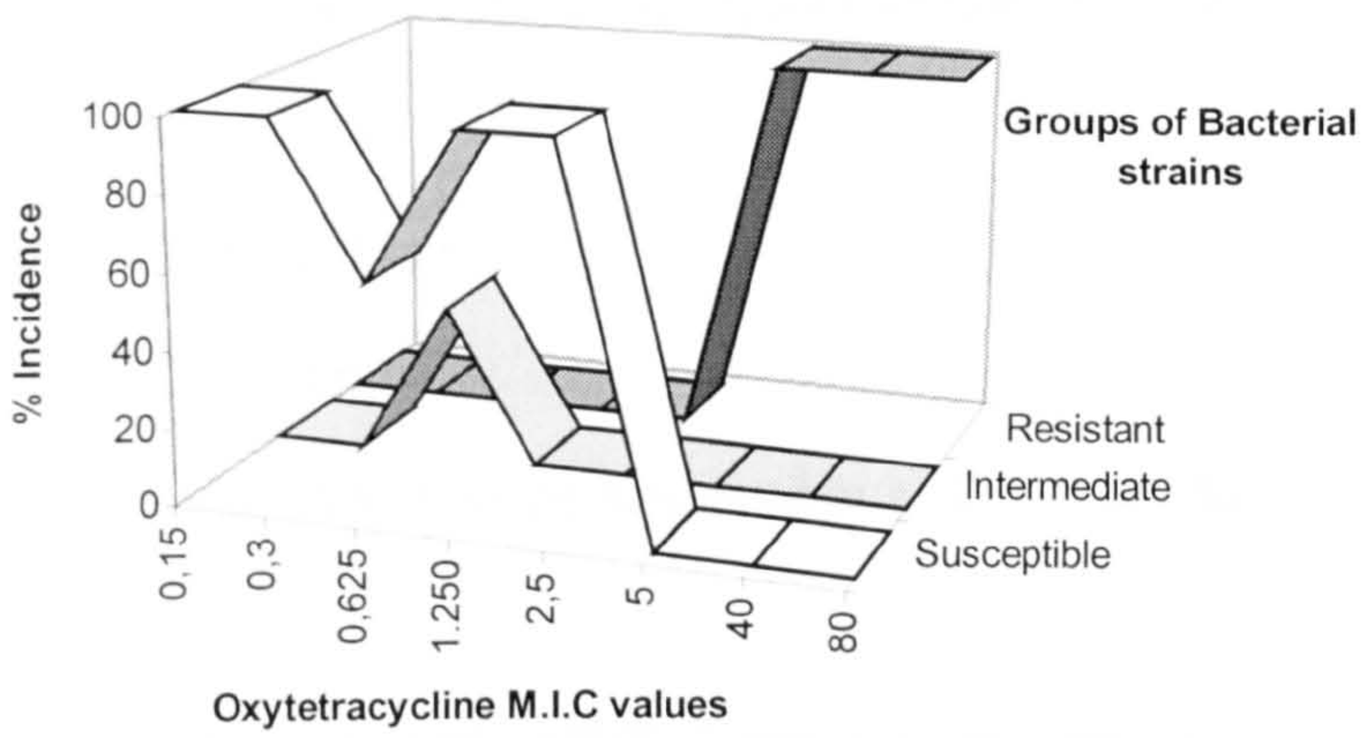
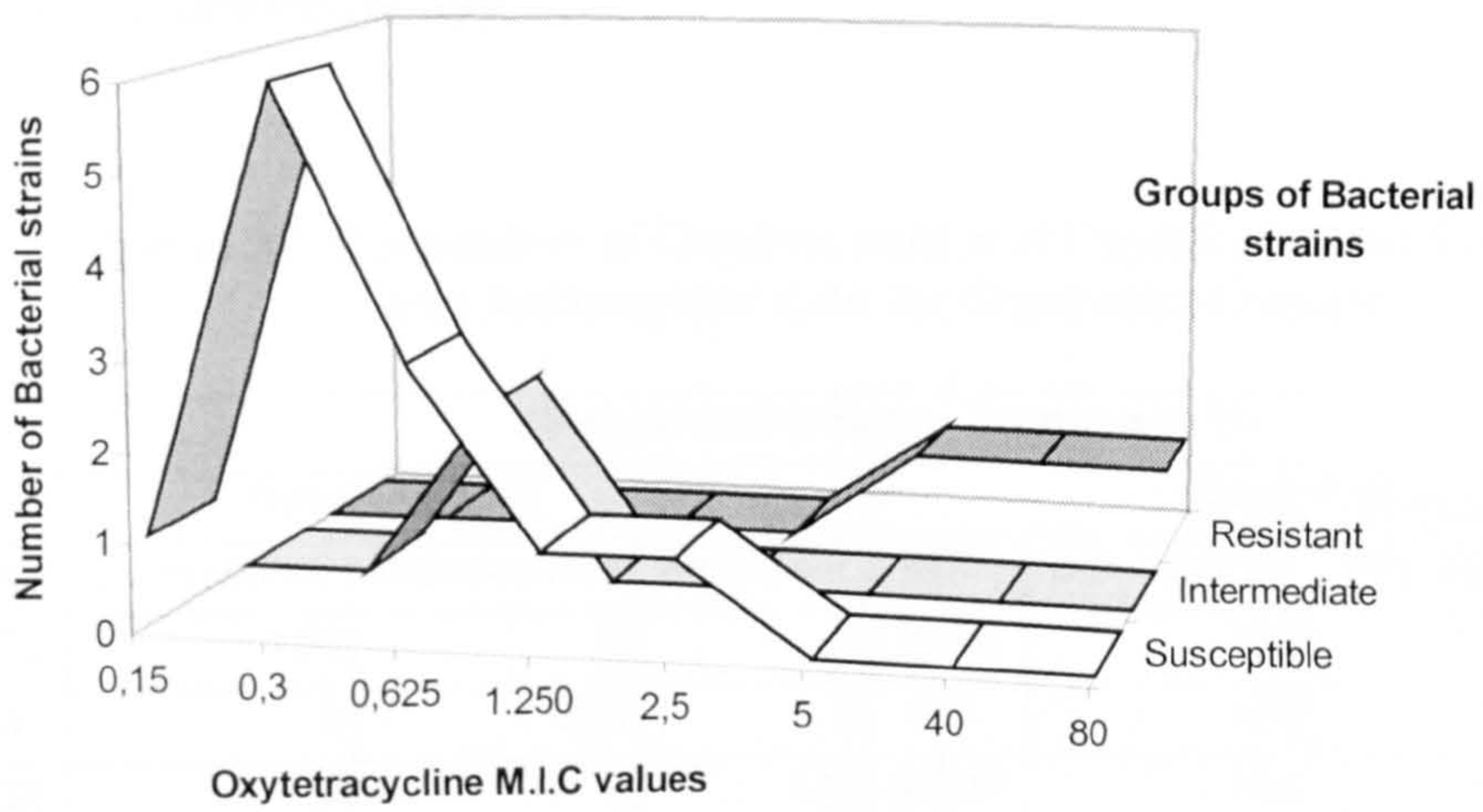


Figure 27: Sea bream: Correlation of Oxytetracycline M.I.C values and Quantitative Antibiogram data



3.1.15.3 Sharpsnout bream

In sharpsnout bream the O.A M.I.C breakpiont values were defined as:

Susceptible: < 0.075 ppm

Intermediate: 0.075 - 0.3 ppm

Resistant: > 0.625 ppm

while all the bacterial strains were susceptible to OTC with M.I.C values less than 1.25 ppm.

In gray mullet bacterial strains (100% Gram + cocci) all were resistant to Oxolinic acid (M.I.C > 80 ppm) and susceptible to Oxytetracycline (M.I.C < 0.15 ppm). Finally in the case of Common dentex isolates (100% Gram + cocci) like the previous species all were resistant to O.A (M.I.C > 2.5) and susceptible to Oxytetracycline (M.I.C < 0.625 ppm). OA resistance in these cases most likely refers to innate resistance rather that a result of antimicrobial chemotherapy.

Table 32: Correlation of Oxolinic acid and Oxytetracycline M.I.C data and Antibiogram data for Sharpsnout bream

Sharpsnout bream - Frequency %							
Oxolinic Acid				**Oxytetracycline**			
MIC	*Susceptible*	*Intermediate*	*Resistant*	**MIC**	*Susceptible*	*Intermediate*	*Resistant*
0.075	100	0	0	**0.15**	100	0	0
0.3	0	100	0	**0.3**	100	0	0
0.625	0	0	100	**0.625**	100	0	0
1.25	0	0	100	**1.250**	100	0	0
Number of Strains							
Oxolinic Acid				**Oxytetracycline**			
MIC	*Susceptible*	*Intermediate*	*Resistant*	**MIC**	*Susceptible*	*Intermediate*	*Resistant*
0.075	1	0	0	**0.15**	1	0	0
0.3	0	2	0	**0.3**	2	0	0
0.625	0	0	1	**0.625**	1	0	0
1.25	0	0	1	**1.250**	1	0	0

Figures: 28, 29, 30, 31: Correlation of Oxolinic acid and Oxytetracycline M.I.C data and Antibiogram data for Sharpsnout bream

Figure 28: Sharpsnout bream: % Incidence of Oxolinic acid M.I.C values in relation to Quantitative Antibiogram data

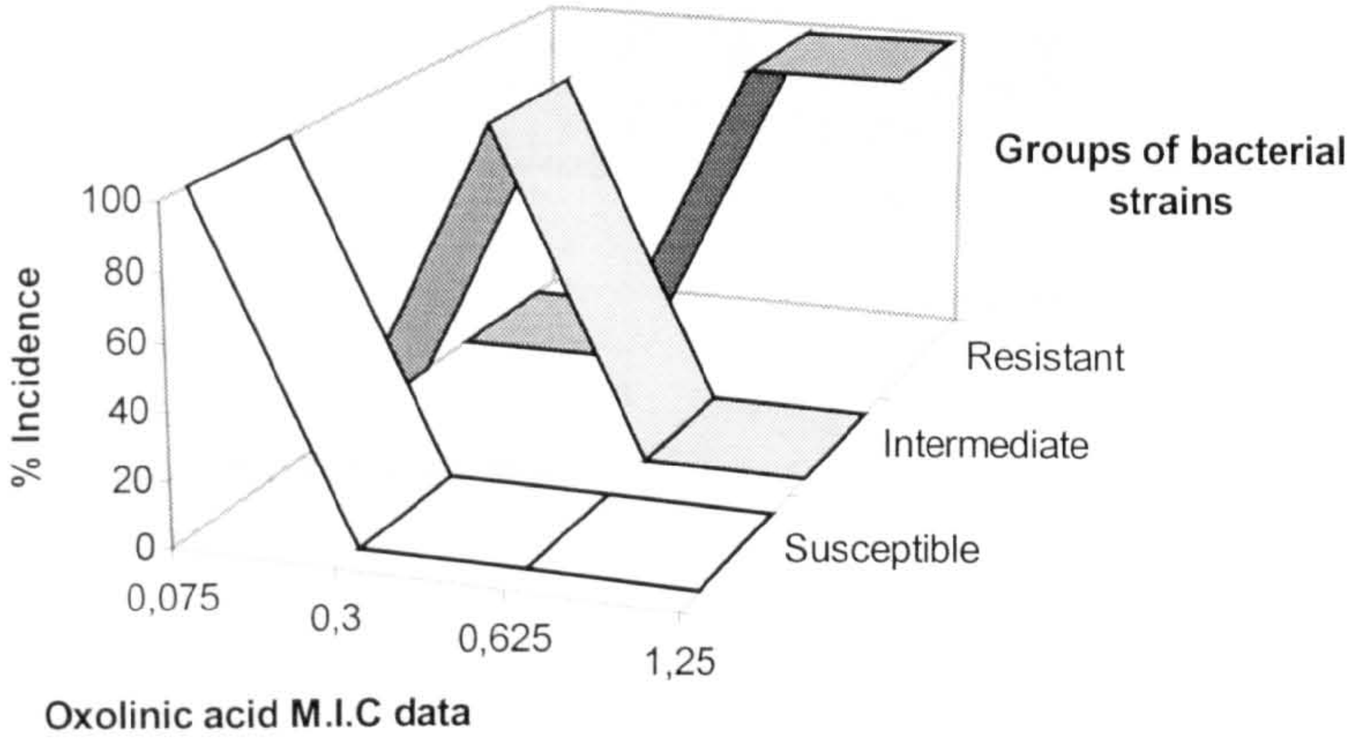


Figure 29: Sharpsnout bream: Correlation of Oxolinic acid M.I.C data and Quantitative Antibiogram data

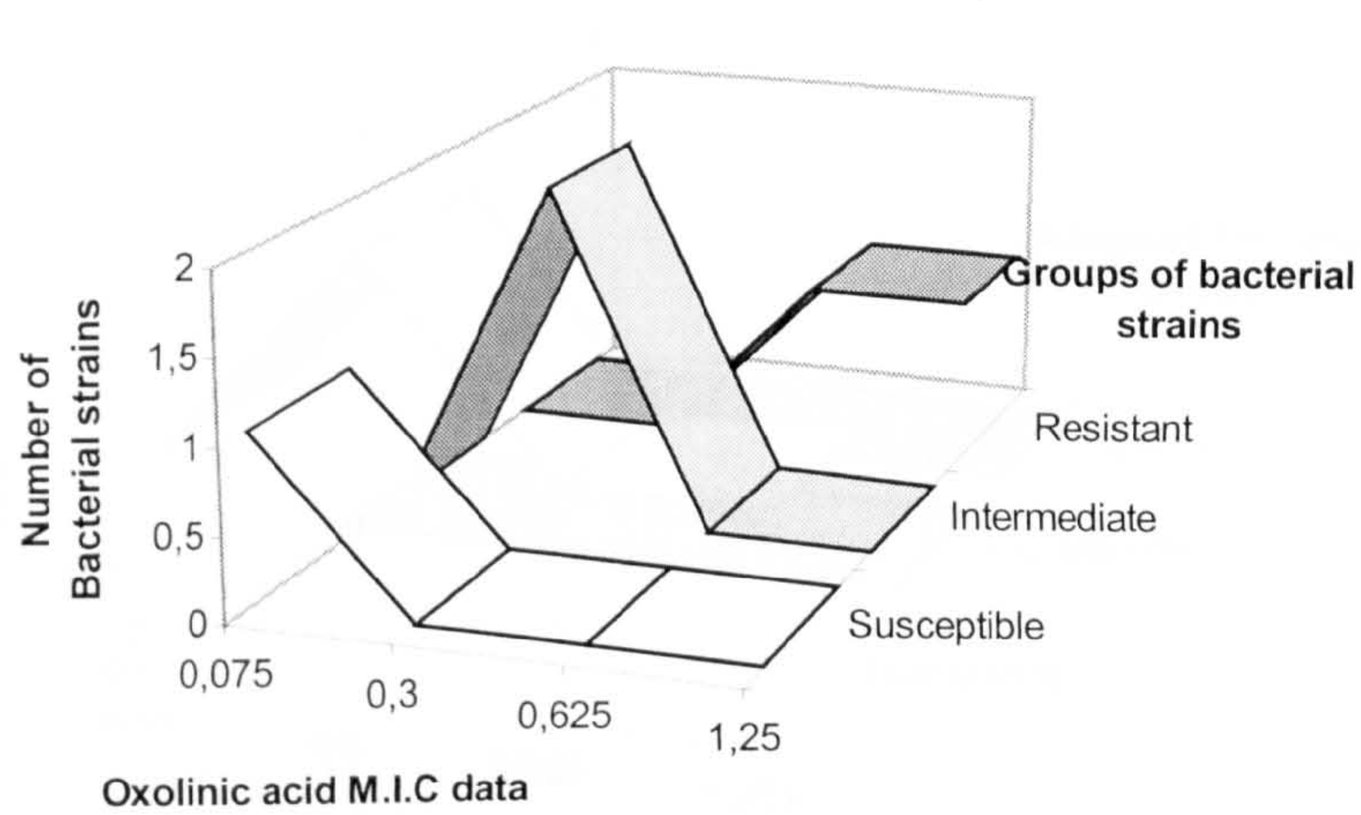


Figure 30: Sharpsnout bream: % Incidence of Oxytetracycline M.I.C values in relation to Quantitative Antibiogram data

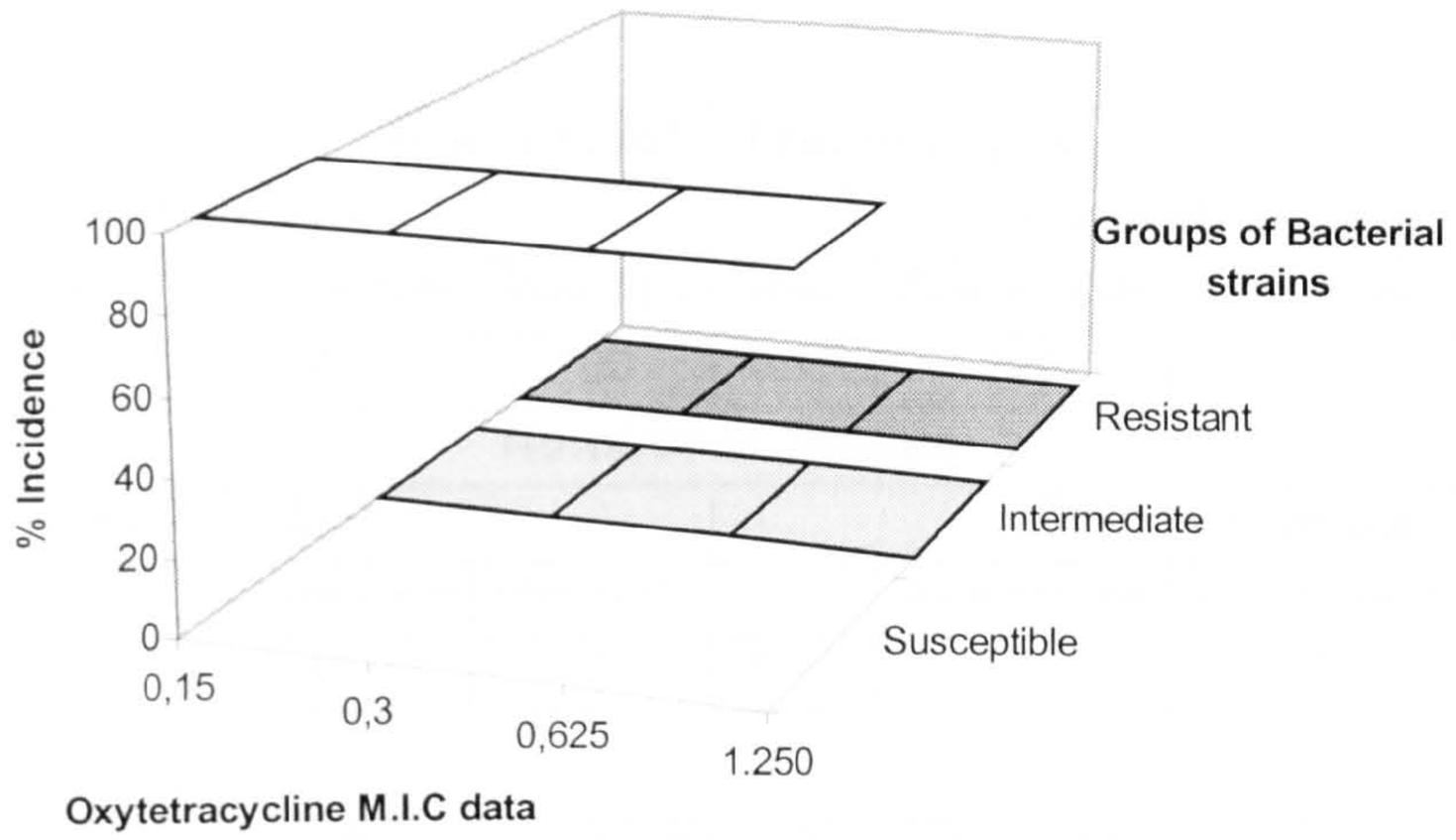
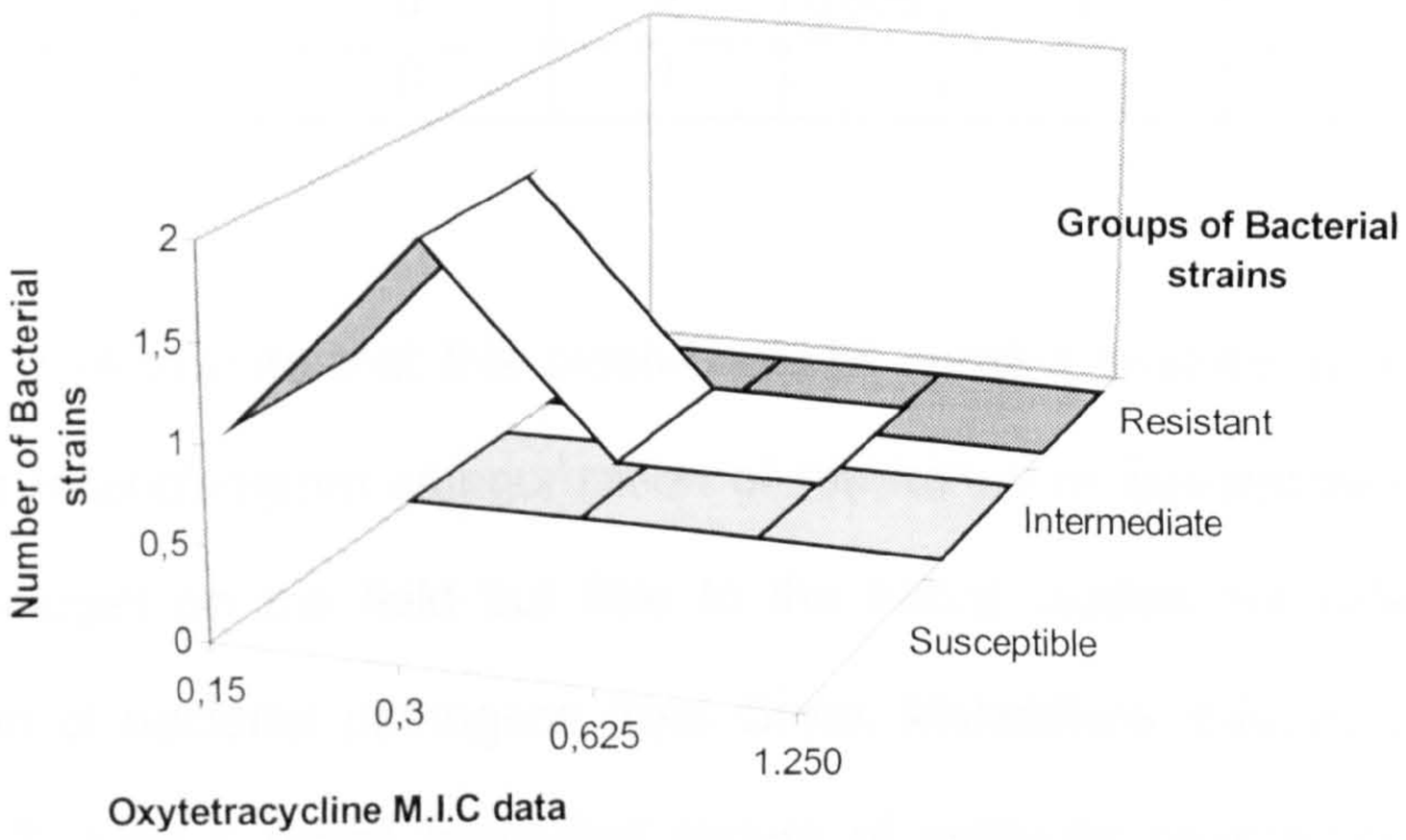


Figure 31: Sharpsnout bream: Correlation of Oxytetracycline M.I.C data and Quantitative Antibiogram data



3.1.15.4 Grey mullet and Common dentex

Table 33: Correlation of Oxolinic acid and Oxytetracycline M.I.C data and Antibiogram data for Grey mullet and Common dentex

Grey mullet - Frequency %							
Oxolinic Acid				**Oxytetracycline**			
mic	*Susceptible*	*Intermediate*	*Resistant*	**mic**	*Susceptible*	*Intermediate*	*Resistant*
80	0	0	100	0.15	100	0	0
Number of Strains							
Oxolinic Acid				**Oxytetracycline**			
mic	*Susceptible*	*Intermediate*	*Resistant*		*Susceptible*	*Intermediate*	*Resistant*
80	0	0	2	0.15	2	0	0

Common dentex - Frequency %							
Oxolinic Acid				**Oxytetracycline**			
mic	*Susceptible*	*Intermediate*	*Resistant*	**mic**	*Susceptible*	*Intermediate*	*Resistant*
2.5	0	0	100	0.625	100	0	0
5	0	0	100				
Number of Strains							
Oxolinic Acid				**Oxytetracycline**			
mic	*Susceptible*	*Intermediate*	*Resistant*		*Susceptible*	*Intermediate*	*Resistant*
2.5	0	0	2	0.625	3	0	0
5	0	0	1				

It is important to note that this cross-bacterial species analysis of MIC and the cross reference to antibiogram categorization of resistance or susceptibility serves more the fish pathologist on the field but little to the future studies but referring to a certain population of bacterial pathogens from Greek Mariculture industry and for the period 1994-1997 gives a broad quantified picture of antibiotic resistance level of the most clinically relevant bacterial pathogens. These data are much more relevant for sea bass which contributed the majority of the strains and is subject to the majority of pathological conditions and therefore the bulk of antibiotic application.

However a specific analysis of MIC analysis is given for the major bacterial pathogen *Vibrio anguillarum* in order to have specific data that could be compared with future epidemiological studies that will attempt to quantify bacterial resistance in Mediterranean Mariculture.

3.1.15.5 *Vibrio anguillarum* MIC-Quantitative Antibiogram correlation

Tables 34: Correlation of OA / OTC Quantitative antibiogram data for *V.anguillarum*

V.anguillarum % distribution	**Oxytetracycline**	**Oxolinic acid**
6-15 mm Resistant	3%	6%
15-25 mm Intermediate	6%	3%
>25 mm Susceptible	91%	91%

For each of the categories described in the Quantitative Antibiogram, we described the % frequencies and in order to establish $M.I.C_{OA}$ $M.I.C_{OTC}$ breakpoints for *Vibrio anguillarum* we employed concentration under which at least 80% of the bacterial isolates of this category belonged in. Employment of this condition made possible to consider a level of 0,3 ppm as $M.I.C_{OA}$ and 0,625 ppm as $M.I.C_{OTC}$ for *V. anguillarum* (Table 35).

Tables 35: Correlation of MIC and OA / OTC data for *V.anguillarum*

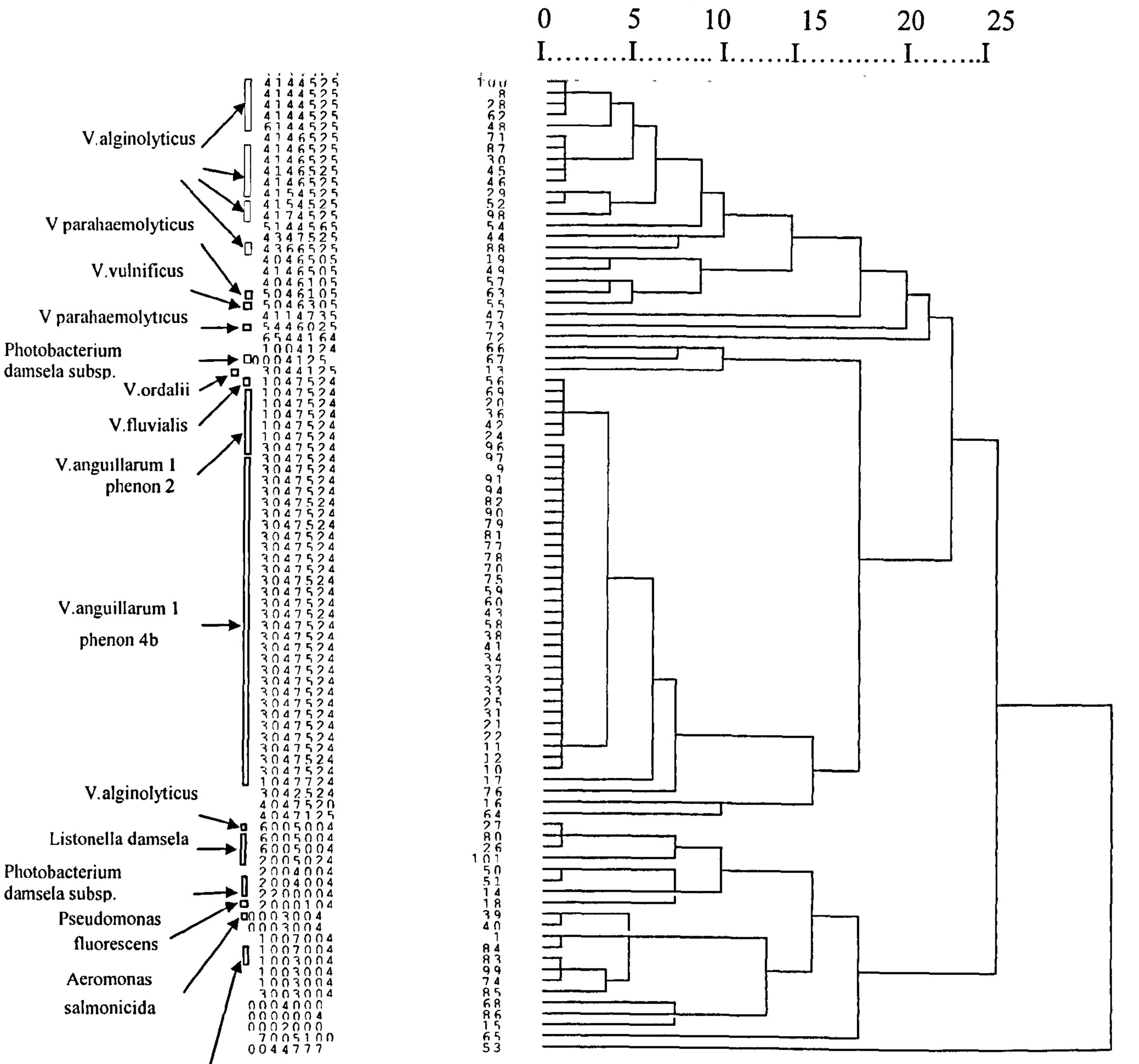
OXYTETRACYCLINE MIC - Vibrio anguillarum (32 strains)			
MIC VALUE	FREQUENCY	PERCENT	CUMMULATIVE PERCENT
0.075	4	12,5%	12,5%
0.15	3	9,4%	21,9%
0.3	11	34,4%	56,3%
0.6	9	28,1%	84,4%
1.25	4	12,5%	96,9%
160	1	3,1%	100,0%
	32	100,0%	

OXOLINIC ACID MIC - Vibrio anguillarum (32 strains)			
MIC VALUE	FREQUENCY	PERCENT	CUMMULATIVE PERCENT
0.075	18	56,3%	56,3%
0.15	6	18,8%	75,0%
0.3	3	9,4%	84,4%
0.6	1	3,1%	87,5%
1.25	3	9,4%	96,9%
80	1	3,1%	100,0%
	32	100,0%	

3.1.16 API 20E profiles: Cluster analysis-Eucleidian distances dendrogram

Different phenotypes of *Vibrio alginolyticus* formed a distinct cluster, while *V.anguillarum* phenon 4b and phenon 2 (Crisez et al 1991) formed close related clusters. Statistical interpretation of API 20E data according to the NORDIC manual and employing the ALO test (ADH / LDC / OX) revealed that Alo test could be used in order to separate *V.anguillarum* 1 phenon 4b and V.anguillarum 1 phenon 2 along with *Listonella damsela* and *V.alginolyticus* different phenotypes. It was not however possible to be employed for the discrimination between *V.parahaemolyticus, V.vulnificus* and *V.alginolyticus* which formed in this type of statistical analysis the same cluster (Figure 32).

Figure 32: Hierarchical Cluster Analysis
Dendrogram using Average Linkage (between groups)



3.2 OXOLINIC ACID KINETICS

3.2.1 Development of Bioassay method for OA detection

Interpretation of Bioassay results consisted of the presentation of the raw data and the statistical analysis of Oxolinic acid residue detection method. In the first step Table 37 presented the zones of inhibition measured in triplicate in three bioassay plates for the detection of method accuracy in the detection of standard dilutions of Oxolinic acid. Each bioassay plate is presented as trial 1,2 and 3 while details of the method like pH and inolulum size are presented. The detection limit of the method detecting standard dilutions of O.A was 125 ppb O.A. The statistical analysis of these trials was presented in the following table (Table 36). In trial2 the concentration of 32ppm pf O.A was not considered because of odd readings.

Table 36: Oxolinic acid residues detection bioassay method

Standard dilutions – Statistical analysis	
Trial 1	**R-squared:** 98.63%, **slope:** 9.2, **Intercept:** 23.05
Trial 2 *	**R-squared:**98.69% , **slope:** 11.01, **Intercept:** 20.71
Trial 3	**R-squared:** 96.41%, **slope:** 11.65,**Intercept:** 20.17

*(*Concentration 32ppm O.A was not considered)*

Table 37: Oxolinic acid residues detection bioassay method Standard dilutions

Bioassay trial 1 - oxolinic acid pH=9,45 sample:50µl							
number	O.A concentration (ppm)	Log O.A concentration	Zone A	Zone B	Zone C	Average	Median
1	32	1,505	35,69	36,66	35,93	36,09	35,93
2	16	1,204	36,05	33,74	33,58	34,46	33,74
3	8	0,903	30,83	31,87	32,53	31,74	31,87
4	4	0,602	25,55	27,71	28,72	27,33	27,71
5	2	0,301	26,27	27,81	26,04	26,71	26,27
6	1	0,000	23,87	22,99	24,07	23,64	23,87
7	0,5	-0,301	21,10	21,48	21,03	21,20	21,10
8	0,25	-0,602	16,53	17,84	18,88	17,75	17,84
9	0,125	-0,903	13,48	13,91	12,77	13,39	13,48
10	0,06	-1,221					
11	0,03	-1,522					
12	0						

Bioassay trial 2 - oxolinic acid pH=9,45 sample:50µl							
number	O.A concentration (ppm)	Log O.A concentration	Zone A	Zone B	Zone C	Average	Median
1	32	1,505	33,31	32,46	34,68	33,48	33,31
2	16	1,204	32,47	32,9	34,83	33,40	32,90
3	8	0,903	31,28	29,43	28,47	29,73	29,43
4	4	0,602	27,49	28,41	28,06	27,99	28,06
5	2	0,301	26,1	22,33	23,65	24,03	23,65
6	1	0,000	21,25	23,23	21,45	21,98	21,45
7	0,5	-0,301	17,83	18,28	19,15	18,42	18,28
8	0,25	-0,602	13,42	14,55	13,79	13,92	13,79
9	0,125	-0,903	8,71	9,47	10,1	9,43	9,47
10	0,06	-1,221					
11	0,03	-1,522					
12	0						

Bioassay trial 3 - oxolinic acid pH=9,7 sample: 50µl							
number	O.A concentration (ppm)	Log O.A concentration	Zone A	Zone B	Zone C	Average	Median
1	32	1,505	37,91	33,89	41,86	37,89	37,91
2	16	1,204	31,93	32,54	32,94	32,47	32,54
3	8	0,903	33,86	30,64	29,41	31,30	30,64
4	4	0,602	28,09	26,91	29,62	28,21	28,09
5	2	0,301	28,65	19,23	24,45	24,11	24,45
6	1	0,000	21,08	25,8	20,82	22,57	21,08
7	0,5	-0,301	17,84	17,32	22,77	19,31	17,84
8	0,25	-0,602	10,93	12,43	11,97	11,78	11,97
9	0,125	-0,903	8,34	8,3	8,49	8,38	8,34
10	0,06	-1,221					
11	0,03	-1,522					
12	0						

In the next stage data from bioassay trials employing "spiked" serum samples were presented. The detection limit of the bioassay for "spiked " serum samples was 125 ppb. The data from these trials (Trial 1, 2) were presented in Table 38 along with method technical data. The statistical analysis of the "spiked "serum trials was presented in Table 39.

Finally, the data from the detection of O.A in "spiked " muscle samples using this bioassay method were presented along with the statistical analysis. The detection limit of the method for muscle samples was 250 ppb O.A. For the statistical analysis only the zones of inhibition regarding O.A concentrations from 250ppb to 32 ppm were considered because the zones of inhibition seemed to reach a plateau at this concentration giving odd readings in higher concentrations. Another test that was performed involved the Operator testing. Bioassay methods rely on the Subjectivity of the Operator and his capacity to read in the same manner small distances down to two decimal points using a digital caliper. Three plates (plate 1, 2 and 3) were employed in this trial using "spiked" muscle samples. In each of these plates readings were taken by two operators (tests A and B). The statistical significance of the difference in the

readings of the two operators was examined (S.P.S.S ®) using Paired T-test. The 2-tailed significance value (p value) was higher than 0.05 suggesting that there were no statistically significant differences between the readings. The data from the trial using "spiked " muscle were presented in the Tables 40,41,42 while the statistical analysis and P-values were demonstrated in the Table 43.

Table 38: Oxolinic acid residues detection bioassay method

"Spiked" serum samples - Statistical analysis	
Trial 1	**R-squared:** 98.85%, **slope:** 11.81, **Intercept:**18.82
Trial 2	**R-squared:** 98.65%, **slope:** 11.97, **Intercept:** 20

Table 39: Oxolinic acid residues detection bioassay method - Spiked serum samples

Bioassay serum (25µl) - oxolinic acid dilution (25µl) TRIAL 1 pH=7.55 sample:50µl							
Number	**O.A concentration (ppm)**	**Log O.A concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**
1	32	1,505	34,94	35,99	34,95	35,29	34,95
2	16	1,204	32,1	32,38	32,52	32,33	32,38
3	8	0,903	29,95	29,65	29,54	29,71	29,65
4	4	0,602	27,07	26,66	26,5	26,74	26,66
5	2	0,301	24,75	23,73	22,92	23,80	23,73
6	1	0,000	19,53	20,52	20,07	20,04	20,07
7	0,5	-0,301	15,82	15,02	16,04	15,63	15,82
8	0,25	-0,602	10,46	10,25	11,47	10,73	10,46
9	0,125	-0,903	7,03			7,03	7,03
10	0,06	-1,221					
11	0,03	-1,522					
12	0						

Bioassay serum(25µl) - oxolinic acid dilution (25µl) TRIAL 2 pH=7.55 sample:50µl							
Number	**O.A concentration (ppm)**	**Log O.A concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**
1	32	1,505	36,96	36,09	36,34	36,46	36,34
2	16	1,204	34,6	34,1	34,12	34,27	34,12
3	8	0,903	31,52	31,21	30,51	31,08	31,21
4	4	0,602	26,96	28,24	27,63	27,61	27,63
5	2	0,301	24,44	24,28	24,13	24,28	24,28
6	1	0,000	20,77	21,95	22,2	21,64	21,95
7	0,5	-0,301	16,95	18,44	17,38	17,59	17,38
8	0,25	-0,602	12,3	11,56	11,65	11,84	11,65
9	0,125	-0,903	7,1	8,16		7,63	7,63
10	0,06	-1,221					
11	0,03	-1,522					
12	0						

Table 40: Oxolinic acid residues detection bioassay method

"Spiked" Muscle samples Trial 1 - Plate 1

Bioassay muscle (50µl) TRIAL 1 plate 1 test A pH=7.55 sample:50µl							
Number	O.A concentration (ppm)	Log O.A concentration	Zone A	Zone B	Zone C	Average	Median
1	64	1,806	32,20	33,53	32,34	32,69	32,34
2	32	1,505	30,82	31,01	33,94	31,92	31,01
3	16	1,204	27,05	29,74	30,38	29,06	29,74
4	8	0,903	26,63	25,37	23,56	25,19	25,37
5	4	0,602	21,57	24,2	22,84	22,87	22,84
6	2	0,301	20,12	19,37	21,46	20,32	20,12
7	1	0,000	16,32	18,44	15,72	16,83	16,32
8	0,5	-0,301	13,47	13,18	15,22	13,96	13,47
9	0,25	-0,602	11,75	10,92	11,29	11,32	11,29
10	0,125	-0,903					
11	0,06	-1,221					
12	0,03	-1,522					
13	0						

Bioassay muscle (50µl) TRIAL 1 plate 1 test B pH=7.55 sample:50µl							
Number	O.A concentration (ppm)	Log O.A concentration	Zone A	Zone B	Zone C	Average	Median
1	64	1,806	30,58	33,10	32,01	31,90	32,01
2	32	1,505	32,01	31,91	32,97	32,30	32,01
3	16	1,204	26,35	29,81	30,3	28,82	29,81
4	8	0,903	26	25,19	24,68	25,29	25,19
5	4	0,602	21,4	24,35	23,34	23,03	23,34
6	2	0,301	20,21	20,09	21,1	20,47	20,21
7	1	0,000	15,62	18,9	16,12	16,88	16,12
8	0,5	-0,301	13,83	13,07	15,63	14,18	13,83
9	0,25	-0,602	11,96	10,97	11,16	11,36	11,16
10	0,125	-0,903					
11	0,06	-1,221					
12	0,03	-1,522					
13	0						

Table 41: Oxolinic acid residues detection bioassay method
"Spiked" Muscle samples Trial 1 - Plate 2

Bioassay muscle (50µl) TRIAL 1 plate 2 test A pH=7.55 sample:50µl							
Number	O.A concentration (ppm)	Log O.A concentration	Zone A	Zone B	Zone C	Average	Median
1	64	1,806	31,75	34,42	31,29	32,49	31,75
2	32	1,505	36,67	30,48	31,04	32,73	31,04
3	16	1,204	26,66	29,06	29,54	28,42	29,06
4	8	0,903	26,47	27,04	24,14	25,88	26,47
5	4	0,602	22,89	24,86	23,05	23,60	23,05
6	2	0,301	20,53	19,28	22,51	20,77	20,53
7	1	0,000	15,92	18	17,03	16,98	17,03
8	0,5	-0,301	14,43	12,61	16,65	14,56	14,43
9	0,25	-0,602	12,71	11,32	11,17	11,73	11,32
10	0,125	-0,903					
11	0,06	-1,221					
12	0,03	-1,522					
13	0						

Bioassay muscle (50µl) TRIAL 1 plate 2 test B pH=7.55 sample:50µl							
Number	O.A concentration (ppm)	Log O.A concentration	Zone A	Zone B	Zone C	Average	Median
1	64	1,806	31,71	33,86	32,26	32,61	32,26
2	32	1,505	32,20	31,70	31,01	31,64	31,70
3	16	1,204	27,03	28,63	30,09	28,58	28,63
4	8	0,903	27,06	26,82	24,67	26,18	26,82
5	4	0,602	23,34	24,58	22,72	23,55	23,34
6	2	0,301	20,21	19,66	21,42	20,43	20,21
7	1	0,000	16,66	18,08	16,71	17,15	16,71
8	0,5	-0,301	13,34	12,19	16,3	13,94	13,34
9	0,25	-0,602	13,18	11,58	12	12,25	12,00
10	0,125	-0,903					
11	0,06	-1,221					
12	0,03	-1,522					
13	0						

Table 42: Oxolinic acid residues detection bioassay method

"Spiked" Muscle samples Trial 1 - Plate 3

Bioassay muscle (50µl) TRIAL 1 plate 3 test A pH=7.55 sample:50µl							
Number	O.A concentration (ppm)	Log O.A concentration	Zone A	Zone B	Zone C	Average	Median
1	64	1,806	33,73	32,93	33,54	33,40	33,54
2	32	1,505	35,50	32,64	33,64	33,93	33,64
3	16	1,204	29,19	29,36	31,81	30,12	29,36
4	8	0,903	26,62	25,76	26,37	26,25	26,37
5	4	0,602	23,39	25,13	24,23	24,25	24,23
6	2	0,301	21,58	20,57	23,56	21,90	21,58
7	1	0,000	16,57	19,07	16,95	17,53	16,95
8	0,5	-0,301	13,82	14,09	15,98	14,63	14,09
9	0,25	-0,602	11,93	11,37	11,69	11,66	11,69
10	0,125	-0,903					
11	0,06	-1,221					
12	0,03	-1,522					
13	0						

Bioassay muscle (50µl) TRIAL 1 plate 3 test B pH=7.55 sample:50µl							
Number	O.A concentration (ppm)	Log O.A concentration	Zone A	Zone B	Zone C	Average	Median
1	64	1,806	33,16	32,83	33,73	33,24	33,16
2	32	1,505	36,26	33,58	32,82	34,22	33,58
3	16	1,204	28,64	30,07	31,28	30,00	30,07
4	8	0,903	26	25,49	26,01	25,83	26,00
5	4	0,602	22,67	24,42	24,1	23,73	24,10
6	2	0,301	20,72	20,45	23,29	21,49	20,72
7	1	0,000	16,44	18,26	15,99	16,90	16,44
8	0,5	-0,301	14,82	14,76	15,66	15,08	14,82
9	0,25	-0,602	11,6	12	12,17	11,92	12,00
10	0,125	-0,903					
11	0,06	-1,221					
12	0,03	-1,522					
13	0						

Table 43: Oxolinic acid residues detection bioassay method

"Spiked" Muscle samples - Statistical analysis		
Plate 1 (A)	Operator 1	**R-squared:** 99.32%,**slope:** 9.37, **Intercept:**17.06
	Operator 2	**R-squared:** 99.76%, **slope:** 9.82, **Intercept:**17.12
Plate 2 (B)	Operator 1	**R-squared:**99.57%, **slope:** 9.75, **Intercept:** 17.44
	Operator 2	**R-squared:** 99.69%, **slope:** 9.48, **Intercept:**17.44
Plate 3 (C)	Operator 1	**R-squared:** 99.46%, **slope:** 10.39, **Intercept:**17.85
	Operator 2	**R-squared:** 99.03%, **slope:**10.3, **Intercept:**17.75

A: (p value = 0.925), B: (p value = 0.599) , C: (p value = 0.315)

The statistical analysis indicated that the % recovery of Oxolinic acid residues in "spiked" serum samples was 100% while the % recovery of O.A residues in "spiked " muscle samples was 92.74%.

3.3 Oxolinic Acid Kinetics in Sea Bream (*Sparus aurata*)

3.3.1.1 High Water Temperature

3.3.1.1.1 Oxolinic Acid depletion in Muscle

The concentration of Oxolinic acid achieved in the muscle of Sea bream kept at high temperature was presented in Figures 33,34 and 35 while Table 44 presented the statistical analysis of the concentration achieved in the muscle and liver. Standard deviation of the muscle concentration of Oxolinic acid was very high apparent fact in this type of population experimentation. The concentration maximum was presented on the 10^{th} day (1607.67ppb). The maximum concentration decreased slightly on the 11^{th} day (1467.21 ppb) and sharply until the 14^{th} day (25.85 ppb) and remained low thereafter. The line representing the mean O.A concentration increased on the 3^{rd} day (637.7 ppb) followed by a moderate decrease on the 10^{th} day (411.72 ppb) and a slight increase on the 11^{th} day (583.05 ppb) decreasing thereafter and reaching consumer safe levels on the 14^{th} day (11.13ppb O.A). The Box-plot presentation indicated that the maximum concentration values on the 3^{rd}, 10^{th}, 11^{th} and 12^{th} referred to outlier (o) and extreme (*) values and the majority of the population exhibited muscle concentration of much lower levels. The 75% percentile value increased on the 3^{rd} day (958.63 ppb) and after a decrease on the 10^{th} day (423.57 ppb) reached a concentration of 719.25 ppb O.A on the 11^{th} day decreasing thereafter. The metabolism of O.A in the muscle suggested to be fairly rapid, reaching consumer safe levels in only four days after the end of the treatment.

3.3.1.1.2 Oxolinic Acid depletion in Liver

On the other hand the kinetics of Oxolinic acid in the liver of Sea bream at high temperatures was presented in the Figures 36,37 and 38. The data were presented in two formats the first including minimum, mean and maximum concentration achieved and the other the box-plot analysis and the population distribution of the concentration achieved. Maximum O.A concentration in the liver of sea bream increased rapidly on the 3rd day (6045.98 ppb) decreasing on the 10th day to a level of 2252.98 ppb and reached the peak concentration on the 11th day (7648.51 ppb). The Box-plot analysis of these data indicated that the peak concentration achieved on the 11th day referred to an extreme value while the maximum concentration present on the 10th day was an outlier value (Figure 38). This analysis indicated that peak concentration was achieved on the 3rd day (median: 1716.09ppb, 755 percentile: 5160.03 ppb O.A) and decreased rapidly thereafter consumer safe levels on the 12th day (maximum concentration: 63.28 ppb).

Table 44: EXPERIMENT I Oxolinic acid kinetics in Sea bream at high temperature after 10 day oral treatment - Statistical interpretation

Statistical Analysis
Oxolinic acid concentration - Sea Bream Muscle - High Temperature

	Day 0	Day 3	Day 10	Day 11	Day 12	Day 14	Day 16	Day 18
Samples	5	9	10	10	9	8	7	5
Minimum concentration(ppb)	6,38	164,56	122,16	62,64	43,19	2,39	0,05	2,96
Mean concentration(ppb)	23,66	637,7	411,72	583,05	226,03	11,13	10,28	12,28
Maximum concentration(ppb)	41,99	1450,73	1607,67	1467,21	776,62	22,85	25,01	29,21
Standard deviation	14,49	440,51	438,32	377,12	234,94	6,49	10,57	10,17
25% percentile	9,84	306,02	170,69	362,72	75,5	5,32	0,50	4,56
Median	23,1	575,52	300,30	534,13	117,94	11,02	7,13	10,46
75% Percentile	37,77	958,63	423,57	719,25	321,57	14,52	20,30	20,91

	Day 20	Day 25						
Samples	9	8						
Minimum concentration(ppb)	9,55	2,06						
Mean concentration(ppb)	22,38	11,9						
Maximum concentration(ppb)	56,69	26,45						
Standard deviation	15,42	9,38						
25% Percentile	10,52	2,6						
Median	17,33	10,97						
75% Percentile	31,73	20,32						

Oxolinic acid concentration - Sea Bream Liver - High Temperature								
	Day 0	Day 3	Day 10	Day 11	Day 12	Day 14	Day 16	Day 18
Samples	5	4	9	7	5	5	5	6
Minimum concentration(ppb)	27,12	503,99	173,37	100,74	36,12	10,40	28,72	14,42
Mean concentration(ppb)	40,65	2495,54	568,17	1536,39	48,79	14,91	32,46	134,50
Maximum concentration(ppb)	53	6045,98	2252,98	7648,51	63,28	19,24	35,64	338,89
Standard deviation	10,4	2518,12	681,92	2705,90	9,91	3,45	2,76	133,42
25% percentile	30,37	610,48	186,89	331,42	40,46	11,72	29,73	20,93
Median	42,07	1716,09	213,56	639,10	48,42	14,74	32,90	101,95
75% Percentile	50,22	5160,03	794,99	823,16	57,3	18,19	34,97	254,74

Sea Bream Liver - High Temperature								
	Day 20	Day 25						
Samples	4	5						
Minimum concentration(ppb)	11,2	12,01						
Mean concentration(ppb)	33,91	92,04						
Maximum concentration(ppb)	88,68	213						
Standard deviation	36,85	107,43						
25% Percentile	11,7	13,13						
Median	17,87	14,58						
75% Percentile	72,15	209,69						

Figure 33: Experiment I - Kinetics of Oxolinic acid in the muscle of Sea bream (*Sparus aurata*) at 22 $\pm$ 1°C

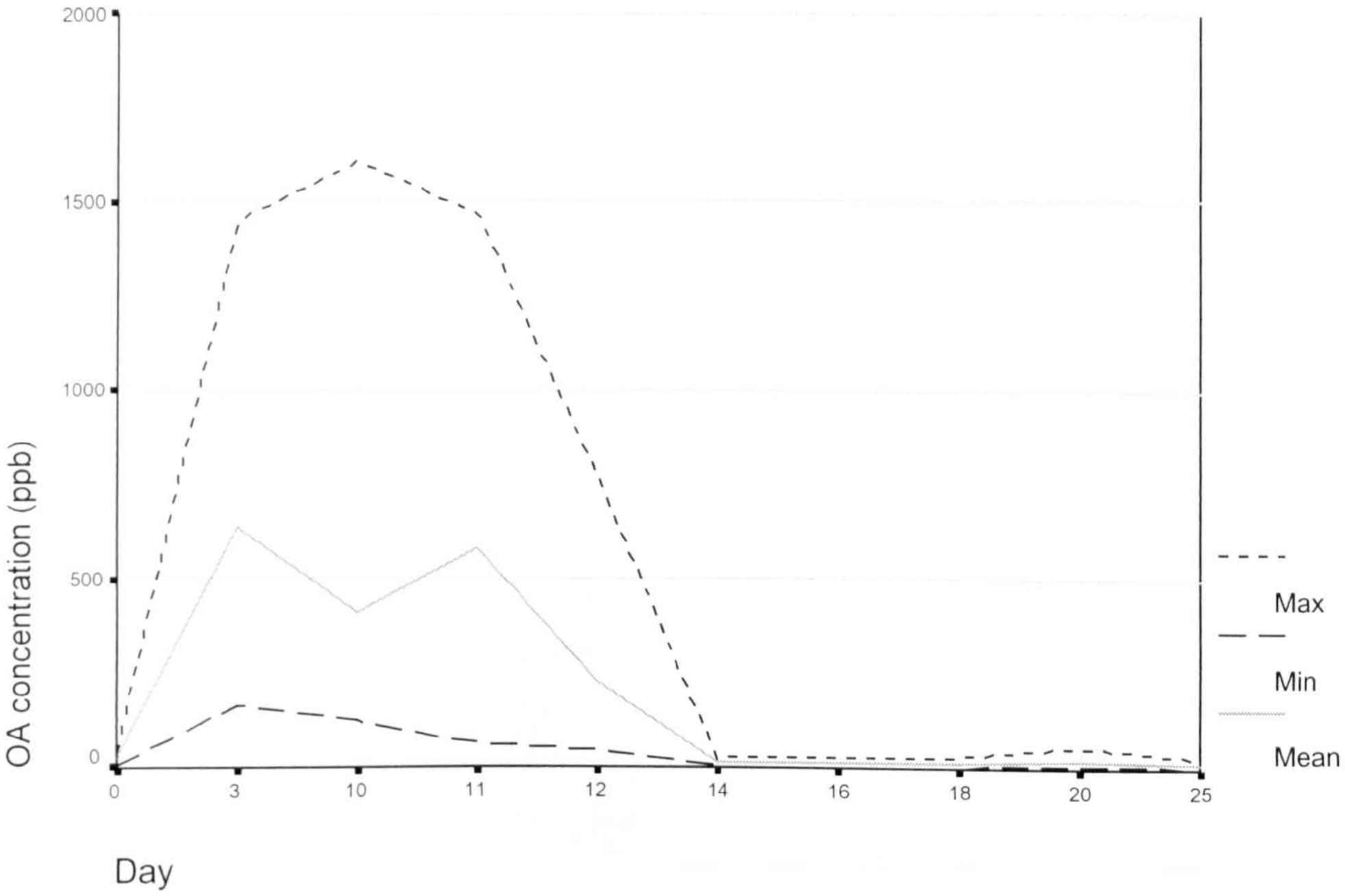


Figure 34: Experiment I - Kinetics of Oxolinic acid in the muscle of Sea bream (*Sparus aurata*) at 22 $\pm$ 1°C – Mean and Standard deviation

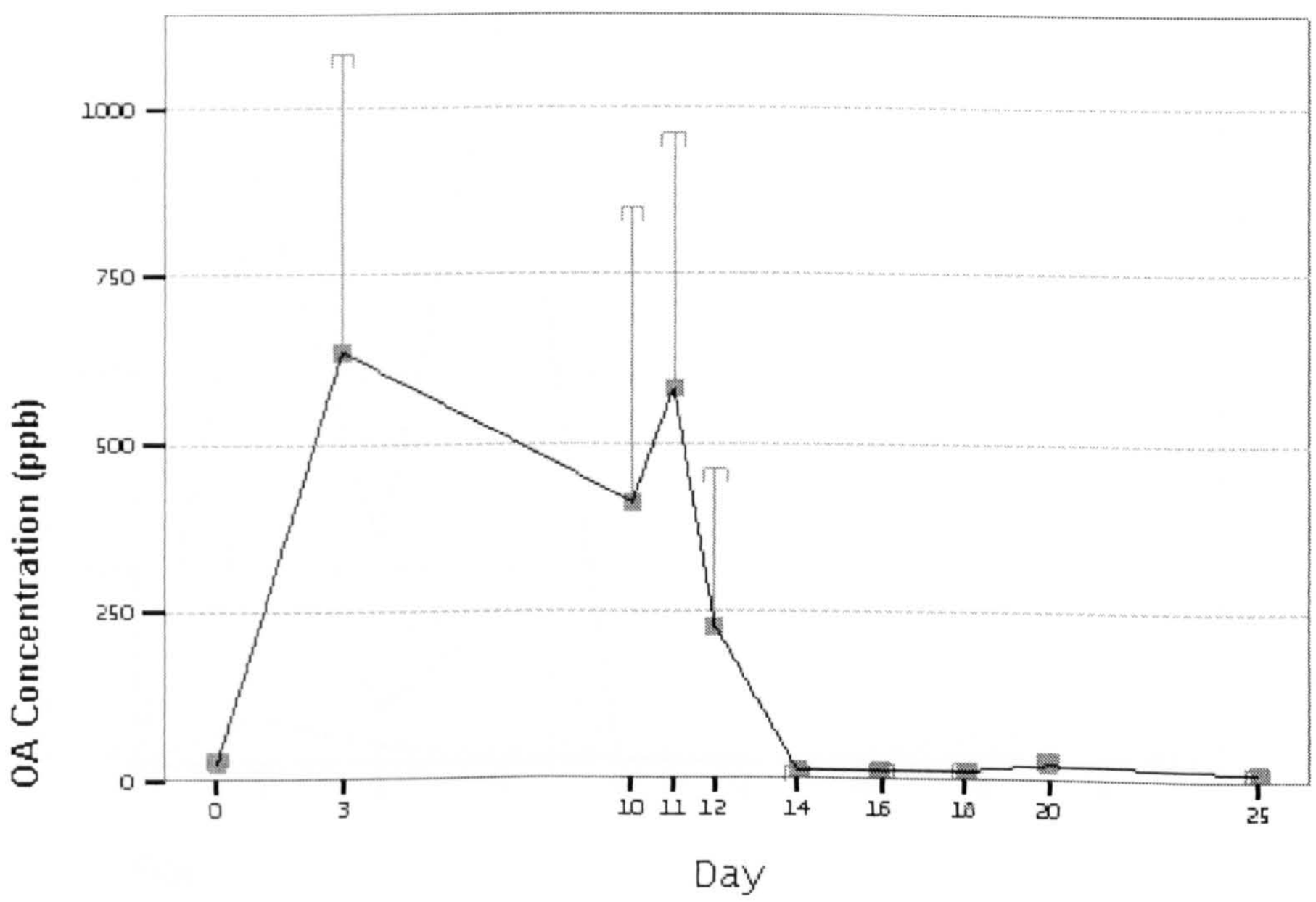


Figure 35: Experiment I - Kinetics of Oxolinic acid in the muscle of Sea bream (*Sparus aurata*) at 22 ± 1°C - Box plot presentation

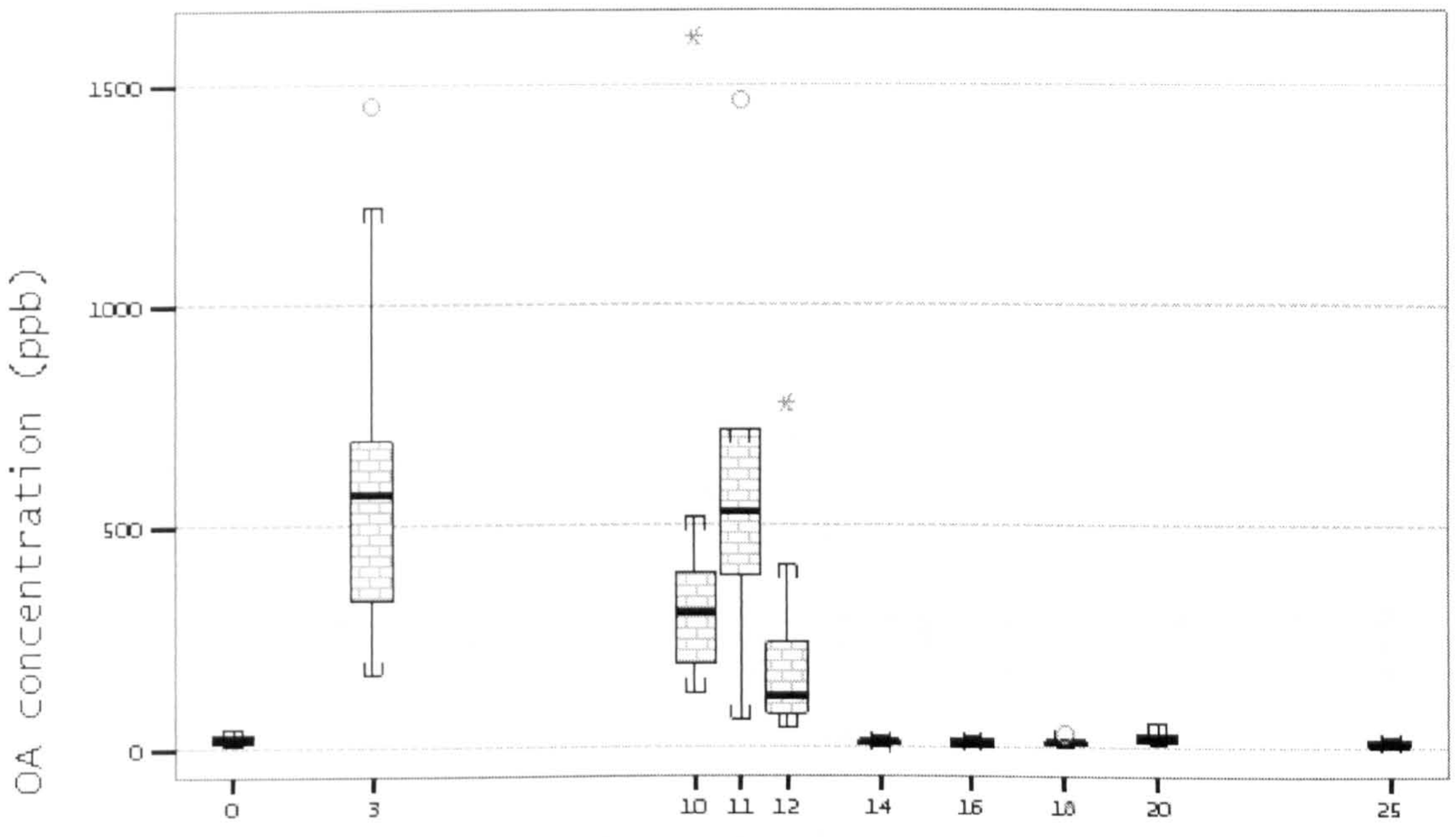


Figure 36: Experiment I - Kinetics of Oxolinic acid in the liver of Sea bream (*Sparus aurata*) at 22 ± 1°C

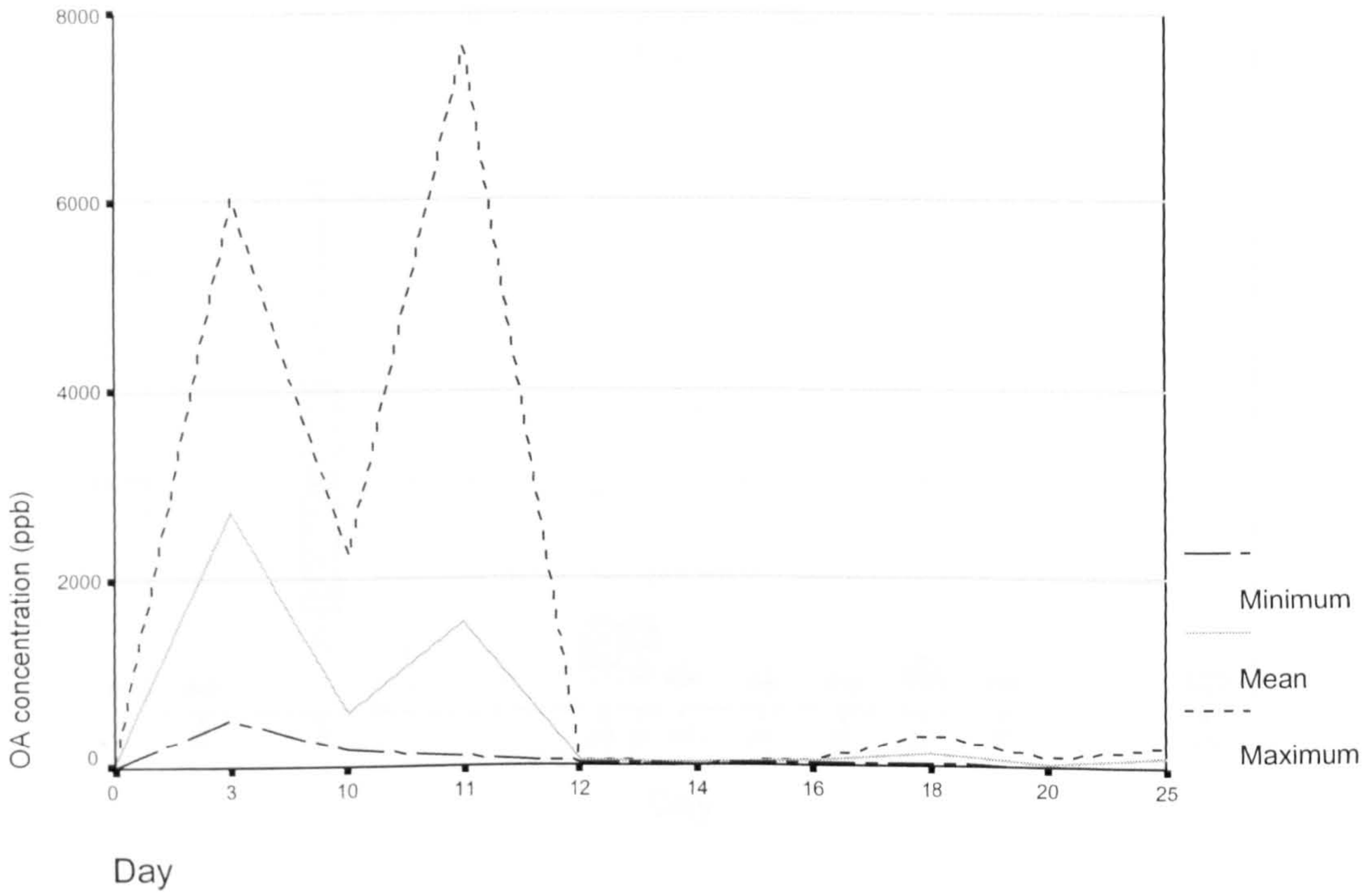


Figure 37: Experiment I - Kinetics of Oxolinic acid in the liver of Sea bream (*Sparus aurata*) at 22 $\pm$ 1°C – Mean and Standard deviation

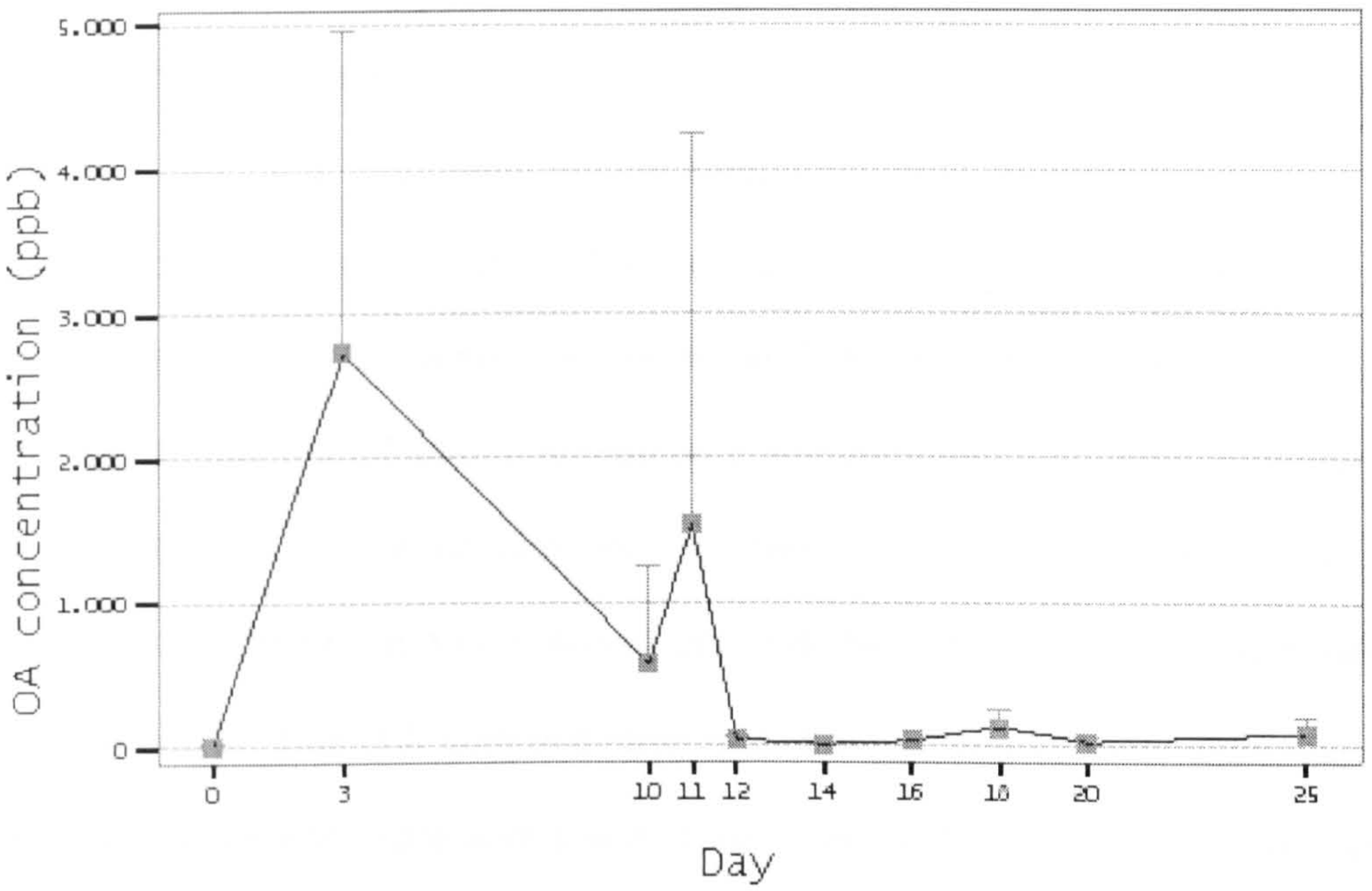
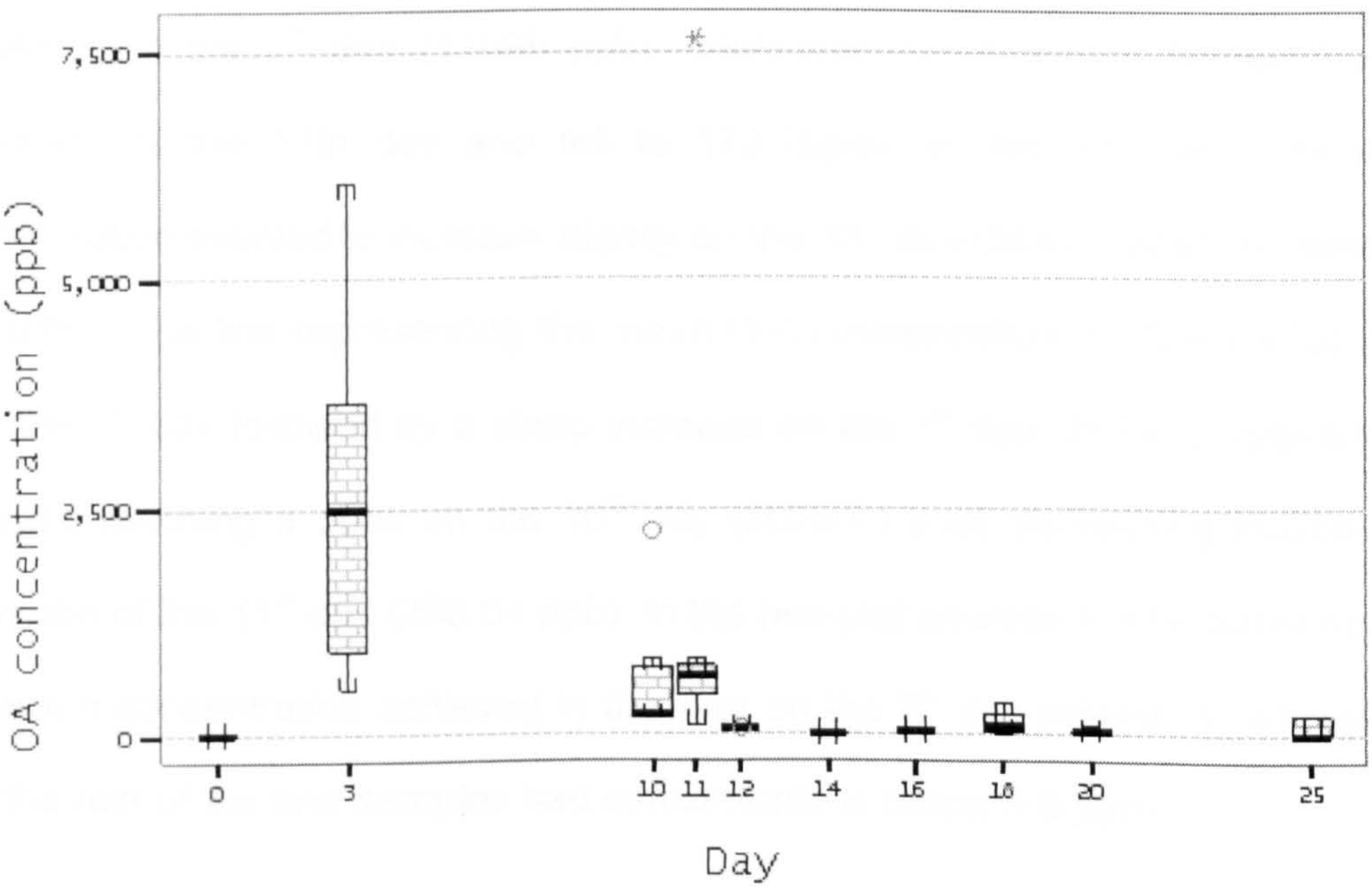


Figure 38: Experiment I - Kinetics of Oxolinic acid in the liver of Sea bream (*Sparus aurata*) at 22 $\pm$ 1°C - Box plot presentation



3.3.1.2 Low Water Temperature

3.3.1.2.1 Oxolinic Acid depletion in Muscle

The concentration maximum was presented in the 7th day (1979.43 ppb). O.A concentration in the muscle seemed to decrease reaching a maximum of 1018.33 ppb on the 10th day. The maximum concentration seemed to increase on the 11th day (1379.73 ppb) and was decreased sharply until the 12th day (110.67 ppb) and remained low thereafter. The line representing the mean O.A concentration exhibited a lag phase up until the 3rd day (42.58 ppb) followed by a sharp increase on the 7th day (654.6 ppb) and a very slight increase up until the 11th day (736.17 ppb). It was evident that the median of O.A concentration increased up until the 10th day (647.46 ppb) decreasing sharply thereafter. The O.A concentration after the 12th day (110.67 ppb) was found to be below the consumer safe levels with 3 samples on the 13th and the 14th day above this concentration being considered as Outliers.

3.3.1.2.2 Oxolinic Acid depletion in Liver

The O.A concentration maximum in the liver of Sea bream at low temperature was presented in the 7th day (11095 ppb), decreased reaching 810.78 ppb O.A on the afternoon of the 11th day and fell to 173.75ppb on the 12th day. The maximum concentration seemed to increase slightly on the 13th day (804.07 ppb) but remained low thereafter. The line representing the mean O.A concentration exhibited a lag phase up until the 3rd day followed by a sharp increase on the 7th day (2628 .2 ppb) a moderate increase reaching a peak on the 10th day (3029.51 ppb) decreasing sharply until the afternoon of the 11th day (235.64 ppb). In the box-plot analysis it was demonstrated that maximum concentration achieved in the liver on the 7th day referred to an outlier value and the rest of the liver samples had concentrations below 3.5 ppm.

The median seemed to increase gradually reaching a peak on the 10th day (2730.94 ppb) and decreased rapidly thereafter. Consumer safe levels were reported from the 13th day of the experiment onward with extreme and outlier values present in a few of the samples taken in the rest days of the experiment.

Table 45: EXPERIMENT II Oxolinic acid kinetics in Sea bream at low temperature after 10 day oral treatment - Statistical interpretation

Statistical Analysis
Oxolinic acid concentration - Sea Bream Muscle - Low Temperature

	Day 0	Day 1	Day 3	Day 7	Day 10	Day 11	Day 11 Aft.	Day 12
Samples	8	9	8	9	4	5	10	10
Minimum concentration(ppb)	3,15	3,64	27,17	44,68	274,84	215,72	67,75	16,36
Mean concentration(ppb)	11,50	42,05	42,58	654,60	647,02	738,17	302,85	48,53
Maximum concentration(ppb)	22,89	120,61	57,43	1979,43	1018,33	1379,73	871,98	110,67
Standard deviation	8,00	36,59	9,83	624,87	346,15	514,49	268,00	28,71
25% percentile	3,98	16,83	33,51	76,26	317,04	322,26	111,59	28,37
Median	9,64	30,78	43,81	597,77	647,46	472,43	207,66	40,44
75% Percentile	20,14	65,23	49,01	1019,93	976,56	1286,96	415,88	66,49

	Day 13	Day 14	Day 16	Day 25	Day 30	Day 35		
Samples	9	10	9	9	10	8		
Minimum concentration(ppb)	9,51	4,61	1,41	8,35	10,37	1,38		
Mean concentration(ppb)	48,48	38,19	11,47	19,02	30,84	8,70		
Maximum concentration(ppb)	142,06	122,12	50,51	38,24	67,84	19,95		
Standard deviation	47,85	34,71	15,29	9,63	19,13	6,07		
25% Percentile	16,18	14,18	3,85	11,36	16,10	3,08		
Median	22,76	30,17	4,78	14,91	23,69	8,25		
75% Percentile	84,81	53,08	13,52	24,81	49,62	12,62		

Oxolinic acid concentration - Sea Bream Liver - Low Temperature

	Day 0	Day 1	Day 3	Day 7	Day 10	Day 11 Aft	Day 12	Day 13
Samples	8	10	9	8	4	10	10	8
Minimum concentration(ppb)	5,50	5,43	26,72	58,96	964,27	75,77	27,03	3,18
Mean concentration(ppb)	29,81	363,09	62,54	2628,02	3029,51	235,64	90,88	135,45
Maximum concentration(ppb)	53,22	1118,83	641,44	11095,46	5691,89	810,78	173,75	804,07
Standard deviation	17,97	418,26	25,32	3649,87	1981,58	217,45	46,96	270,94
25% percentile	15,54	21,19	39,14	105,12	1317,86	93,46	50,36	22,45
Median	26,79	204,84	60,46	1776,40	2730,94	184,34	96,96	51,01
75% Percentile	50,30	691,89	87,82	3217,72	5039,73	257,63	124,87	61,33

	Day 14	Day 16	Day 25	Day 30	Day 35			
Samples	9	8	9	10	8			
Minimum concentration(ppb)	15,03	4,65	14,49	8,89	2,77			
Mean concentration(ppb)	54,79	28,76	68,33	38,31	100,71			
Maximum concentration(ppb)	147,53	61,91	229,87	80,01	478,92			
Standard deviation	51,53	19,94	78,85	19,54	161,40			
25% Percentile	17,67	11,18	19,41	26,56	7,38			
Median	27,49	27,25	39,15	37,79	40,58			
75% Percentile	101,01	45,18	112,74	47,94	138,25			

Figure 39: Experiment II - Kinetics of Oxolinic acid in the muscle of Sea bream (*Sparus aurata*) at 17 $\pm$ 1°C

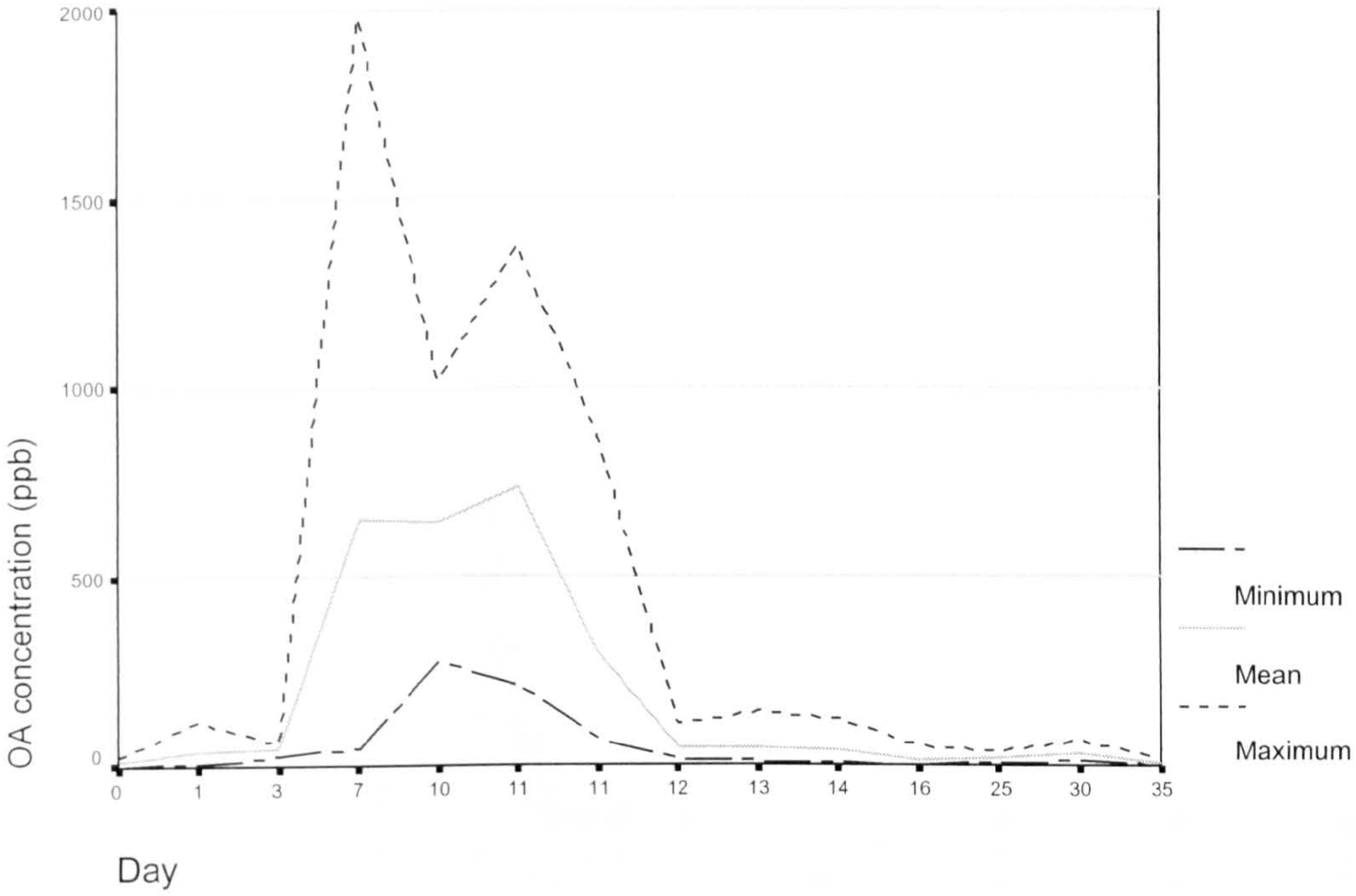


Figure 40: Experiment II - Kinetics of Oxolinic acid in the muscle of Sea bream (*Sparus aurata*) at 17 $\pm$ 1°C - Mean and Standard deviation

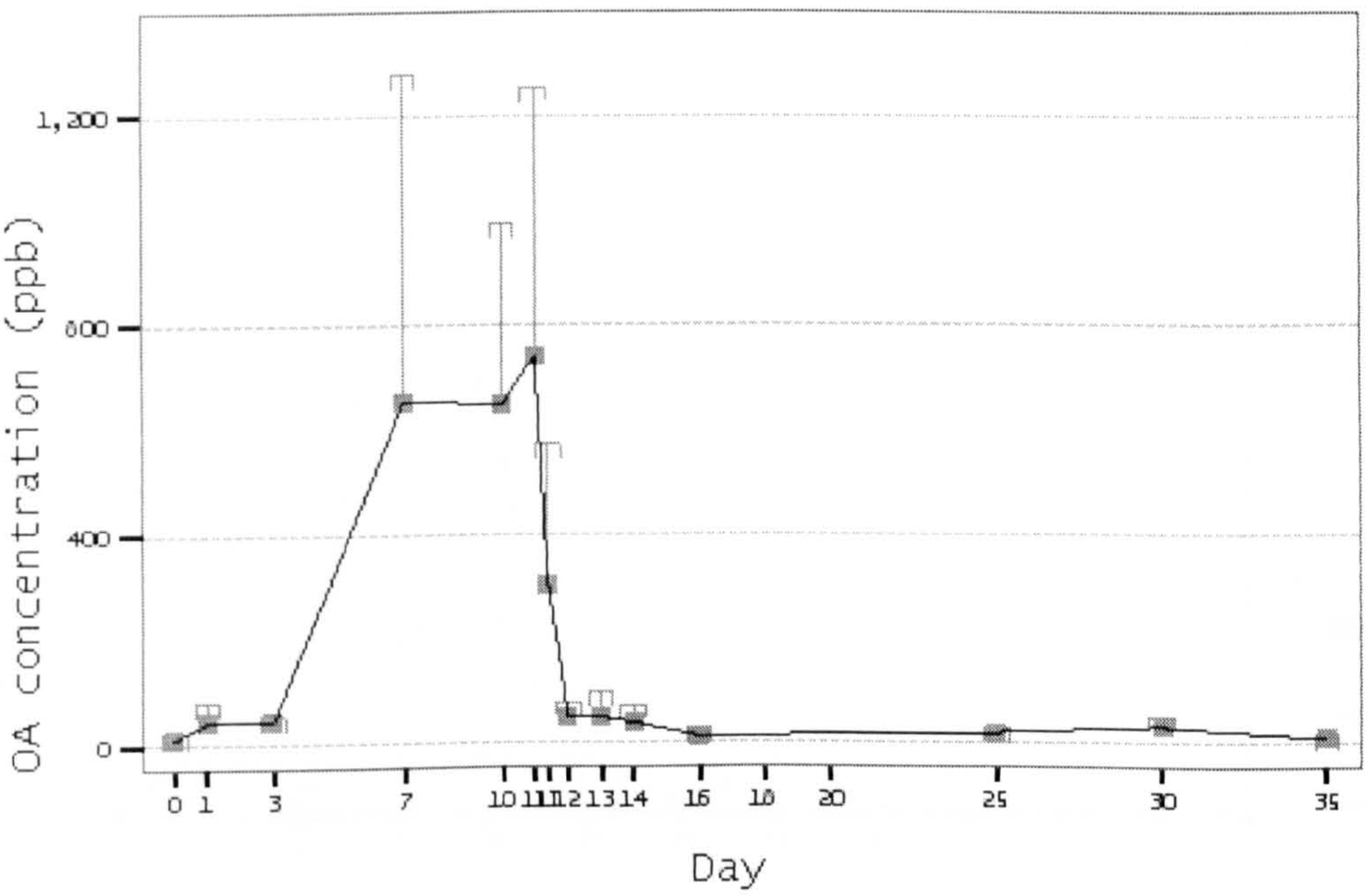


Figure 41: Experiment II - Kinetics of Oxolinic acid in the muscle of Sea bream (*Sparus aurata*) at 17 ± 1°C - Box - plot presentation

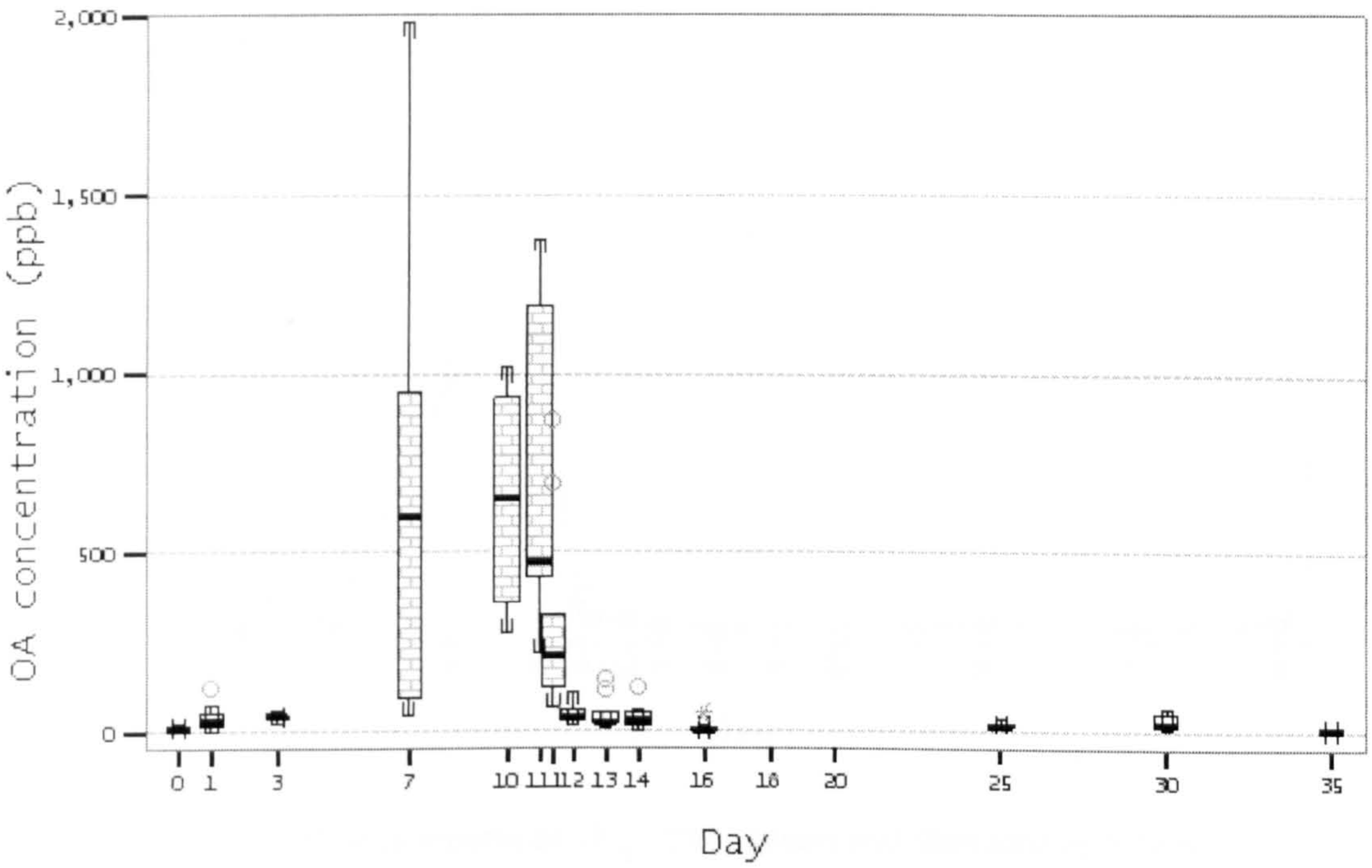


Figure 42: Experiment II - Kinetics of Oxolinic acid in the Liver of Sea bream (*Sparus aurata*) at 17 ± 1°C.

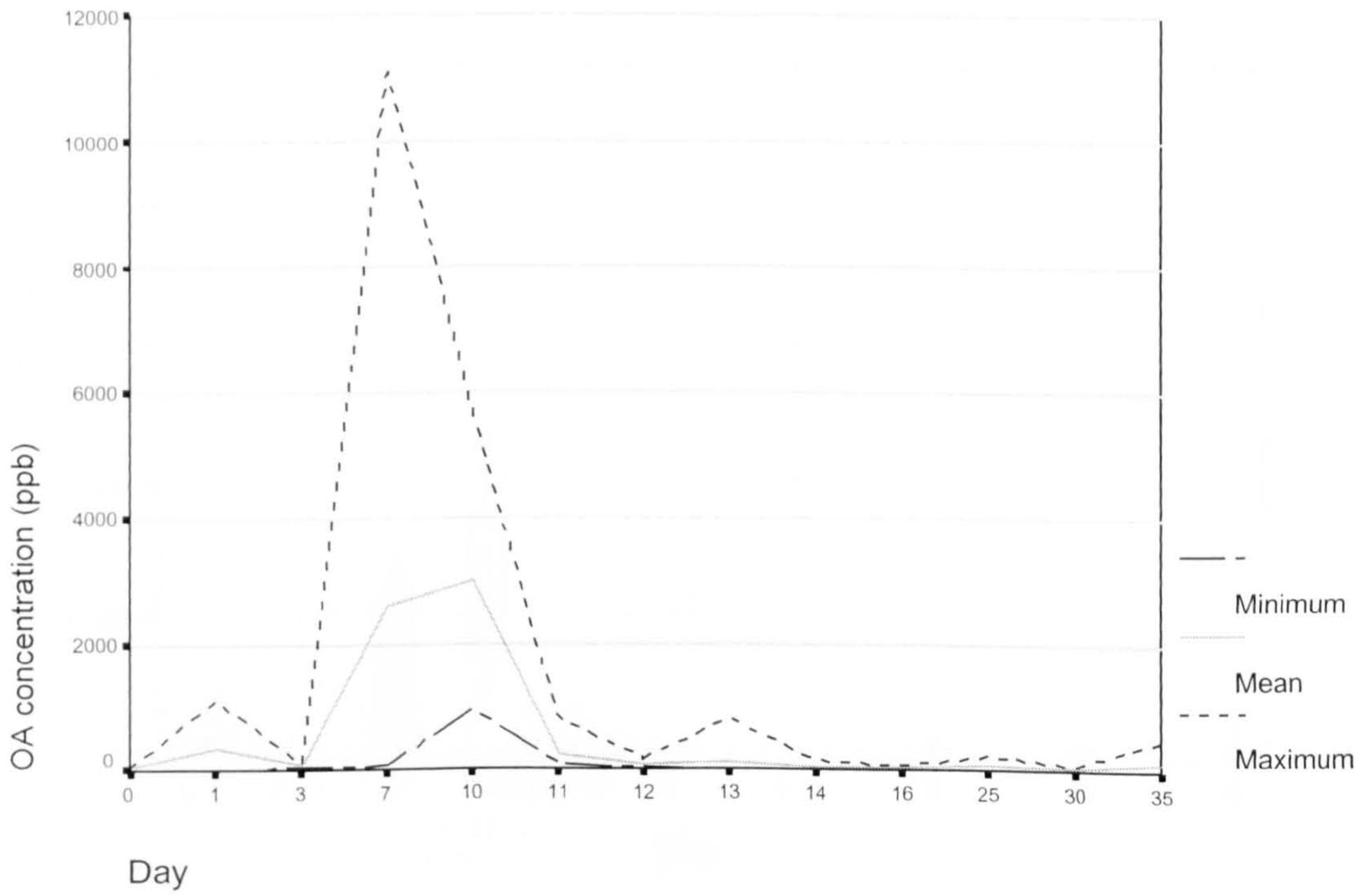
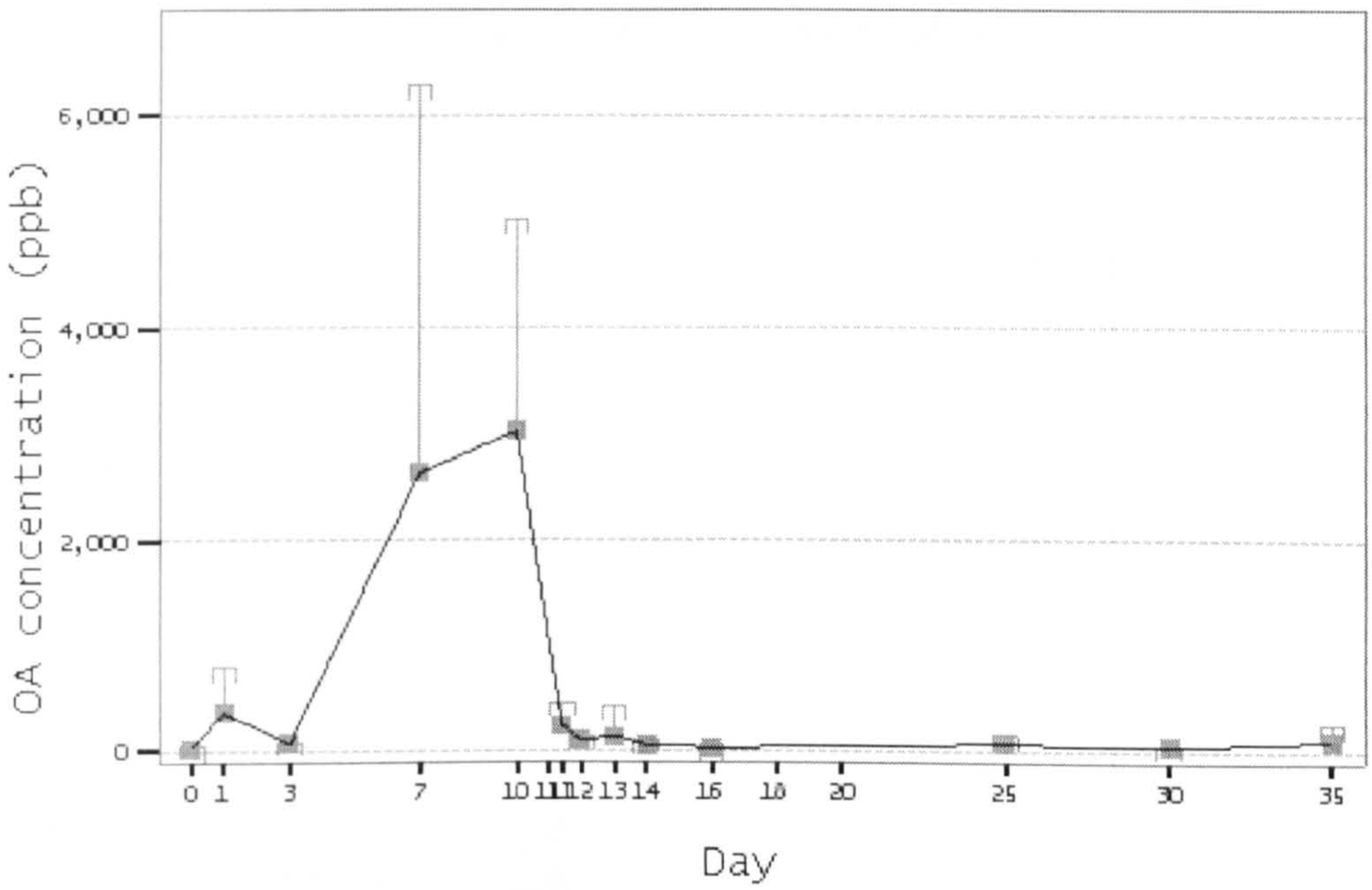
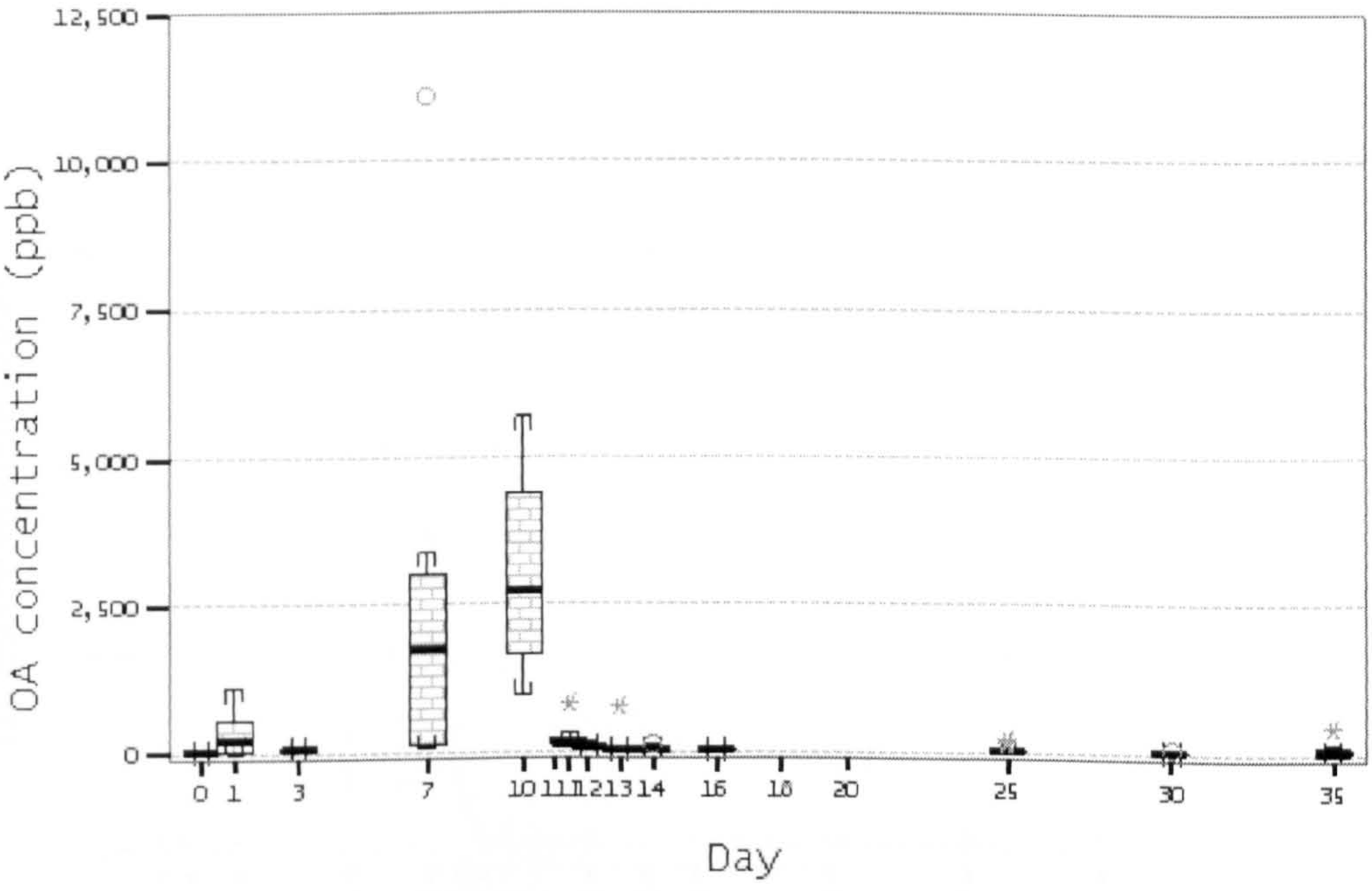


Figure 43: Experiment II - Kinetics of Oxolinic acid in the liver of Sea bream



(*Sparus aurata*) at 17 $\pm$ 1°C – Mean and Standard deviation

Figure 44: Experiment II - Kinetics of Oxolinic acid in the liver of Sea bream (*Sparus aurata*) at 17 $\pm$ 1°C - Box-plot presentation



3.3.2 Comparisons

3.3.2.1 Muscle Depletion versus liver Depletion

Figure 45: Comparison of OA mean concentration in the muscle and liver of sea bream at high temperature

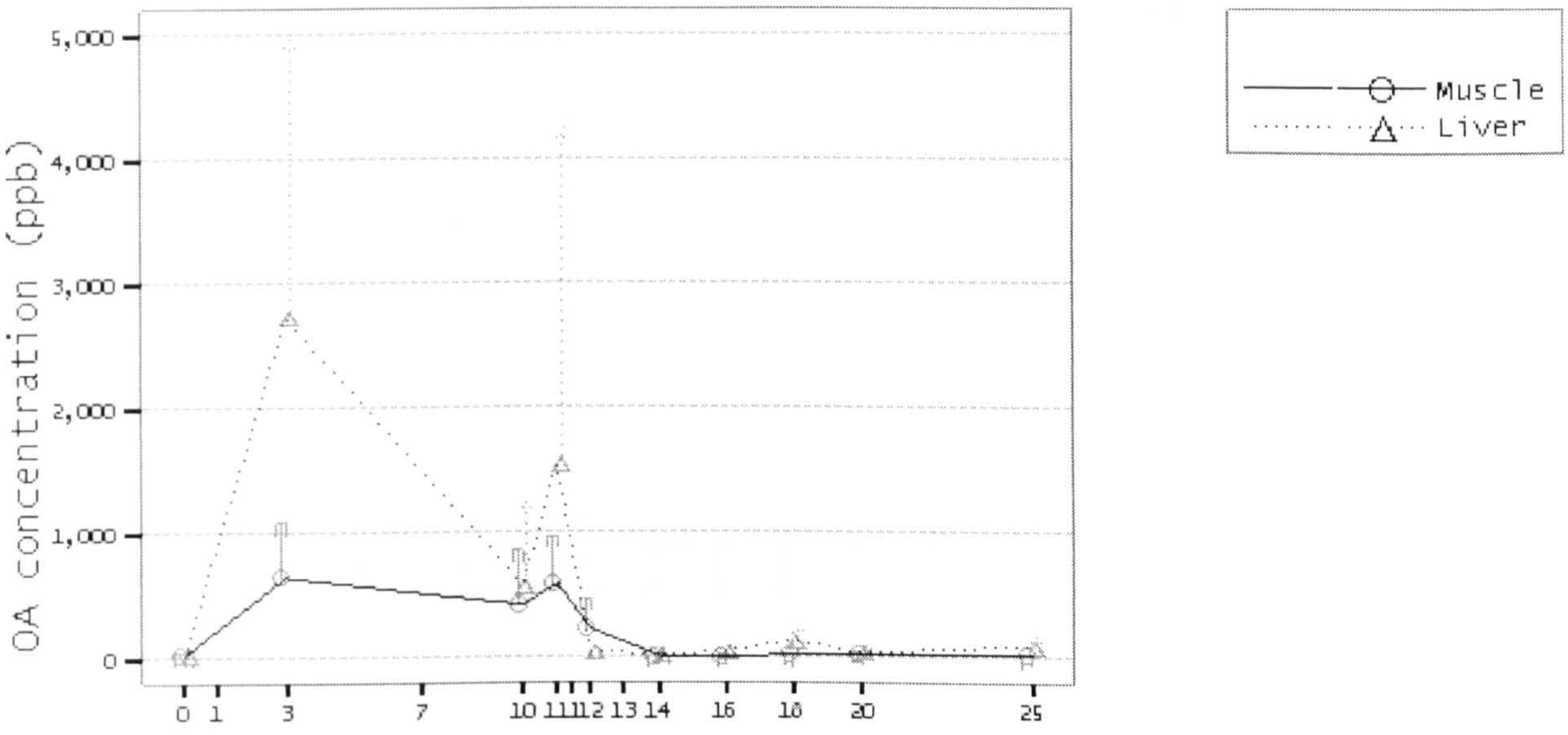
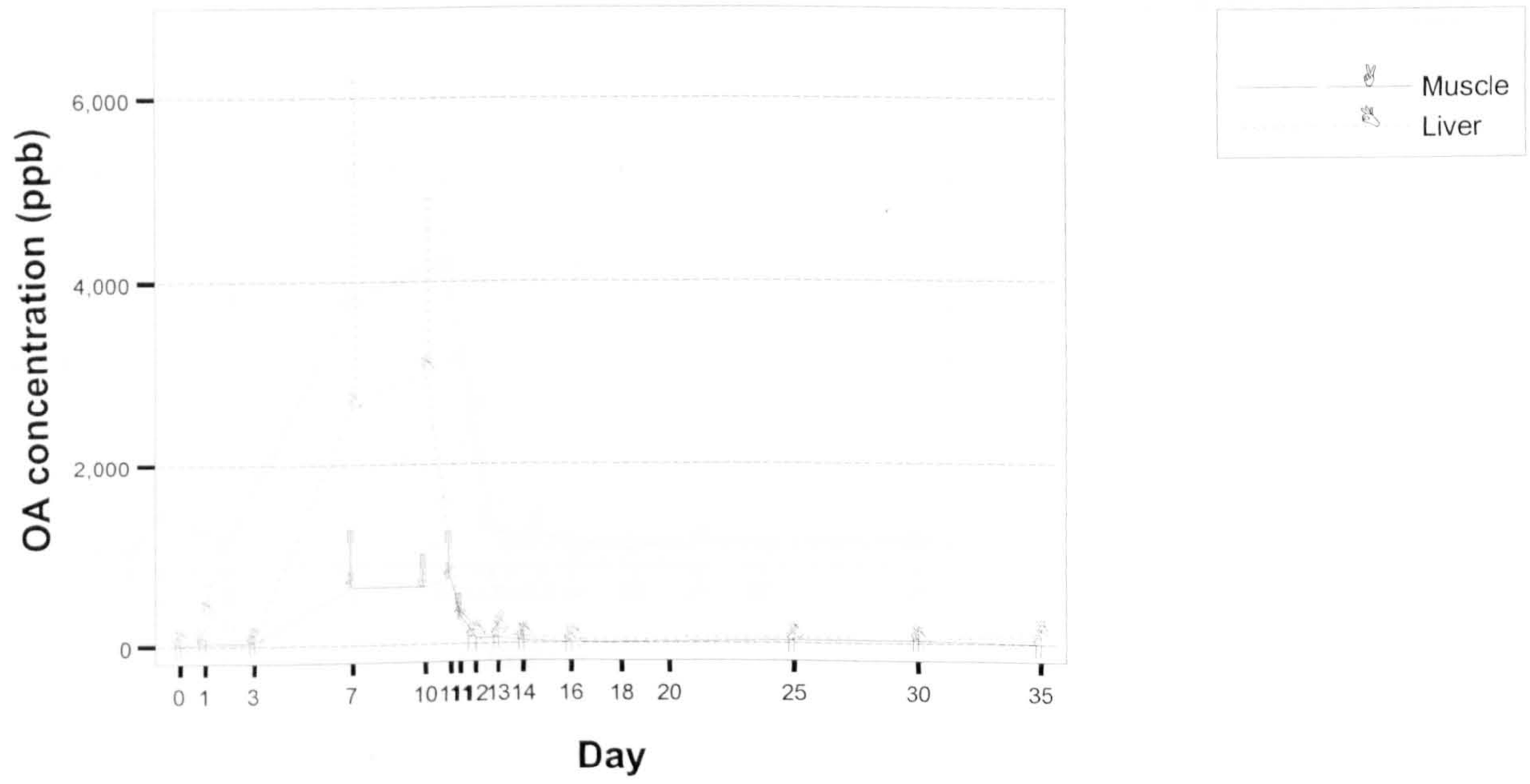


Figure 46: Comparison of OA mean concentration in the muscle and liver of sea bream at low temperature



3.3.2.2 Depletion at High versus Low temperature

Figure 47: Comparison of OA mean concentration in the muscle of sea bream at high and low temperature

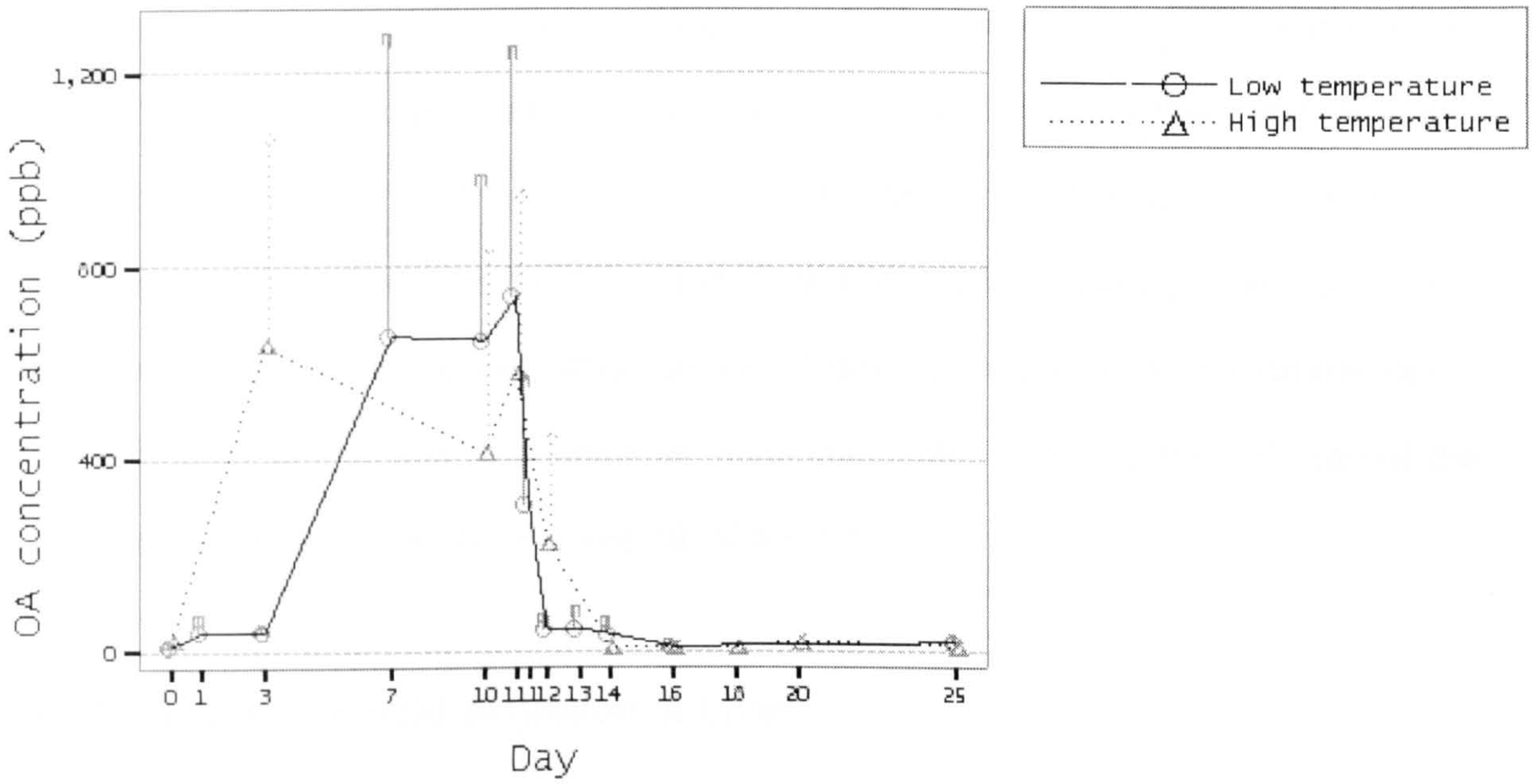
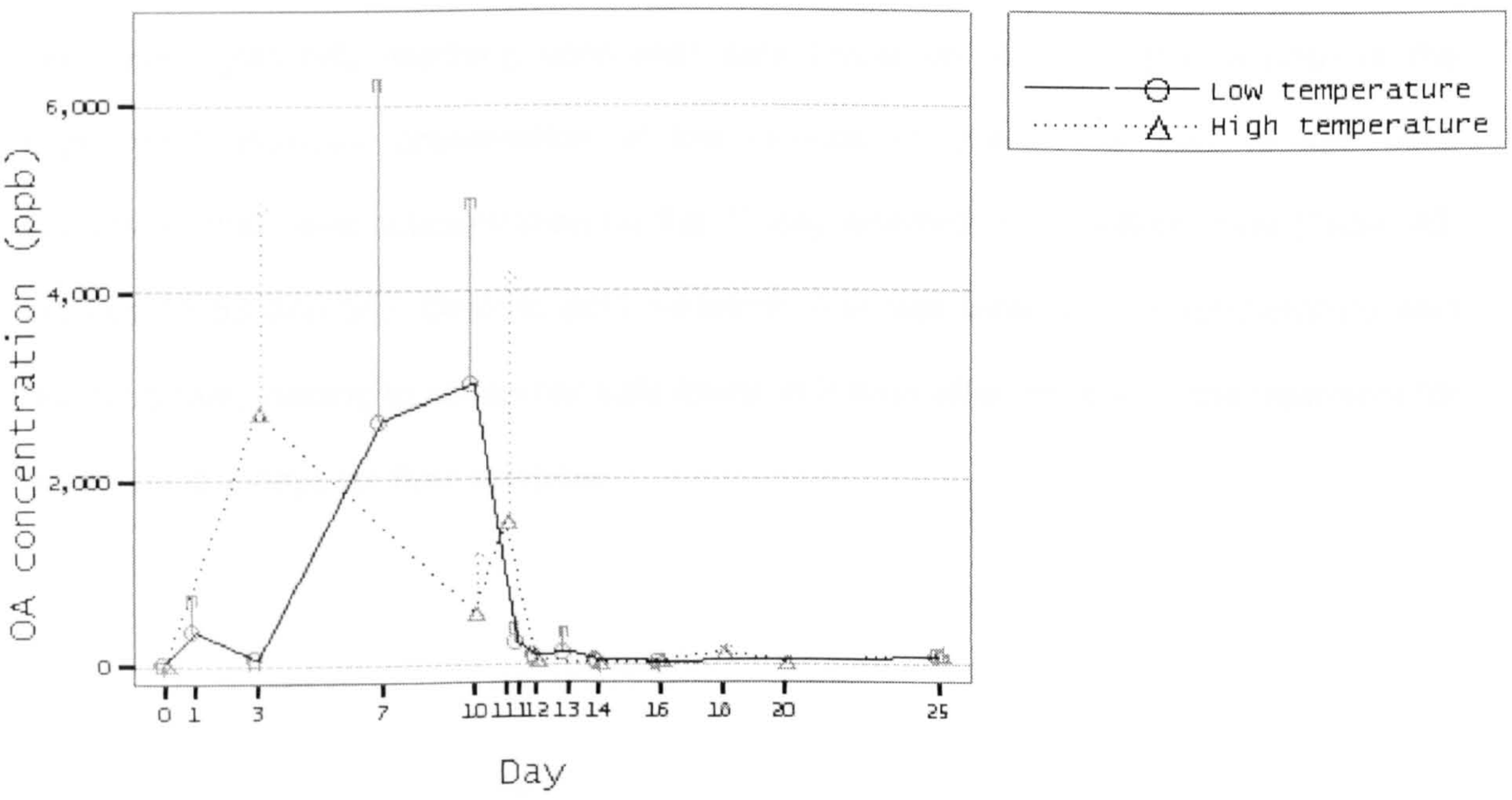


Figure 48: Comparison of OA mean concentration in the liver of sea bream at high and low temperature



3.4 Oxolinic Acid Kinetics in Sea Bass (*Dicentrarchus labrax*)

3.4.1.1 High Water Temperature

3.4.1.1.1 Oxolinic Acid depletion in Muscle

Maximum O.A concentration in the muscle of sea bass at high temperature was reached on the 7th day of the oral treatment (995 ppb) decreasing gradually thereafter, reaching consumer safe levels on the 14th day (4.05 ppb). Mean and Median concentration of O.A. reached a peak on the 11th day (250.09 and 352.58) decreasing thereafter. In the box-plot analysis, peak concentration on the 7th day considered was as extreme value. Concentration of OA was decreased to "consumer safe" levels by the 13th day of the experiment (78DD)(Table 46, Figures 49, 50and 51).

3.4.1.1.2 Oxolinic Acid depletion in Liver

Study of mean concentration revealed gradual increase to peak concentration of 718 ppb on the 10th day. O.A Concentration in the liver of sea bass at high temperature increased sharply from 718 ppb on the 1st day reaching almost double, in relation to the muscle, peak concentration on the 7th day (1861.8 ppb). Maximum concentration decreased gradually reaching consumer safe levels on the 13th day (8 ppb) of the experiment. Box-plot presentation of the kinetics of O.A in the liver of sea bass suggested that peak concentration on the 7th day referred to an outlier value (Table 46, Figures 52,53 and 54). Oxolinic acid metabolism in sea bass at high temperature was relatively fast, leading to consumer safe levels in 3 days after the end of the treatment for muscle and 2 days for liver samples.

Figure 49: Experiment III- Kinetics of Oxolinic acid in the muscle of Sea bass (*Dicentrarchus labrax*) at 26 ± 1°C

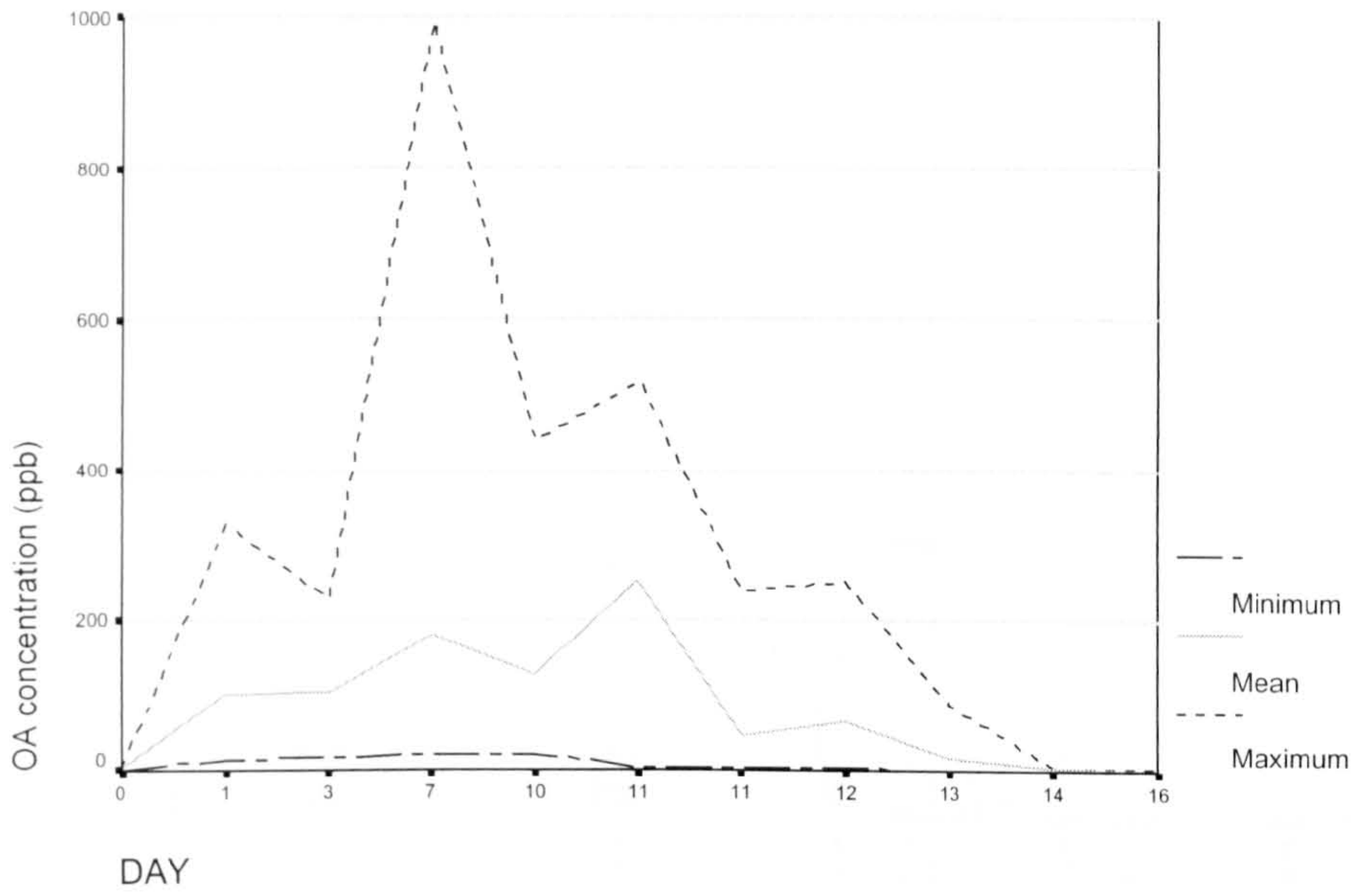
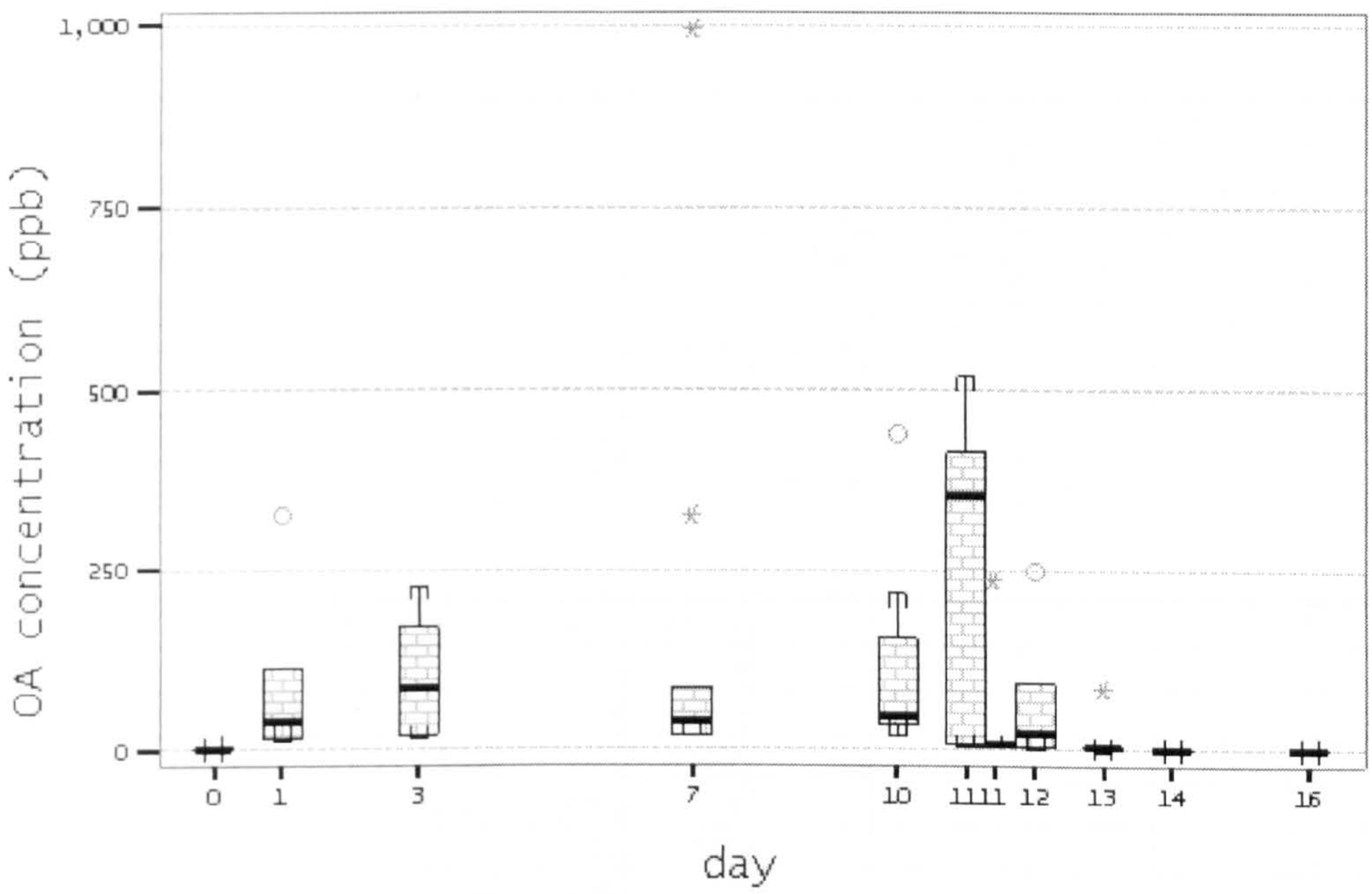


Figure 50: Experiment III- Kinetics of Oxolinic acid in the muscle of Sea bass

(*Dicentrarchus labrax*) at 26 ± 1°C – Mean and Standard deviation

Figure 51: Experiment III- Kinetics of Oxolinic acid in the muscle of Sea bass



(*Dicentrarchus labrax*) at 26 $\pm$ 1°C – Box plot presentation

Table 46: EXPERIMENT III Oxolinic acid kinetics in Sea bass at high temperature after 10 day oral treatment - Statistical interpretation

Statistical Analysis *Oxolinic acid concentration - Sea Bass Muscle - High Temperature*								
	Day 0	Day 1	Day 3	Day 7	Day 10	Day 11	Day 11 Aft.	Day 12
Samples	4	5	5	9	7	9	6	6
Minimum concentration(ppb)	1,35	14,09	15,55	19,36	20,95	3,92	3,75	3,24
Mean concentration(ppb)	2,79	102,43	104,39	178,94	126,62	250.096	47,16	66,64
Maximum concentration(ppb)	5,08	325,62	227,65	994,55	437,89	518,83	238,28	250,9
Standard deviation	1,67	131,17	92,97	320,96	152,97	209,37	93,71	96,28
25% percentile	1,45	15,37	18,94	22,06	27,25	9,72	5,07	4,96
Median	2,38	41,55	85,66	41,28	47,17	352,58	10,78	23,62
75% Percentile	4,5	219,92	199,21	208,38	216,61	430,13	69,95	132,42

	Day 13	Day 14	Day 16					
Samples	7	4	6					
Minimum concentration(ppb)	0	0	0					
Mean concentration(ppb)	15,82	1,76	1,47					
Maximum concentration(ppb)	88,33	4,05	2,76					
Standard deviation	32,1	1,83	0,99					
25% Percentile	1,19	0,05	0,6					
Median	4,9	1,64	1,5					
75% Percentile	7,2	3,6	2,3					

Oxolinic acid concentration - Sea Bass Liver - High Temperature								
	Day 0	Day 1	Day 3	Day 7	Day 10	Day 11	Day 11 Aft.	Day 12
Samples	7	8	10	9	7	8	9	8
Minimum concentration(ppb)	2,7	20,93	35.056	14,25	22,14	20,18	13,98	1,86
Mean concentration(ppb)	8,16	224,79	435,04	444,84	717,54	451	93,76	34
Maximum concentration(ppb)	16,07	717,57	1336,78	1861,82	1366,16	1275,64	226,41	91,61
Standard deviation	5,7	275,52	456,38	606,56	537,27	454,9	82,43	27,34
25% percentile	2,75	30,52	68,13	23,76	33,02	138,08	19,26	11,78
Median	6,81	80,74	225,69	152,18	859,66	220,75	60,8	33,44
75% Percentile	14..26	501,18	832,45	698,6	1174,44	874,95	173,61	41,45

Sea Bass Liver - High Temperature								
	Day 13	Day 14	Day 16					
Samples	5	9	7					
Minimum concentration(ppb)	1,62	0,15	0					
Mean concentration(ppb)	3,53	7,28	3,02					
Maximum concentration(ppb)	7,75	26	11,94					
Standard deviation	2,43	8,46	5,17					
25% Percentile	1,97	0,453	0					
Median	2,61	7,34	0,4					
75% Percentile	5,56	11,4	8,92					

Figure 52: Experiment III- Kinetics of Oxolinic acid in the liver of Sea bass (*Dicentrarchus labrax*) at 26 ± 1°C

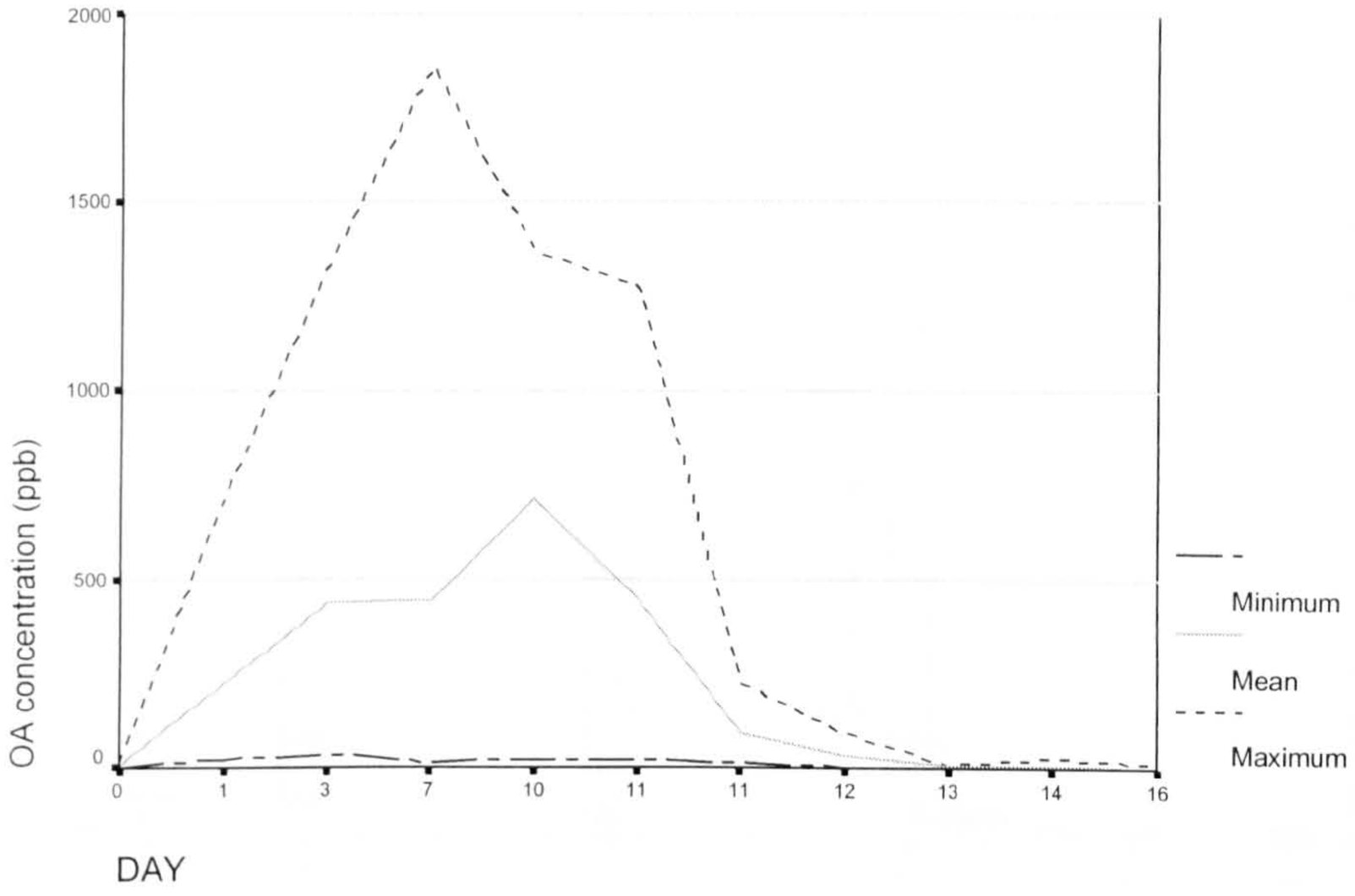


Figure 53: Experiment III- Kinetics of Oxolinic acid in the liver of Sea bass (*Dicentrarchus labrax*) at 26 ± 1°C – Mean and Standard deviation

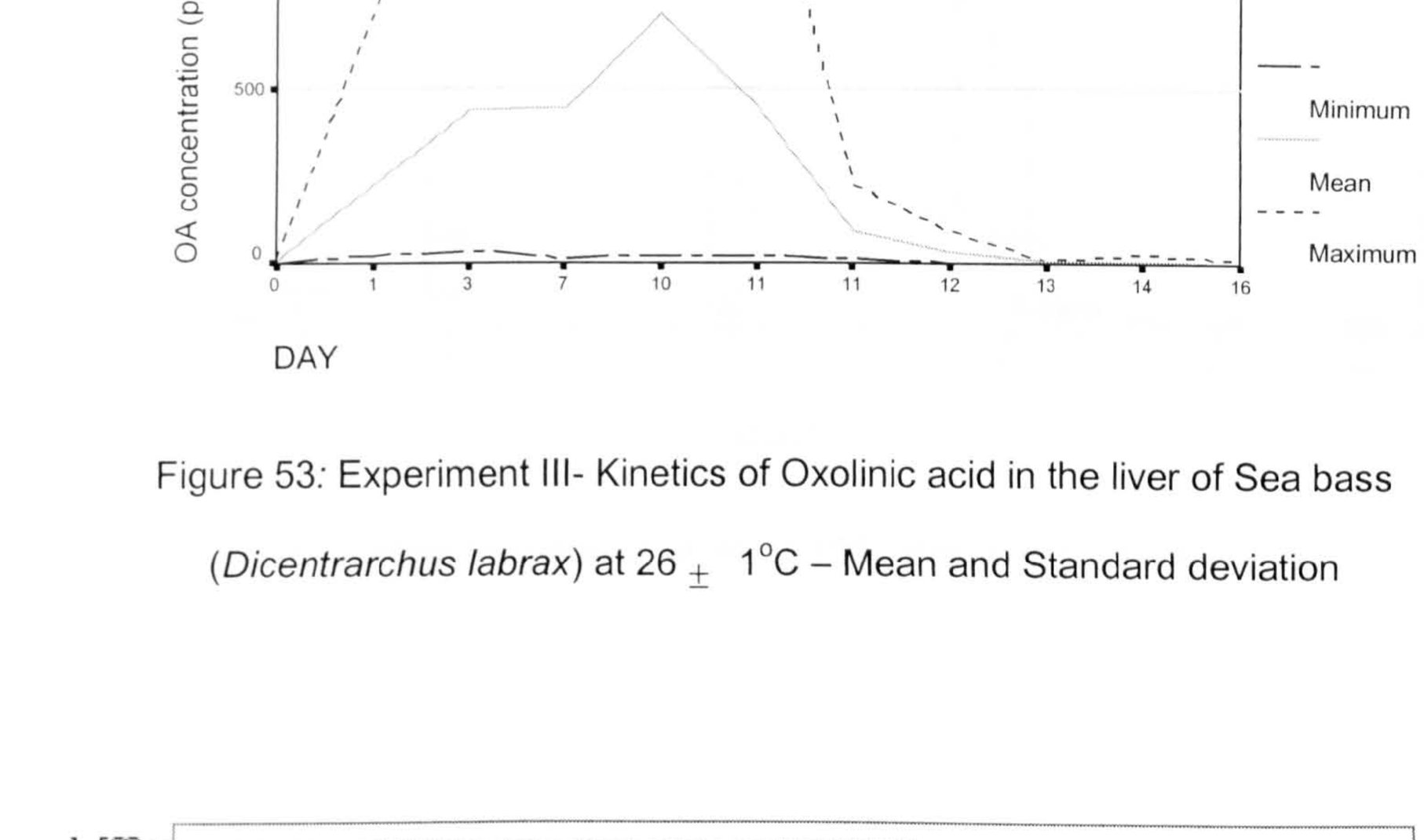
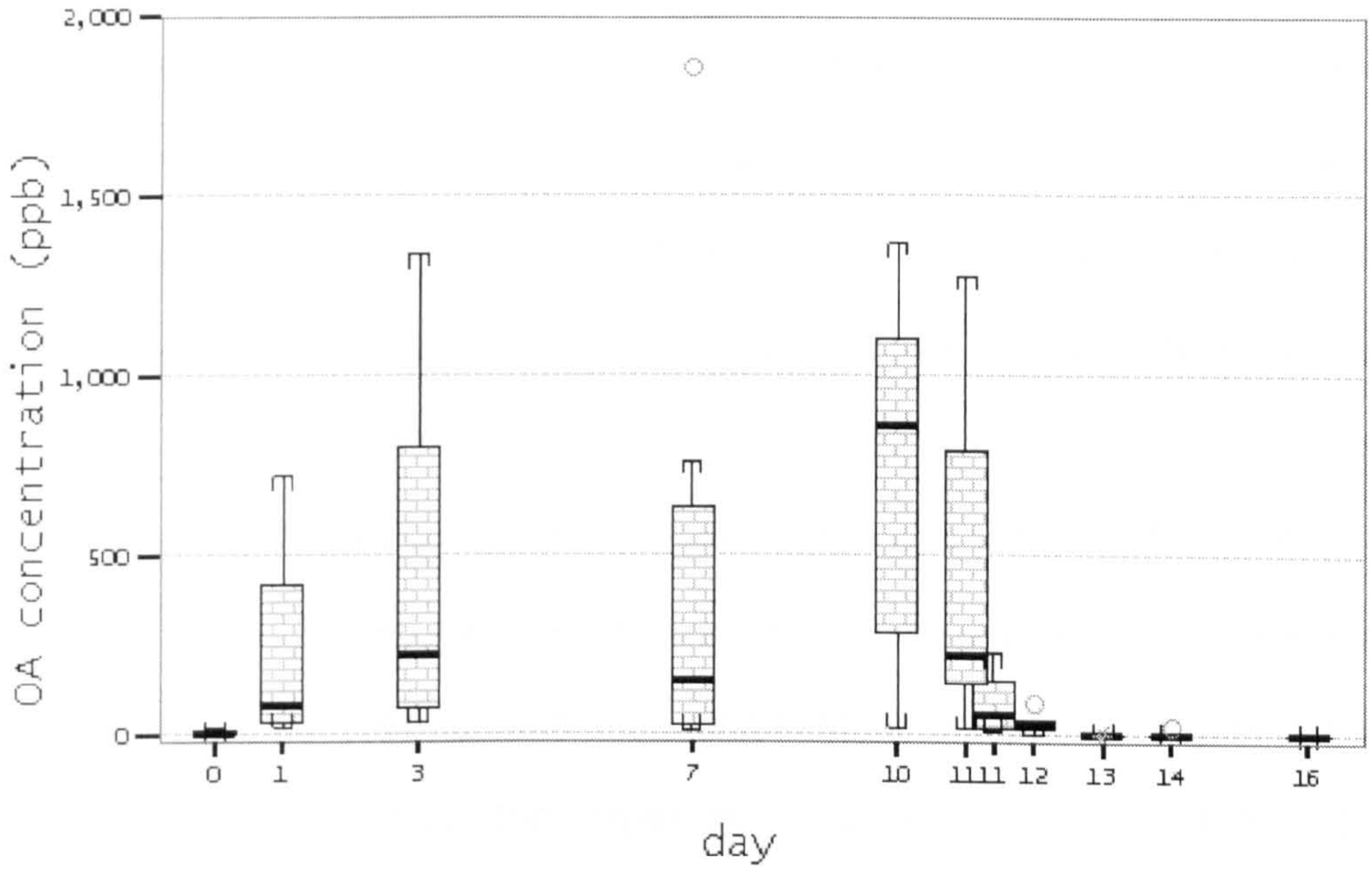


Figure 54: Experiment III- Kinetics of Oxolinic acid in the liver of Sea bass



(*Dicentrarchus labrax*) at 26 $\pm$ 1°C– Box–plot presentation

3.4.1.2 Low Water Temperature

3.4.1.2.1 Oxolinic Acid depletion in Muscle

Standard deviation of the O.A muscle concentration was very high. Concentration maximum was presented in the 10th day (1321.78 ppb). Then it was decreased sharply until the afternoon of the 11th day (84.54 ppb) and remained low until the 16th day (21.74 ppb) when an "odd" increase, lead to moderate peak concentration on the 18th day (814.1 ppb). O.A concentration decreased to consumer safe levels from the 30th day (37.17 ppb) onwards. An O.A concentration plateau was exhibited between 1st (1109.86 ppb) and 3rd day (1107.46 ppb) as well as a slight decrease was evident on the 7th day (844.02 ppb) of the feeding with the antimicrobial compound. Figure 51 presented boxplot analysis throughout the experimental period. In the first 11 days of the experiment, median O.A concentration in the muscle of Sea bass increased gradually achieving highest concentration on the 7th day. Extreme concentration was present in a sample of muscle on the 3rd day. Extreme value existed in one sample on the 14th day. All samples had O.A concentrations under 100ppb and while the general trend was a gradual decline, median concentration persisted around 30ppb until the 13th day and then decreased to around 10ppb. Although initial graphs indicated a sharp increase in O.A muscle concentration on day 18 it was revealed in the following Figure that all samples gave concentrations below 100ppb with the exception of only one sample on the 18th day (around 800ppb)(Table 47, Figures 55,56 and 57).

3.4.1.2.2 Oxolinic Acid depletion in Liver

The concentration of Oxolinic acid obtained in the liver of sea bass that was treated orally for 10 days at water temperature 18 $\pm$ 1 $\circ$ C was presented in the following Figures (58,59 and 60). Maximum and mean concentration fluctuated for a period of twenty days (Table 47). Maximum concentration was achieved on the 7th day (2954

ppb) fluctuating to 1516 ppb and the 1st and 599 ppb on the 3rd day. Maximum concentration decreased gradually to 577 ppb on the afternoon of the 11th day, increased on the 12th day (1694 ppb) and was reduced to 564ppb on the 16th day before presenting an "odd" peak (1285 ppb) on the 18th day. Finally in the following days concentration was decreased sharply until the 20th day and gradually thereafter to reach consumer safe levels on the 30th day (91 ppb). Box –plot presentation of the kinetics of O.A in the liver of Sea bass indicated that maximum concentration reported on days 12 and 18 referred to extreme values. It was evident therefore that consumer safe levels of O.A concentration in the muscle and liver of sea bass at prevailing water temperature of 18◦ C were reached 20 days after the cessation of the oral treatment (after 340 degree days). Maximum O.A concentration achieved in the liver was twice the concentration achieved in the muscle of sea bass at both high and low temperature (Figures 61 and 62) Peak mean concentration in the liver was 2.6 times the muscle concentration at low temperature and 2.9 times the muscle concentration achieved at high temperature. Oxolinic acid metabolism at low temperature was much slower and twenty days after the end of the treatment were needed for consumer safe levels to be reached for both muscle and liver samples as opposed to fast metabolism present at high temperature, when 4 days post treatment were sufficient (Figures 63 and 64). Peak mean concentration in the muscle at low temperature was 1.7 times the concentration achieved at high temperature, while mean concentration achieved in the liver at low temperature was 1.5 times the concentration achieved at high temperature. Sea bream assimilated OA better than sea bass achieving higher concentrations in both tissues and both temperature areas. Depletion was similar at high temperature but seem to take longer in sea bass at low temperature (Figures 65,66,67 and 68).

Table 47: EXPERIMENT IV Oxolinic acid kinetics in Sea bass at low temperature after 10 day oral treatment - Statistical interpretation

Statistical Analysis *Oxolinic acid concentration - Sea Bass Muscle - Low Temperature*								
	Day 0	Day 1	Day 3	Day 7	Day 10	Day 11	Day 11 Aft.	Day 12
Samples	10	6	9	9	9	8	9	7
Minimum concentration(ppb)	0,00	8,02	8,73	80,36	32,46	2,78	8,24	11,82
Mean concentration(ppb)	9,73	332,42	245,11	433,21	410,96	174,79	43,27	33,85
Maximum concentration(ppb)	17,27	1109,86	1107,46	844,02	1321,78	610,39	84,54	63,38
Standard deviation	5,62	488,14	342,91	310,49	479,65	226,75	36,03	18,80
25% percentile	6,00	13,26	44,24	143,38	87,39	14,15	9,77	13,65
Median	9,40	37,37	141,69	391,32	134,45	74,74	31,69	32,30
75% Percentile	15,68	867,64	312,48	803,98	875,82	368,63	82,65	47,97

	Day 13	Day 14	Day 16	Day 18	Day 20	Day 25	Day 30	Day 35
Samples	6	9	9	5	9	8	10	8
Minimum concentration(ppb)	11,07	6,17	3,06	10,23	24,61	21,77	9,78	2,74
Mean concentration(ppb)	29,28	14,89	11,89	192,96	67,96	79,21	20,10	12,39
Maximum concentration(ppb)	49,07	38,76	21,74	814,10	137,50	173,64	37,17	22,63
Standard deviation	14,18	10,10	7,79	349,79	32,44	50,68	9,56	7,49
25% Percentile	13,74	7,80	4,30	10,71	46,99	30,60	11,93	6,80
Median	32,55	13,15	9,34	18,65	65,22	85,10	16,88	10,15
75% Percentile	39,14	18,03	20,15	462,37	83,20	101,51	29,01	20,80

Oxolinic acid concentration - Sea Bass Liver - Low Temperature								
	Day 0	Day 1	Day 3	Day 7	Day 10	Day 11	Day 11 Aft.	Day 12
Samples	10	6	8	6	9	8	9	8
Minimum concentration(ppb)	7,99	28,82	34,03	176,90	25,08	9,83	31,01	19,17
Mean concentration(ppb)	17,18	901,97	227,39	1112,10	409,66	213,92	124,64	278,77
Maximum concentration(ppb)	31,48	1567,12	598,60	2954,24	1518,80	711,82	577,23	1694,35
Standard deviation	7,75	546,43	226,46	1011,89	541,82	300,10	174,91	575,00
25% percentile	10,67	432,80	45,35	433,08	62,44	31,35	37,45	32,27
Median	16,37	992,50	110,67	730,61	126,87	61,35	51,69	72,50
75% Percentile	23,70	1339,33	454,38	1909,88	775,11	540,12	134,43	178,25

Sea Bass Liver - Low Temperature								
	Day 13	Day 14	Day 16	Day 18	Day 20	Day 25	Day 30	Day 35
Samples	8	8	9	9	8	7	10	9
Minimum concentration(ppb)	40,20	15,83	22,36	45,78	38,86	24,75	10,24	15,83
Mean concentration(ppb)	206,60	237,97	239,41	275,57	84,67	67,31	31,52	37,96
Maximum concentration(ppb)	1057,53	597,23	564,88	1285,34	204,63	135,25	91,37	79,90
Standard deviation	346,87	234,37	236,12	426,54	52,00	49,70	22,40	19,65
25% Percentile	47,12	27,09	31,05	50,21	52,24	26,62	20,43	19,18
Median	84,02	197,82	68,27	81,10	69,55	39,26	27,66	37,21
75% Percentile	163,58	437,51	479,47	409,66	93,67	132,93	33,26	46,75

Figure 55: Experiment IV - Kinetics of Oxolinic acid in the muscle of Sea bass (*Dicenrarchus labrax*) at 18 ± 1 °C

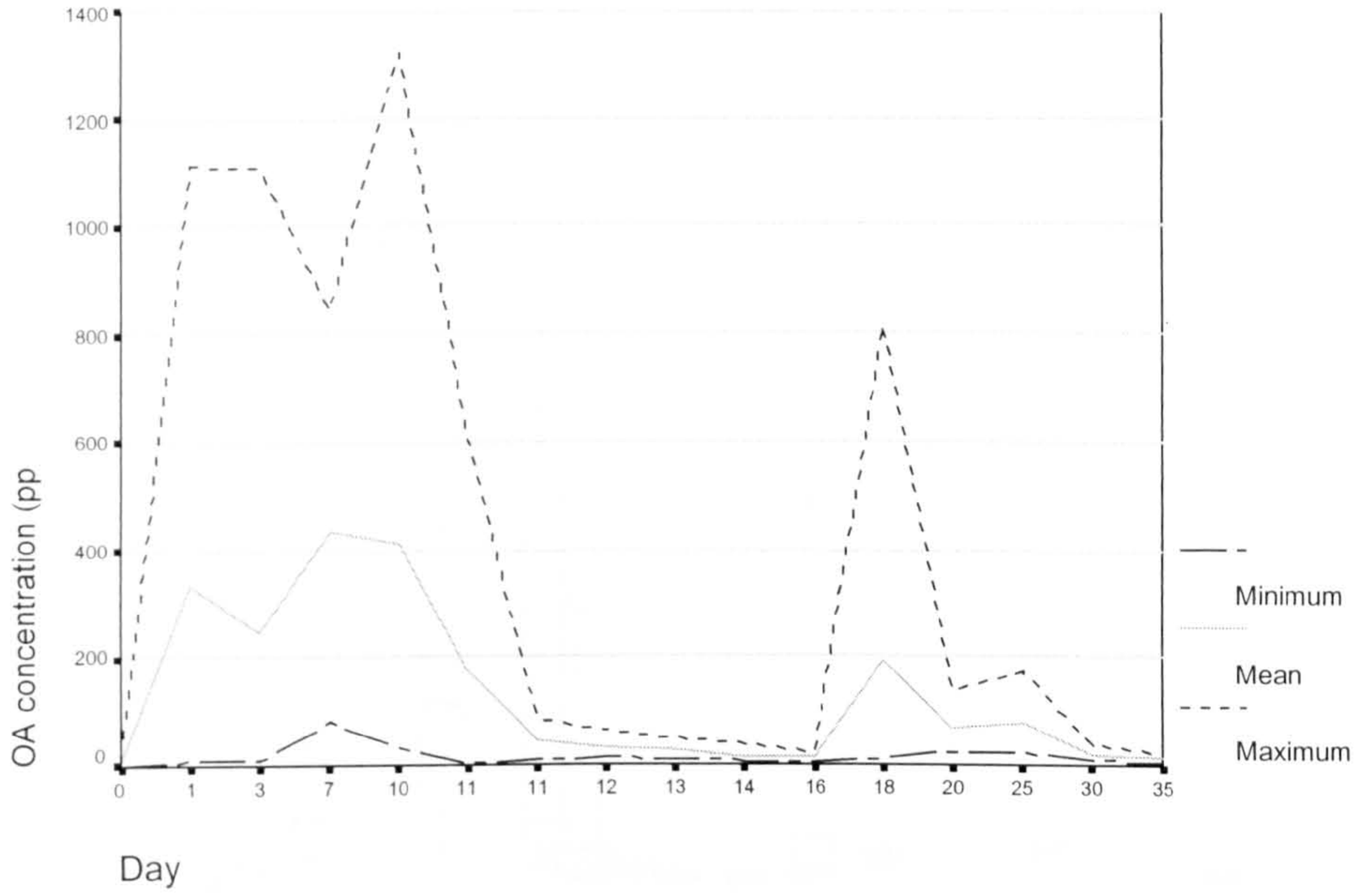


Figure 56: Experiment IV - Kinetics of Oxolinic acid in the muscle of Sea bass (*Dicenrarchus labrax*) at 18 ± 1°C - Mean and Standard deviation

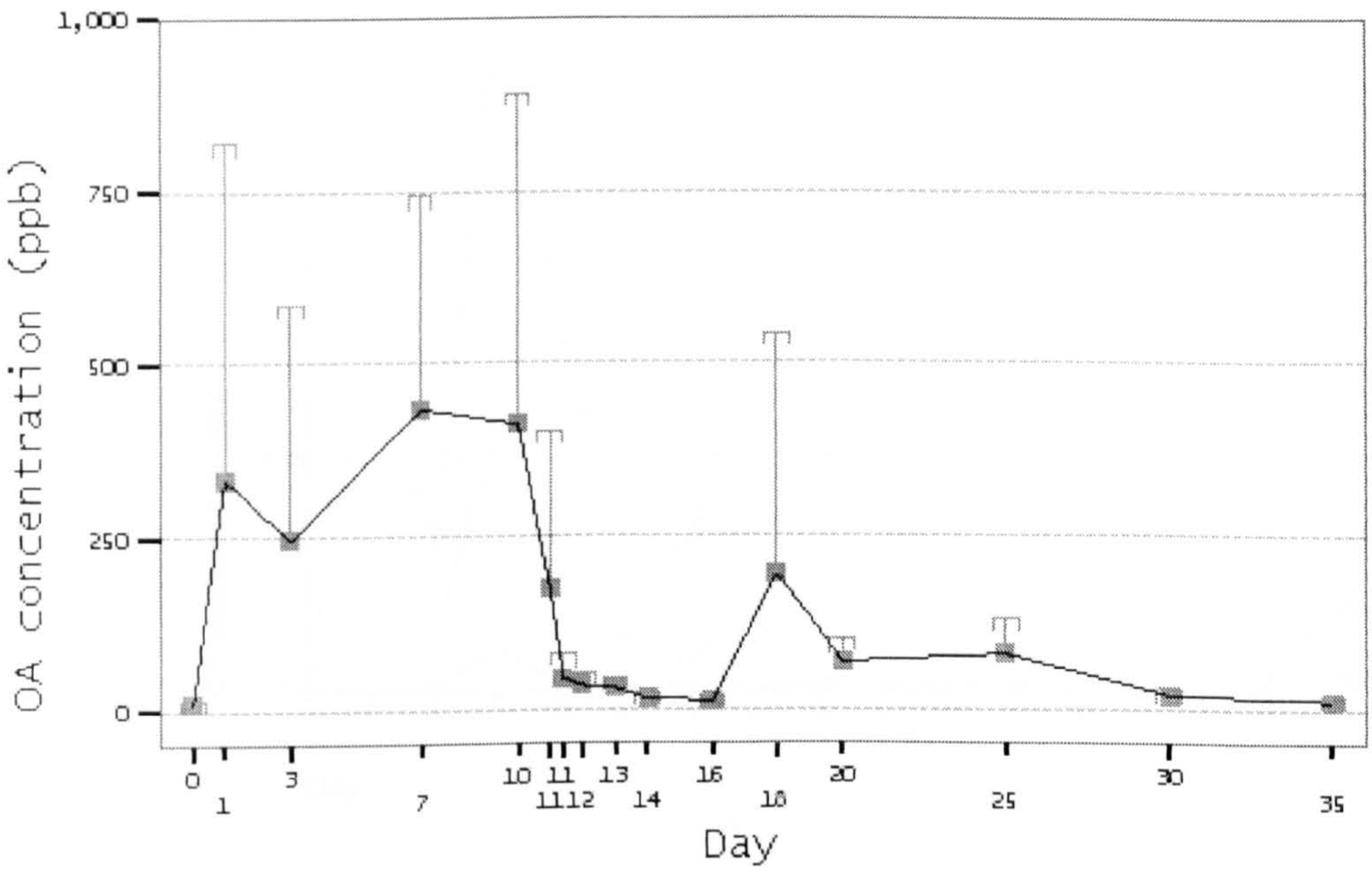


Figure 57: Experiment IV - Kinetics of Oxolinic acid in the muscle of Sea bass (*Dicenrarchus labrax*) at 18 $\pm$ 1°C - Box-plot presentation

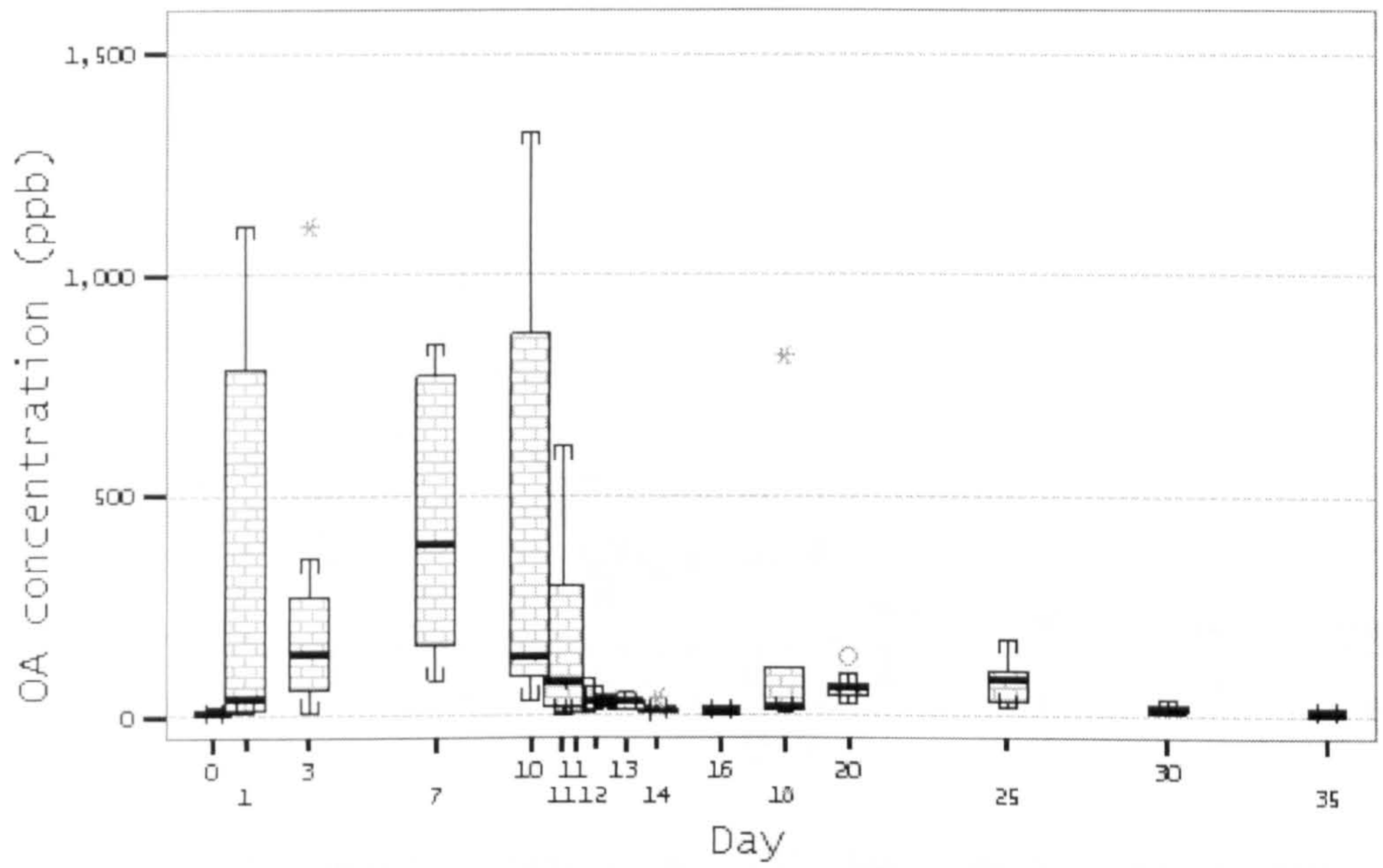


Figure 58: Experiment IV - Kinetics of Oxolinic acid in the liver of Sea bass (*Dicenrarchus labrax*) at 18 $\pm$ 1°C

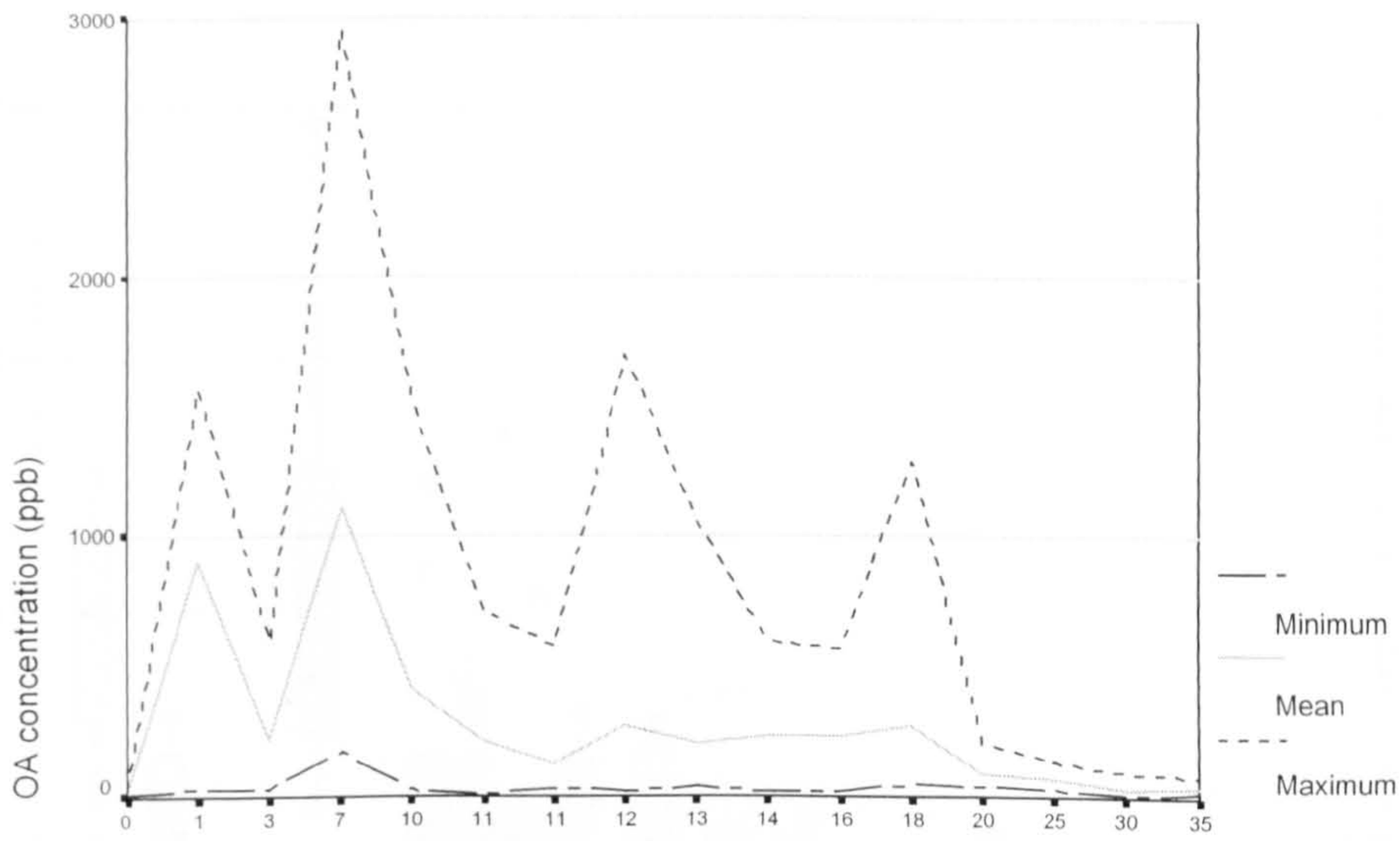
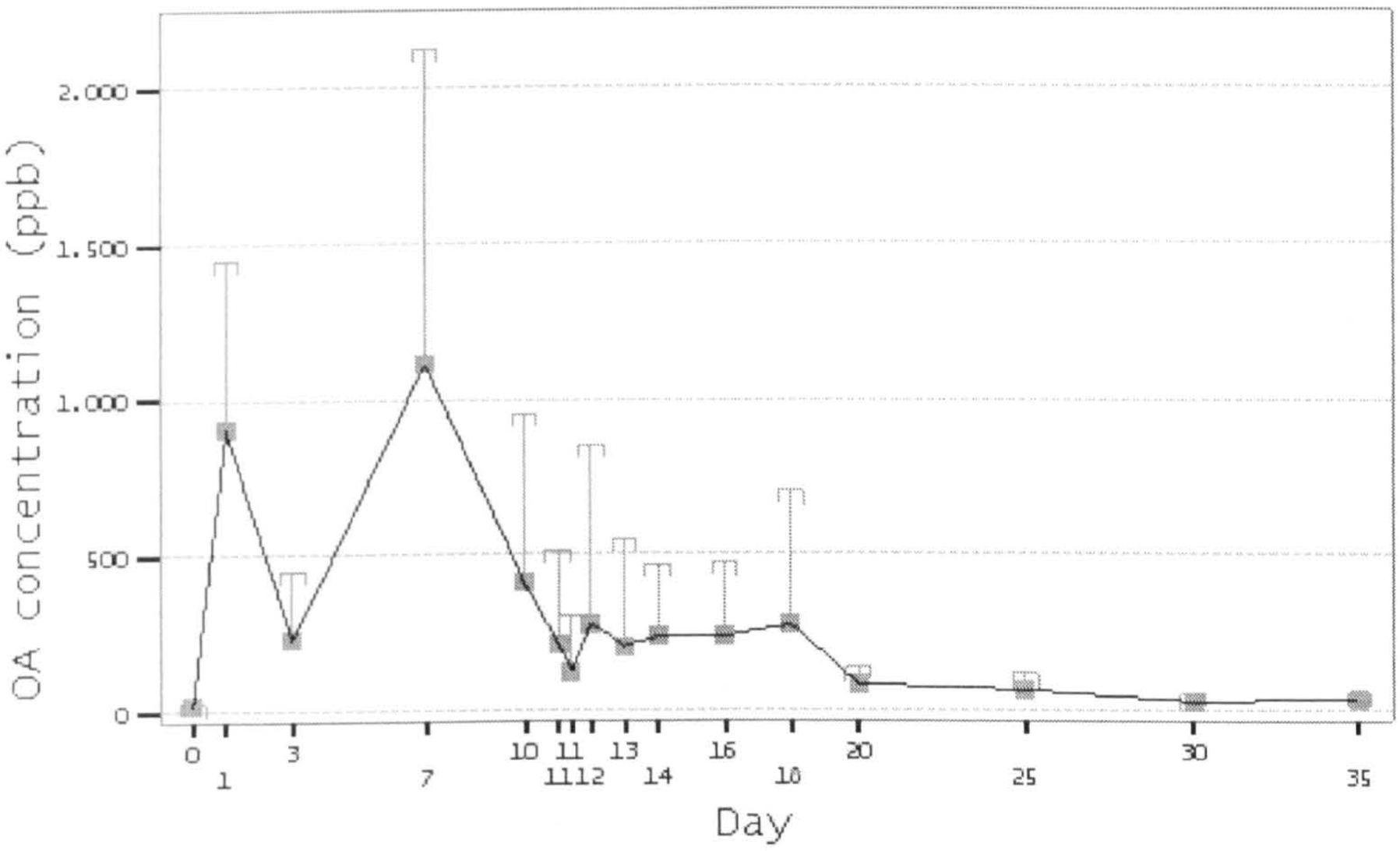
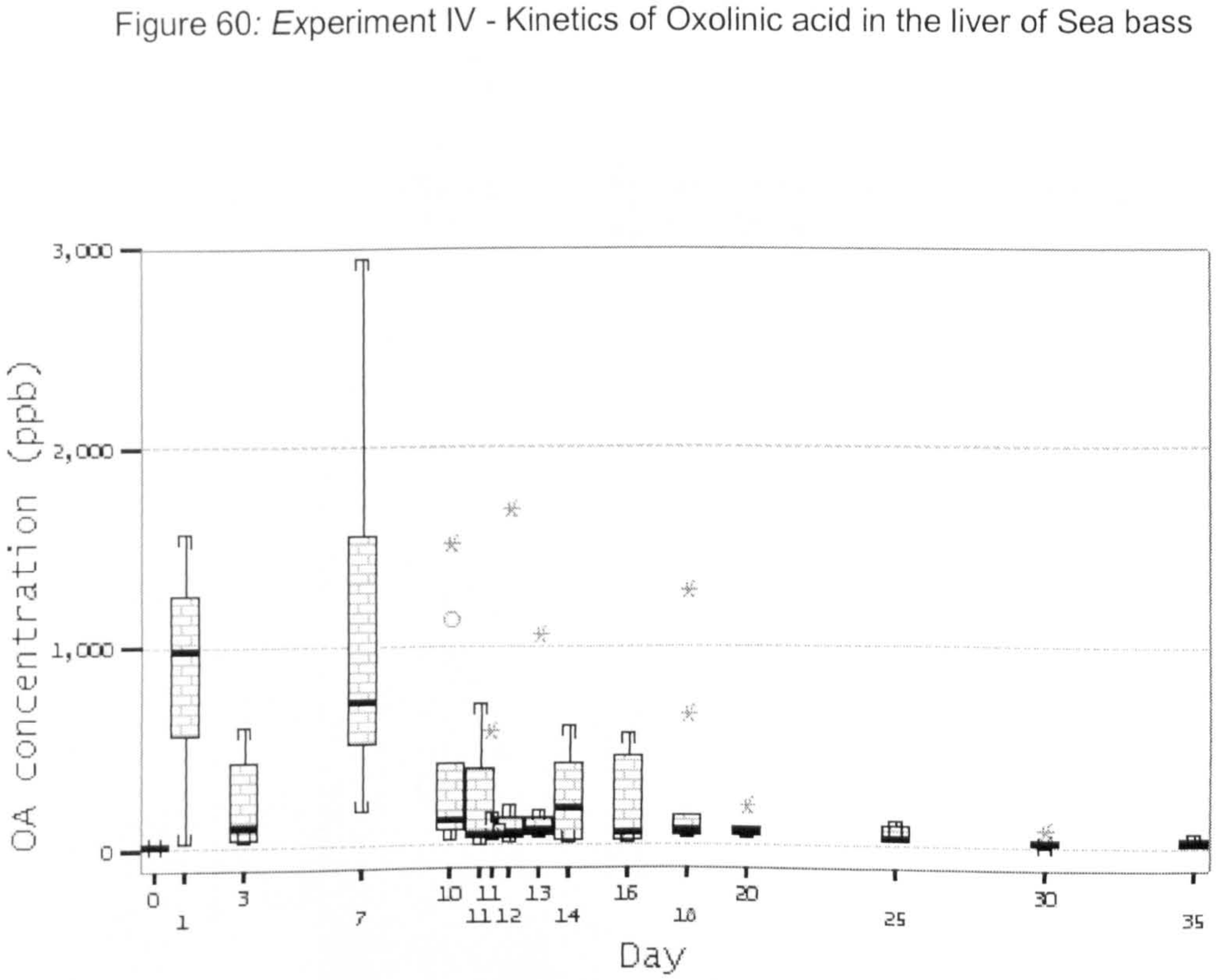


Figure 59: Experiment IV - Kinetics of Oxolinic acid in the liver of Sea bass



(Dicenrarchus labrax) at 18 $\pm$ 1°C - Mean and Standard deviation

Figure 60: *Ex*periment IV - Kinetics of Oxolinic acid in the liver of Sea bass



(*Dicenrarchus labrax*) at 18 $\pm$ 1°C - Box-plot presentation

3.4.2 Comparisons

3.4.2.1 Muscle Depletion versus liver Depletion

Figure 61: Comparison of OA mean concentration in the muscle and liver of sea bass at high temperature

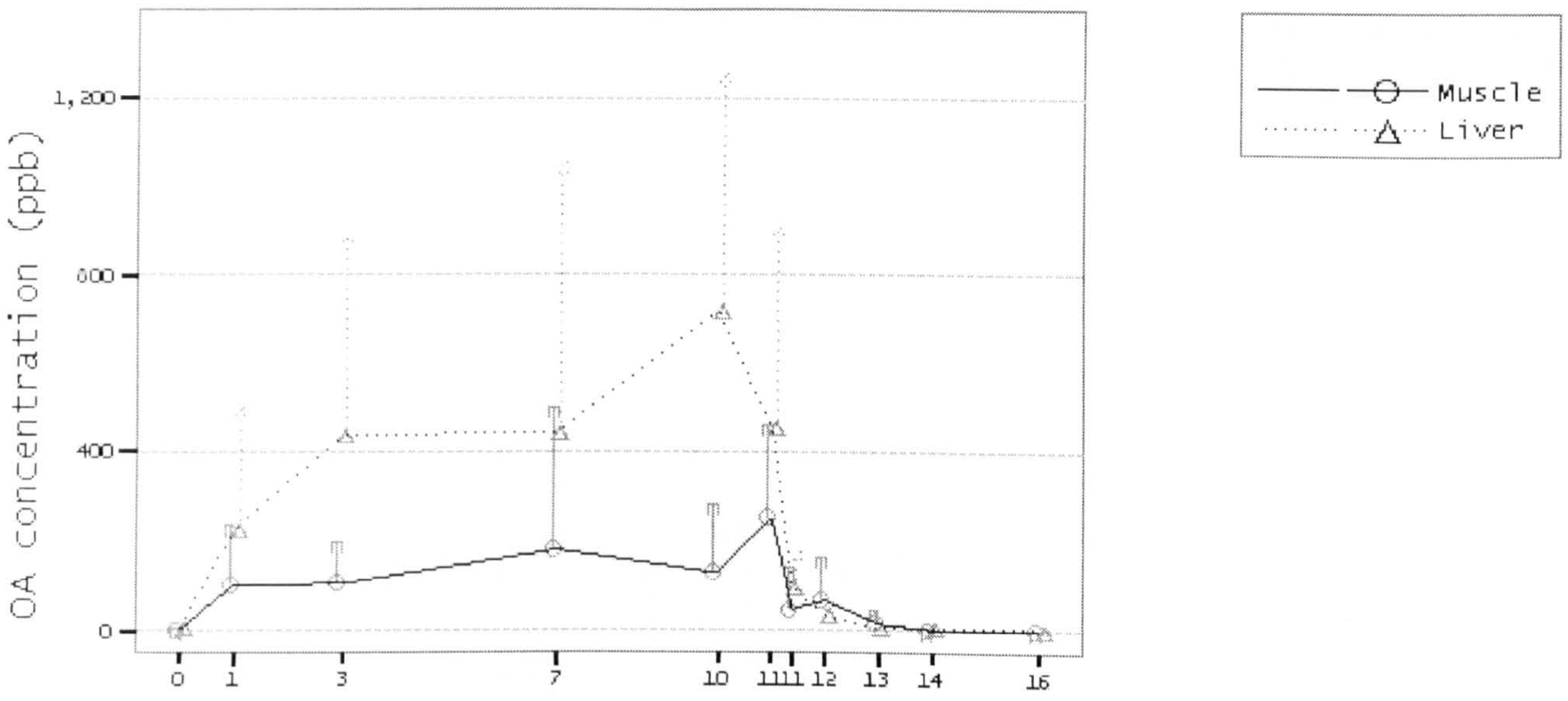
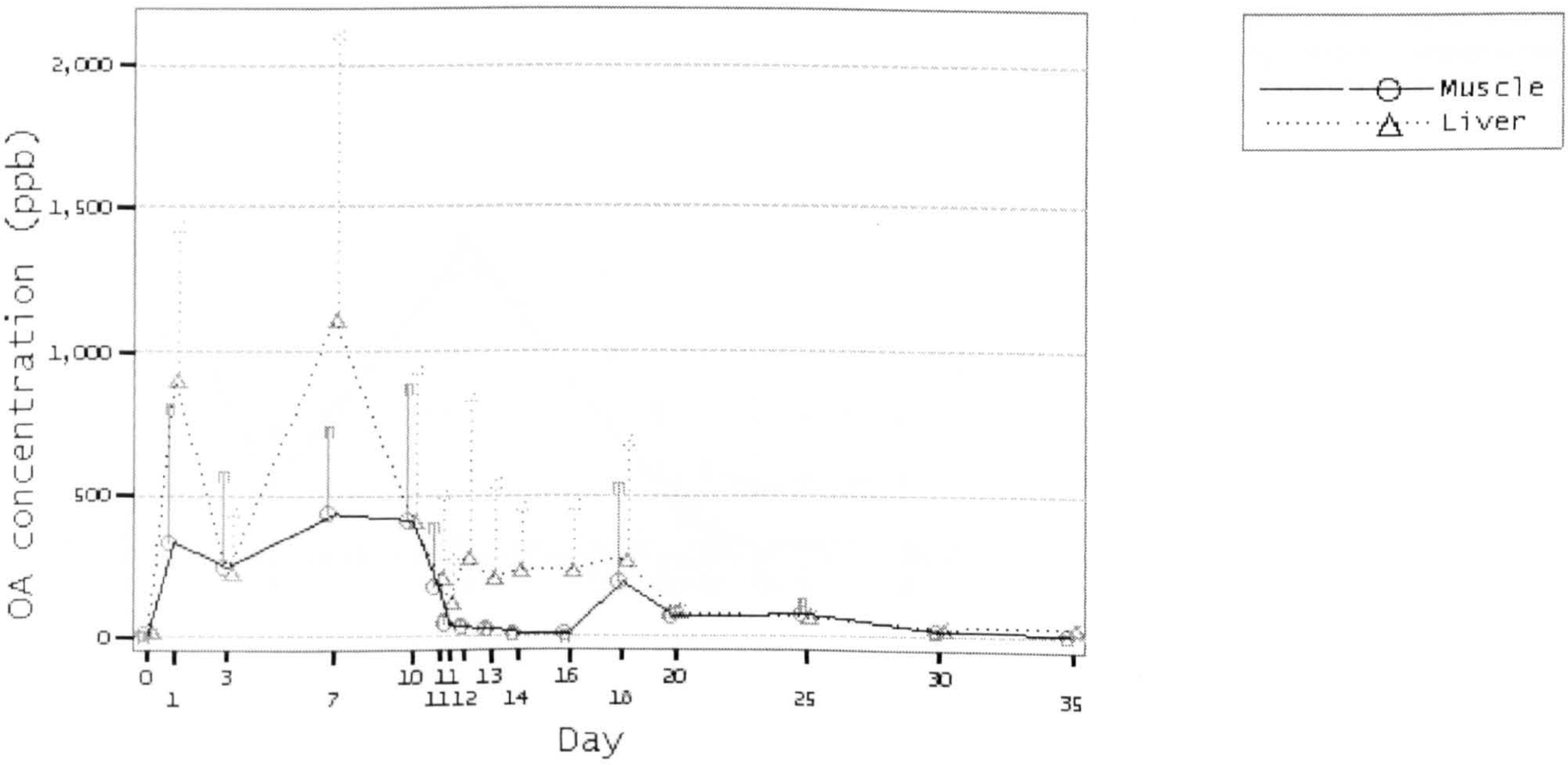


Figure 62: Comparison of OA mean concentration in the muscle and liver of sea bass at low temperature



3.4.2.2 Depletion at High versus Low temperature

Figure 63: Comparison of OA mean concentration in the muscle of sea bass at low and high temperature

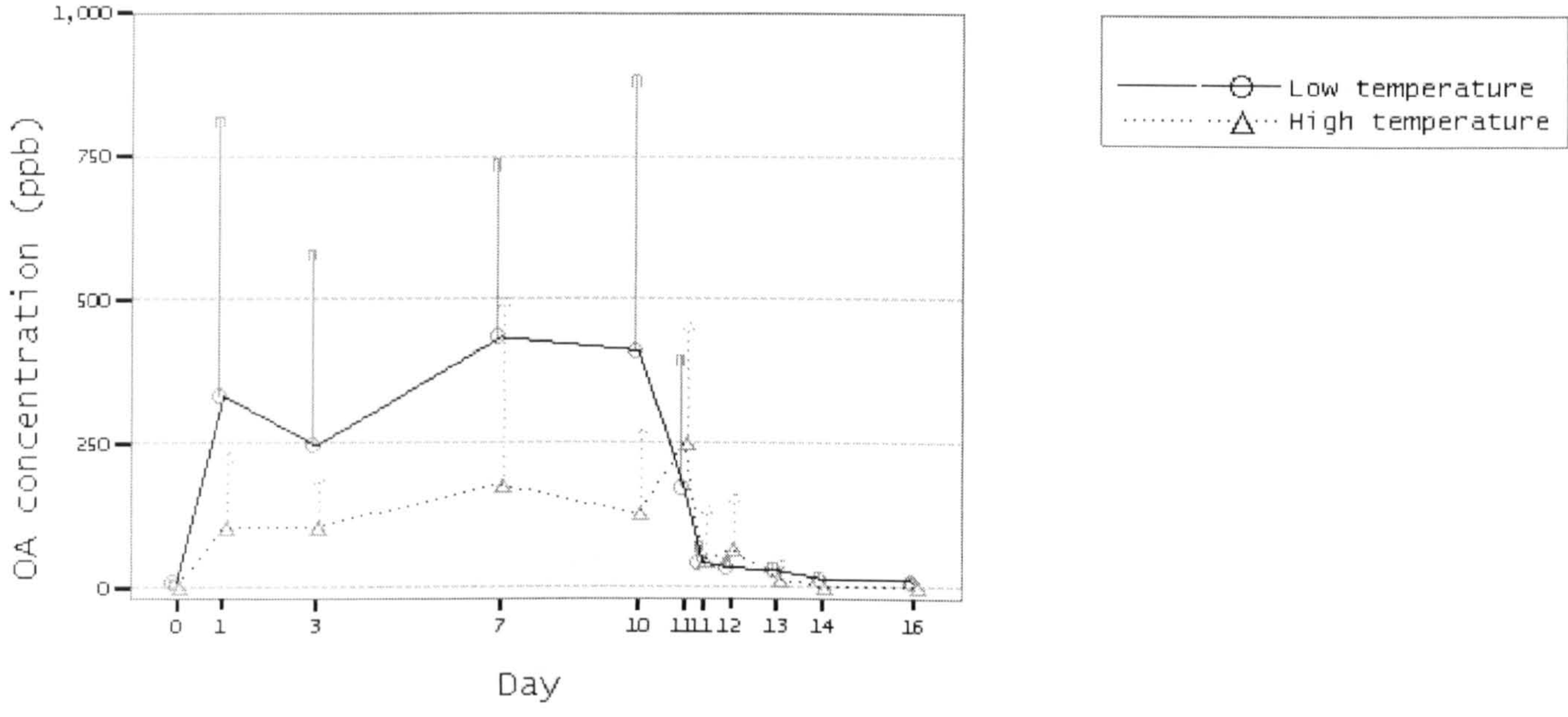
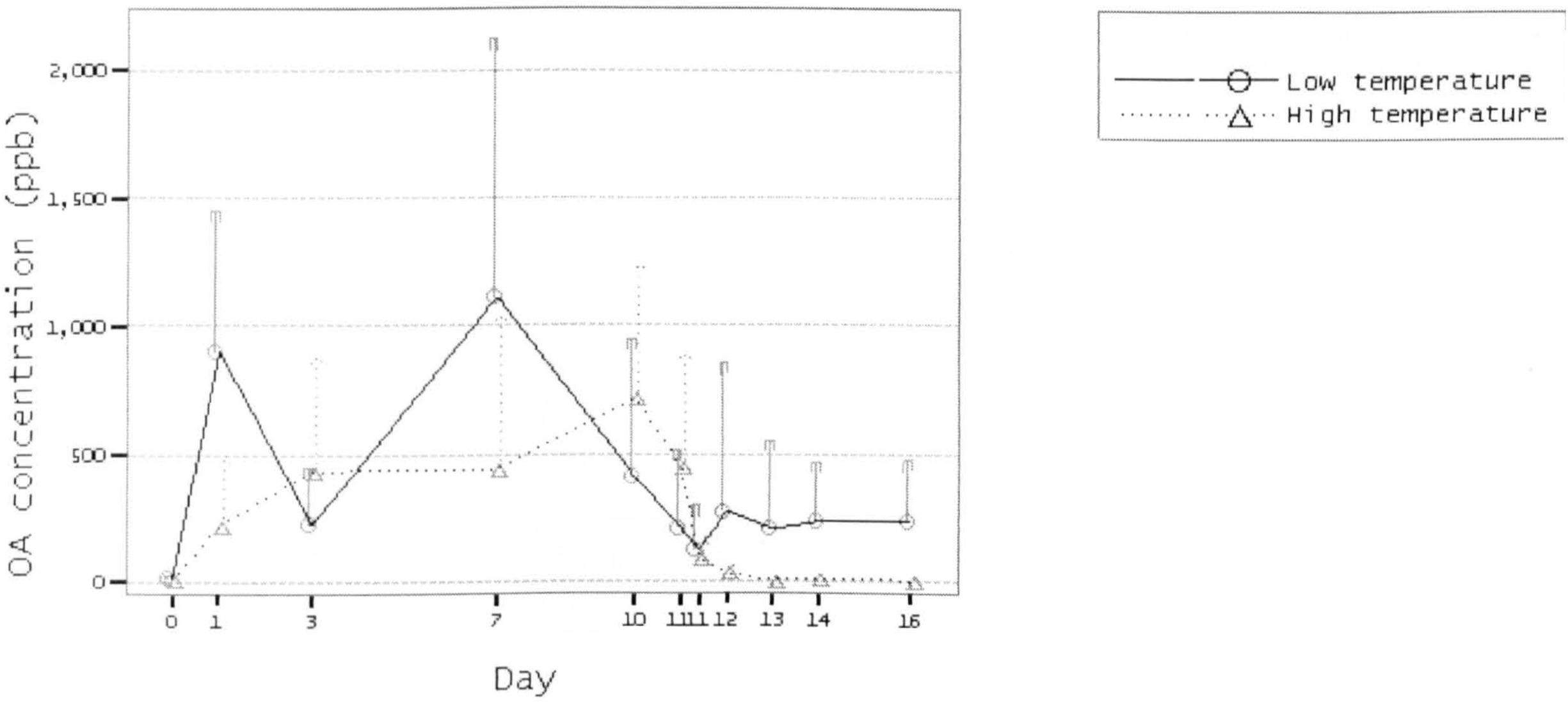


Figure 64: Comparison of OA mean concentration in the liver of sea bass at low and high temperature



3.5 Oxolinic Acid species comparisons

Figure 65: Comparison of OA mean concentration in the muscle of sea bream and sea bass at high temperature

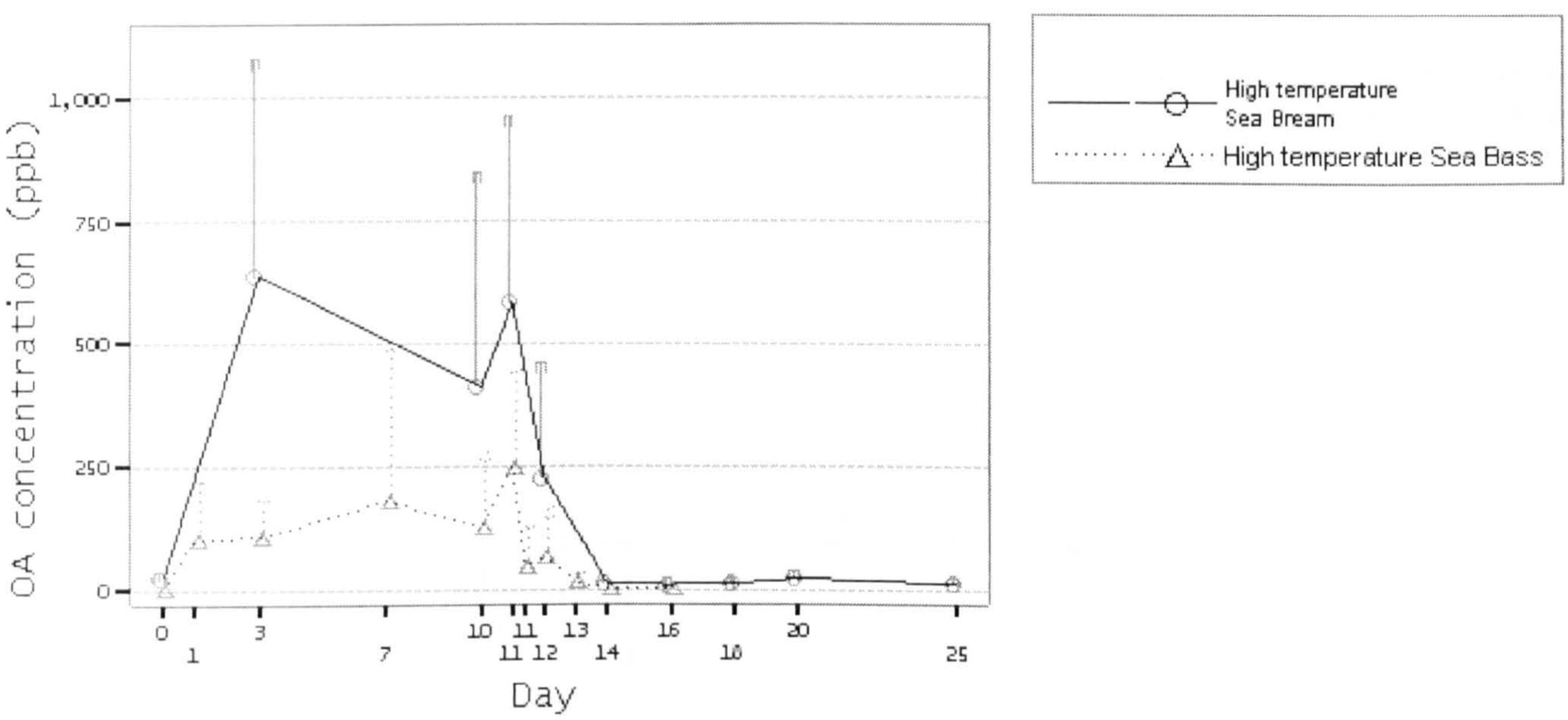


Figure 66: Comparison of OA mean concentration in the liver of sea bream and sea bass at high temperature

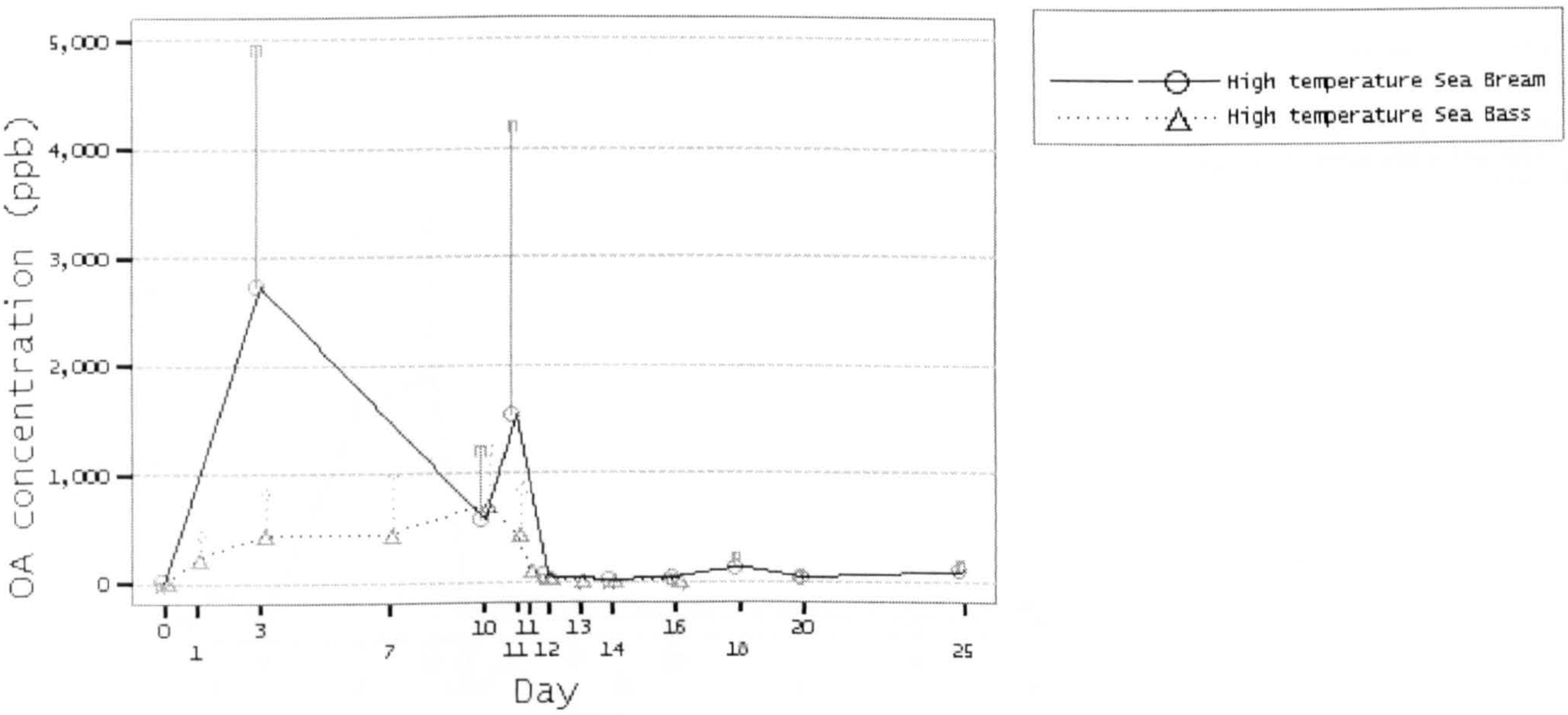


Figure 67: Comparison of OA mean concentration in the muscle of sea bream and sea bass at low temperature

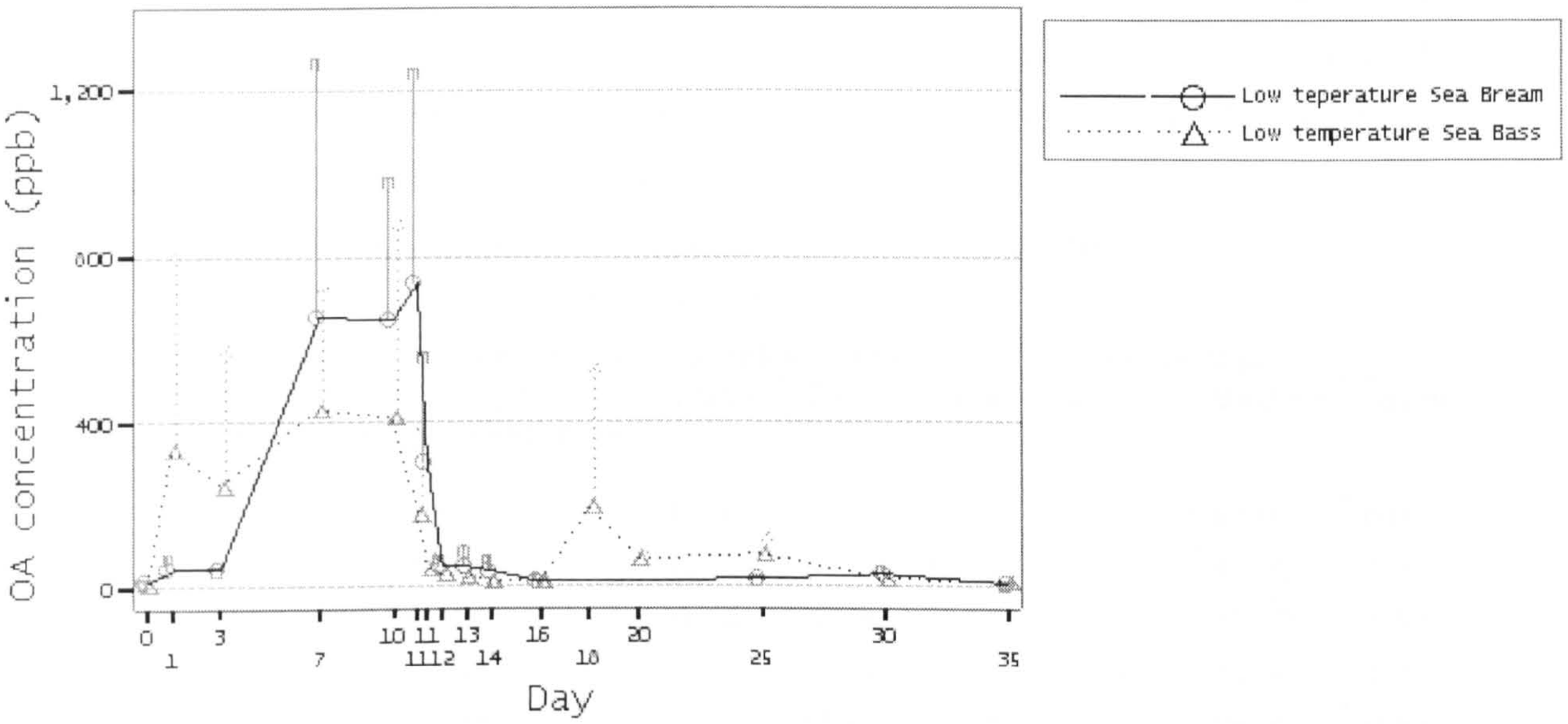
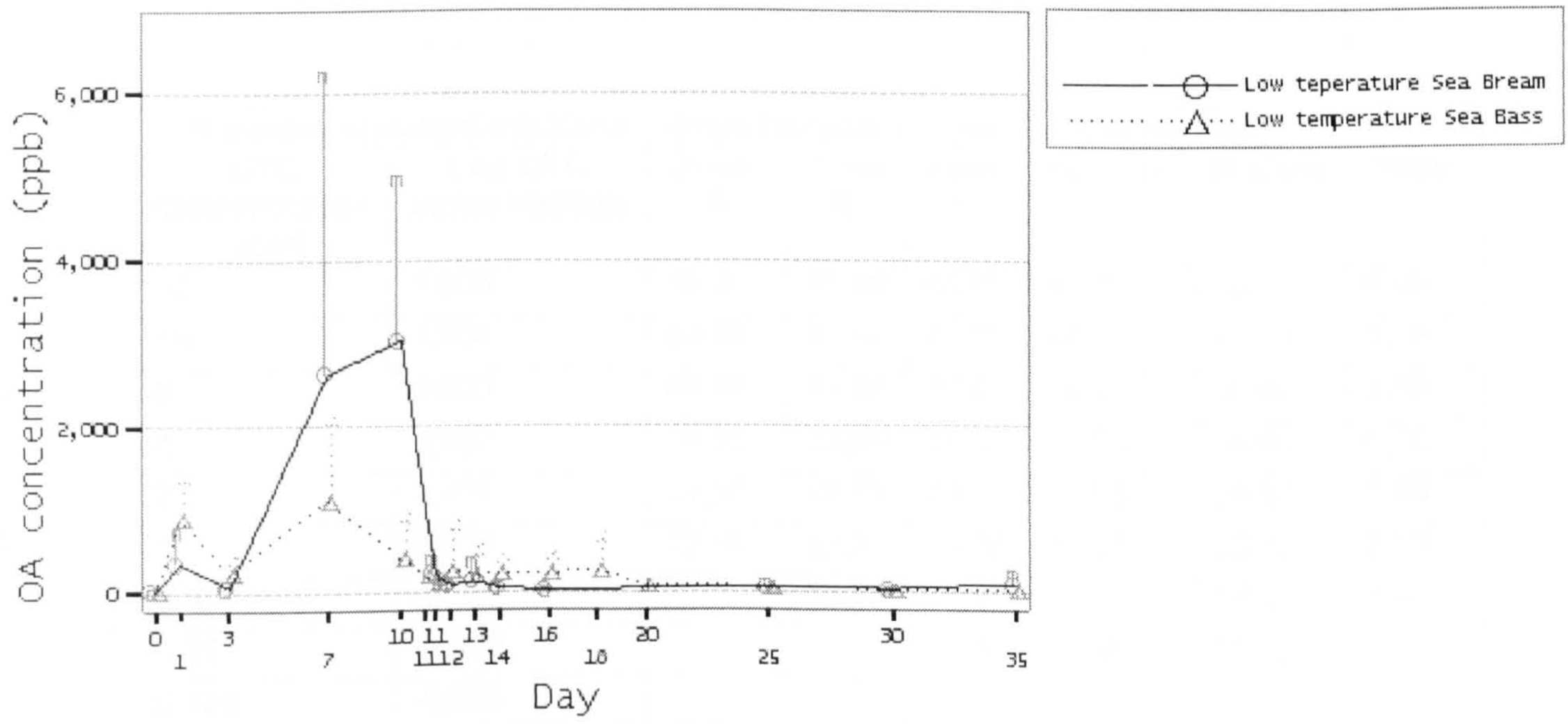


Figure 68: Comparison of OA mean concentration in the liver of sea bream and sea bass at low temperature



3.6 OXYTETRACYCLINE KINETICS

3.6.1 Development of Bioassay method for OTC detection

This bioassay method was developed the same way like O.A bioassay method. Standard dilutions of Oxytetracycline were initially tested and zones of inhibition have been recorded. Table 48 presented the data from the two trials where OTC standard dilutions while, Table 49 presented the statistical analysis of these data. The detection limit of the method (standard dilutions) was 250ppb of Oxytetracycline.

Table 48: Oxytetracycline residues detection bioassay method

Standard dilutions

Bioassay standard dilutions – Oxytetracycline – trial 1 - sample:50µl								
Number	**OTC concentration (ppm)**	**Log OTC concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**	**Stdv**
1	32	1,505	47,00	47,00	47,00	47,00	47,00	0,00
2	16	1,204	43,00	44,00	42,00	43,00	43,00	1,00
3	8	0,903	39,00	40,00	37,00	38,67	39,00	1,53
4	4	0,602	32,50	34,00	34,90	33,80	34,00	1,21
5	2	0,301	29,50	29,00	28,50	29,00	29,00	0,50
6	1	0,000	23,00	25,00	27,00	25,00	25,00	2,00
7	0,5	-0,301	17,00	16,50	18,50	17,33	17,00	1,04
8	0,25	-0,602	12,00	10,90		11,45	11,45	0,78
9	0,125	-0,903						
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Bioassay standard dilutions – Oxytetracycline – trial 2 - sample:50µl								
Number	**OTC concentration (ppm)**	**Log OTC concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**	**Stdv**
1	32	1,505	45,00	45,00	45,00	45,00	45,00	0,00
2	16	1,204	43,50	42,50	42,00	42,67	42,50	0,76
3	8	0,903	40,00	40,00	39,00	39,67	40,00	0,58
4	4	0,602	34,00	33,00	34,50	33,83	34,00	0,76
5	2	0,301	31,00	29,00	29,50	29,83	29,50	1,04
6	1	0,000	22,00	22,40	26,00	23,47	22,40	2,20
7	0,5	-0,301		18,00	16,00	17,00	17,00	1,41
8	0,25	-0,602			11,50	11,50	11,50	
9	0,125	-0,903						
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Bioassay standard dilutions – Oxytetracycline – trial 3 - sample:50µl								
Number	**OTC concentration (ppm)**	**Log OTC concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**	**Stdv**
1	32	1,505	51,00	50,00	46,00	49,00	50,00	2,65
2	16	1,204	47,00	47,00	46,00	46,67	47,00	0,58
3	8	0,903	40,00	43,00	40,00	41,00	40,00	1,73
4	4	0,602	33,50	33,50	34,50	33,83	33,50	0,58
5	2	0,301	30,00	26,00	25,00	27,00	26,00	2,65
6	1	0,000	22,50		26,00	24,25	24,25	2,47
7	0,5	-0,301	14,00	12,50	16,00	14,17	14,00	1,76
8	0,25	-0,602						
9	0,125	-0,903						
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Table 49: Statistical Analysis - OTC Bioassay - standard dilutions

OTC STANDARD DILUTIONS					
Log OTC concentration	Average trial 1	Average trial 2	Average trial 3	Average Total	Stdv
1,505	47,00	45,000	49,00	47,00	2,00
1,204	43,00	42,670	46,67	44,11	2,22
0,903	38,67	39,670	41,00	39,78	1,17
0,602	33,80	33,830	33,83	33,82	0,02
0,301	29,00	29,830	27,00	28,61	1,45
0,000	25,00	23,470	24,25	24,24	0,77
-0,301	17,33	17,000	14,17	16,17	1,74
-0,602	11,45	11,500		11,48	0,04

Table 50: Oxytetracycline residues detection bioassay method

Standard dilutions – Inter-operator variability

Bioassay standard dilutions - oxytetracycline – operator A - sample:50µl								
Number	OTC concentration (ppm)	Log OTC concentration	Zone A	Zone B	Zone C	Average	Median	Stdv
1	32	1,505	43,80	44,50	42,00	43,43	43,80	1,29
2	16	1,204	41,00	42,00	42,00	41,67	42,00	0,58
3	8	0,903	38,00	38,00	35,00	37,00	38,00	1,73
4	4	0,602	33,00	33,00	35,00	33,67	33,00	1,15
5	2	0,301	28,00	29,50	28,00	28,50	28,00	0,87
6	1	0,000	25,00	23,50	22,00	23,50	23,50	1,50
7	0,5	-0,301	16,00	16,50	15,00	15,83	16,00	0,76
8	0,25	-0,602	12,50	11,50	12,00	12,00	12,00	0,50
9	0,125	-0,903						
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Bioassay standard dilutions - oxytetracycline – operator B - sample:50µl								
Number	OTC concentration (ppm)	Log OTC concentration	Zone A	Zone B	Zone C	Average	Median	Stdv
1	32	1,505	44,00	44,50	43,00	43,83	44,00	0,76
2	16	1,204	41,00	42,00	40,00	41,00	41,00	1,00
3	8	0,903	37,00	38,50	37,00	37,50	37,00	0,87
4	4	0,602	33,50	35,00	32,00	33,50	33,50	1,50
5	2	0,301	29,00	30,00	30,00	29,67	30,00	0,58
6	1	0,000	24,00	23,00	21,50	22,83	23,00	1,26
7	0,5	-0,301	19,00	18,00	18,50	18,50	18,50	0,50
8	0,25	-0,602	10,50	10,50	10,00	10,33	10,50	0,29
9	0,125	-0,903						
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Bioassay standard dilutions - oxytetracycline – operator C - sample:50µl								
Number	**OTC concentration (ppm)**	**Log OTC concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**	**Stdv**
1	32	1,505	43,50	44,00	43,00	43,50	43,50	0,50
2	16	1,204	40,00	41,00	40,00	40,33	40,00	0,58
3	8	0,903	37,00	38,00	39,00	38,00	38,00	1,00
4	4	0,602	33,00	34,00	32,00	33,00	33,00	1,00
5	2	0,301	29,50	28,00	29,00	28,83	29,00	0,76
6	1	0,000	26,00	25,00	25,50	25,50	25,50	0,50
7	0,5	-0,301	16,50	17,50	16,00	16,67	16,50	0,76
8	0,25	-0,602	12,50	12,50	14,00	13,00	12,50	0,87
9	0,125	-0,903						
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Table 51: OTC Bioassay - Statistical Analysis - OTC Standard Dilutions

Inter-Operator variability

OTC Standard Dilutions – Inter-Operator variability – Statistical analysis					
Operator A	**Operator B**	**Operator C**	**Average**	**Log**	
12	10,33	13,00	11,777	-0,602	
15,83	18,5	16,67	17,000	-0,301	
23,5	22,83	25,50	23,943	0,000	
28,5	29,67	28,83	29,000	0,301	
33,67	33,5	33,00	33,390	0,602	
37	37,5	38,00	37,500	0,903	
41,67	41	40,33	41,000	1,200	
43,43	43,83	43,50	43,587	1,500	
98,15	97,67	97,7	98,250		R-squared
15,65	15,65	14,8	15,370		slope
22,39	22,59	23,18	22,720		intercept

Table 52: Oxytetracycline residues detection bioassay method
Spiked serum samples

Bioassay – Oxytetracycline - "spiked" serum – trial1 - sample:50µl								
number	**OTC concentration (ppm)**	**Log OTC concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**	**Stdv**
1	32	1,505	51,44	52,54	51,98	51,99	51,98	0,55
2	16	1,204	45,49	46,62	44,72	45,61	45,49	0,96
3	8	0,903	40,34	39,92	40,96	40,41	40,34	0,52
4	4	0,602	32,06	33,45	35,06	33,52	33,45	1,50
5	2	0,301	28,82	30,82	28,81	29,48	28,82	1,16
6	1	0,000	19,37	18,68	18,81	18,95	18,81	0,37
7	0,5	-0,301	14,90	15,29	14,51	14,90	14,90	0,39
8	0,25	-0,602			8,02	8,02	8,02	
9	0,125	-0,903						
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Bioassay – Oxytetracycline - "spiked" serum – trial2 - sample:50µl								
number	**OTC concentration (ppm)**	**Log OTC concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**	**Stdv**
1	32	1,505	49,08	53,15	52,29	51,51	52,29	2,15
2	16	1,204	45,47	46,13	46,80	46,13	46,13	0,67
3	8	0,903	41,15	42,33	42,62	42,03	42,33	0,78
4	4	0,602	32,86	37,21	33,32	34,46	33,32	2,39
5	2	0,301	25,53	27,82	26,79	26,71	26,79	1,15
6	1	0,000	20,10	23,16	21,36	21,54	21,36	1,54
7	0,5	-0,301	15,45	13,95	13,05	14,15	13,95	1,21
8	0,25	-0,602	7,97	8,40	8,99	8,45	8,40	0,51
9	0,125	-0,903						
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Bioassay – Oxytetracycline - "spiked" serum – trial3 - sample:50µl								
number	**OTC concentration (ppm)**	**Log OTC concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**	**Stdv**
1	32	1,505	49,76	53,18	52,04	51,66	52,04	1,74
2	16	1,204	45,90	48,70	46,82	47,14	46,82	1,43
3	8	0,903	39,13	42,45	42,90	41,49	42,45	2,06
4	4	0,602	31,92	35,64	34,69	34,08	34,69	1,93
5	2	0,301	26,29	25,97	25,13	25,80	25,97	0,60
6	1	0,000	20,17	23,18	20,00	21,12	20,17	1,79
7	0,5	-0,301	14,98	16,07	15,41	15,49	15,41	0,55
8	0,25	-0,602	8,50	8,36	8,00	8,29	8,36	0,26
9	0,125	-0,903						
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Table 53: OTC Bioassay – Spiked serum samples - Statistical Analysis

OTC Spiked Serum sample Analysis					
Log OTC concentration	Average trial 1	Average trial 2	Average trial 3	Average Total	Stdv
1,505	51,99	51,510	51,66	51,72	0,25
1,204	45,61	46,130	47,14	46,29	0,78
0,903	40,41	42,030	41,69	41,38	0,85
0,602	33,52	34,460	34,08	34,02	0,47
0,301	29,48	26,710	25,80	27,33	1,92
0,000	18,95	21,540	21,12	20,54	1,39
-0,301	14,90	14,510	15,49	14,97	0,49
-0,602	8,02	8,450	8,29	8,25	0,22

Table 54: Oxytetracycline residues detection bioassay method
Spiked serum samples – Inter-Operator Variability

Bioassay – Oxytetracycline "spiked serum" Operator A - sample:50µl – pH=5.6								
number	**OTC concentration (ppm)**	**Log OTC concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**	**Stdv**
1	32	1,505	42,50	40,00	39,00	40,50	40,00	1,80
2	16	1,204	38,00	38,00	38,00	38,00	38,00	0,00
3	8	0,903	35,00	33,00	31,50	33,17	33,00	1,76
4	4	0,602	30,00	30,00	29,00	29,67	30,00	0,58
5	2	0,301	25,00	26,00	25,50	25,50	25,50	0,50
6	1	0,000	20,00	21,00	21,00	20,67	21,00	0,58
7	0,5	-0,301	14,00	14,00	14,00	14,00	14,00	0,00
8	0,25	-0,602	11,50	11,00	10,00	10,83	11,00	0,76
9	0,125	-0,903	7,00	7,50	8,00	7,50	7,50	0,50
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Bioassay – Oxytetracycline "spiked serum" Operator B - sample:50µl								
number	**OTC concentration (ppm)**	**Log OTC concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**	**Stdv**
1	32	1,505	41,00	40,00	40,00	40,33	40,00	0,58
2	16	1,204	37,00	36,00	36,00	36,33	36,00	0,58
3	8	0,903	35,00	33,00	32,50	33,50	33,00	1,32
4	4	0,602	29,50	29,00	28,50	29,00	29,00	0,50
5	2	0,301	27,50	26,50	27,00	27,00	27,00	0,50
6	1	0,000	21,50	19,00	18,00	19,50	19,00	1,80
7	0,5	-0,301	15,50	16,00	15,00	15,50	15,50	0,50
8	0,25	-0,602	11,50	10,50	10,00	10,67	10,50	0,76
9	0,125	-0,903	8,00	8,00		8,00	8,00	0,00
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Bioassay – Oxytetracycline "spiked serum" Operator C - sample:50µl								
number	**OTC concentration (ppm)**	**Log OTC concentration**	**Zone A**	**Zone B**	**Zone C**	**Average**	**Median**	**Stdv**
1	32	1,505	39,50	40,00	39,50	39,67	39,50	0,29
2	16	1,204	37,50	36,50	36,00	36,67	36,50	0,76
3	8	0,903	33,50	35,50	34,00	34,33	34,00	1,04
4	4	0,602	30,50	29,50	29,50	29,83	29,50	0,58
5	2	0,301	26,00	26,00	25,00	25,67	26,00	0,58
6	1	0,000	21,50	21,00	21,00	21,17	21,00	0,29
7	0,5	-0,301	14,50	14,00	16,00	14,83	14,50	1,04
8	0,25	-0,602	11,00	12,00	12,00	11,67	12,00	0,58
9	0,125	-0,903	8,00			8,00	8,00	
10	0,06	-1,221						
11	0,03	-1,522						
12	0							

Table 55: OTC Bioassay – Spiked serum

Inter operator variability - Statistical Analysis

OTC Spiked serum – Inter-Operator variability – Statistical analysis					
Spiked serum	**Operator A**	**Operator B**	**Operator C**	**Average**	**log**
	7,5	8	8,000	7,83	-0,903
	10,83	10,67	11,670	11,06	-0,602
	14	15,5	14,830	14,78	-0,301
	20,67	19,5	21,170	20,45	0
	25,5	27	25,670	26,06	0,301
	29,67	29	29,830	29,5	0,602
	33,17	33,5	34,330	33,67	0,903
	38	36,33	36,670	37	1.204
	40,5	40,33	39,670	40,17	1.505
r-squared	99,31	98,99	99,070	99,31	
slope	14,44	13,94	13,800	14,06	
intercept	20,07	20,22	20,490	20,26	

The data and the statistical analysis from the examination of "spiked" serum samples were presented in Tables 52 to 55. The recovery of the method for serum samples was 91,4%.

3.7 Oxytetracycline Kinetics in Sea Bream (*Sparus aurata*)

3.7.1.1 High water temperature

3.7.1.1.1 Oxytetracycline depletion in muscle

The analysis of the OTC in muscle samples indicated that except on the 3rd day, feeding response of the medicated diet was almost optimal. The depletion of the drug in muscle of gilthead sea bream showed biphasic elimination since the analysis of the mean OTC concentration displayed two distinct peaks at days 7 (0.104 ppm) and 11 (0.122 ppm) (Figures 69,70,71and Table 56). Muscle tissues retained the lowest OTC levels when compared to other examined tissues. The OTC muscle maximum concentration achieved an early peak (175 ppm) on the first day and after a sharp drop on the 3rd day, was gradually increased to reach maximum concentration of 0.275 ppm on the 11th day. In subsequent days, OTC concentration fell quickly (<0.069 ppm). The maximum average OTC levels were evident at the conclusion of the medication. Detectable levels were found until 15 days after treatment ceased.

3.7.1.1.2 Oxytetracycline depletion in Liver

In this analysis, extreme and outlier values present on the 1st and 5th day were excluded. Biphasic elimination was also evident in OTC depletion from gilthead sea bream liver (Figure 72,73,74 and Table 56). The mean OTC liver concentration presented a peak on the 5th day (3.088 ppm) and maximum concentration reached 4.7 ppm on the 10th day decreasing sharply on the 11th day (< 500 ppb). Liver tissues presented the highest OTC concentrations with detectable levels until 15 d following the completion of the treatment.

3.7.1.1.3 Oxytetracycline depletion in serum

The OTC depletion pattern in gilthead sea bream serum was not similar to the profile of the drug in muscle and liver, displaying only one peak (Figure 75,76,77 and Table 56). Mean and maximum OTC serum concentration increased rapidly up until the 5th day (980 ppb / 1940 ppb) and decreased gradually reaching the method's detection limit on the 21st day.

Table 56: EXPERIMENT V Oxytetracycline kinetics in Sea bream at high temperature after 10 day oral treatment - Statistical interpretation

Statistical Analysis *Oxytetracycline concentration - Sea Bream Muscle - High Temperature*											
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 12	Day 13	Day 14	Day 17
Samples	6	9	7	6	5	7	7	6	7	7	7
Minimum concentration(ppb)	1,74	12,01	11,72	42,93	50,69	29,89	21,53	22,50	24,33	21,56	8,87
Mean concentration(ppb)	1,91	58,40	31,19	65,37	103,64	73,72	121,85	62,01	69,36	42,16	16,17
Maximum concentration(ppb)	2,80	170,51	51,74	120,10	164,07	144,44	275,99	106,60	92,85	88,59	24,99
Standard deviation	0,43	56,39	15,45	28,65	47,67	38,55	101,35	35,67	23,98	23,72	6,27
25% percentile	1,74	13,30	13,31	44,28	57,71	48,41	26,56	29,29	52,72	24,94	1,01
Median	1,74	35,70	34,83	58,11	101,63	63,79	91,33	56,46	74,61	34,35	13,96
75% Percentile	2,00	106,16	42,45	81,22	150,57	99,45	248,60	100,54	88,85	55,74	24,73

Oxytetracycline concentration - Sea Bream Muscle - High Temperature											
	Day 21	Day 23	Day 25								
Samples	7	8	7								
Minimum concentration(ppb)	7,03	7,41	9,33								
Mean concentration(ppb)	12,40	12,29	12,02								
Maximum concentration(ppb)	31,89	18,85	18,45								
Standard deviation	8,68	4,22	3,06								
25% percentile	8,06	9,10	9,97								
Median	9,89	11,01	11,21								
75% Percentile	10,49	16,86	12,90								

Oxytetracycline concentration - Sea Bream Liver - High Temperature											
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 12	Day 13	Day 14	Day 17
Samples	7	9	6	6	7	7	6	7	7	7	6
Minimum concentration(ppb)	1,74	319,15	37,04	1327,84	81,28	343,60	276,53	141,48	157,25	183,41	30,77
Mean concentration(ppb)	4,30	1020,62	72,29	3088,47	652,10	1824,58	420,04	64,20	325,24	373,20	52,50
Maximum concentration(ppb)	9,37	3065,02	133,68	6099,96	2365,03	4665,21	547,54	1138,12	531,46	719,83	86,87
Standard deviation	3,42	796,16	40,24	1696,27	803,76	1770,21	119,17	355,22	130,03	179,57	21,42
25% percentile	1,74	676,73	38,10	1799,24	96,31	414,87	289,10	230,33	186,10	242,71	34,64
Median	1,74	781,80	57,44	2841,02	314,17	649,96	429,52	329,40	369,10	319,60	48,40
75% Percentile	8,47	1001,96	114,79	4123,46	774,23	3533,53	543,85	745,29	388,32	485,61	70,19

Oxytetracycline concentration - Sea Bream Liver - High Temperature											
	Day 21	Day 23	Day 25								
Samples	7	8	3								
Minimum concentration(ppb)	15,24	52,04	39,67								
Mean concentration(ppb)	48,64	111,50	74,40								
Maximum concentration(ppb)	83,19	154,70	128,13								
Standard deviation	26,62	30,19	47,19								
25% Percentile	25,76	94,69	39,67								
Median	52,17	118,42	55,39								
75% Percentile	76,75	126,60									

Oxytetracycline concentration - Sea Bream Serum (BIOASAY) - High Temperature											
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 12	Day 13	Day 14	Day 17
Samples		6	5	5	6	5		7	5	5	5
Minimum concentration(ppb)		100,00	151,00	653,00	349,00	342,00		287,00	219,00	197,00	100,00
Mean concentration(ppb)		474,00	843,00	981,80	807,00	787,40		610,71	526,60	421,80	191,00
Maximum concentration(ppb)		1150,00	1632,00	1940,00	1402,00	1188,00		957,00	763,00	709,00	304,00
Standard deviation		360,88	628,42	540,46	465,32	302,10		272,08	196,35	212,30	102,04
25% percentile		208,75	217,00	671,50	379,00	539,00		358,00	372,00	214,50	100,00
Median		445,50	905,00	813,00	756,00	812,00		567,00	540,00	446,00	155,00
75% Percentile		631,00	1438,00	1376,00	1243,00	1023,00		937,00	674,50	617,00	300,00

Oxytetracycline concentration - Sea Bream Serum (BIOASAY) - High Temperature											
	Day 21										
Samples	5										
Minimum concentration(ppb)	100,00										
Mean concentration(ppb)	100,00										
Maximum concentration(ppb)	100,00										
Standard deviation	0,00										
25% Percentile											
Median											
75% Percentile											

Figure 69: EXPERIMENT V – Pharmacokinetics of Oxytetracycline in the muscle of sea bream (*Sparus aurata)* at 24 ± 1◦ C

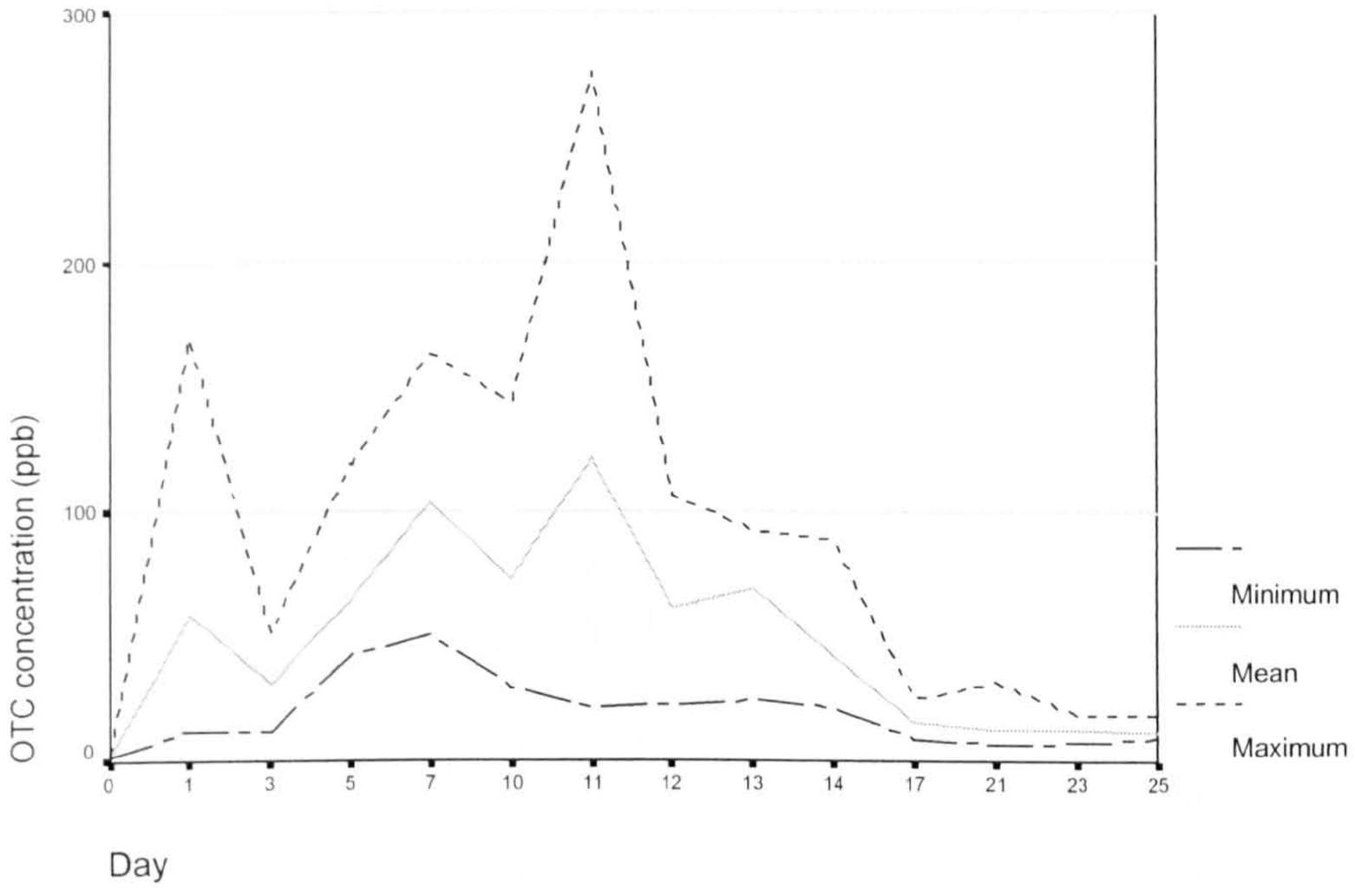


Figure 70: EXPERIMENT V – Pharmacokinetics of Oxytetracycline in the muscle of sea bream (*Sparus aurata)* at 24 ± 1◦ C - Mean and Standard deviation

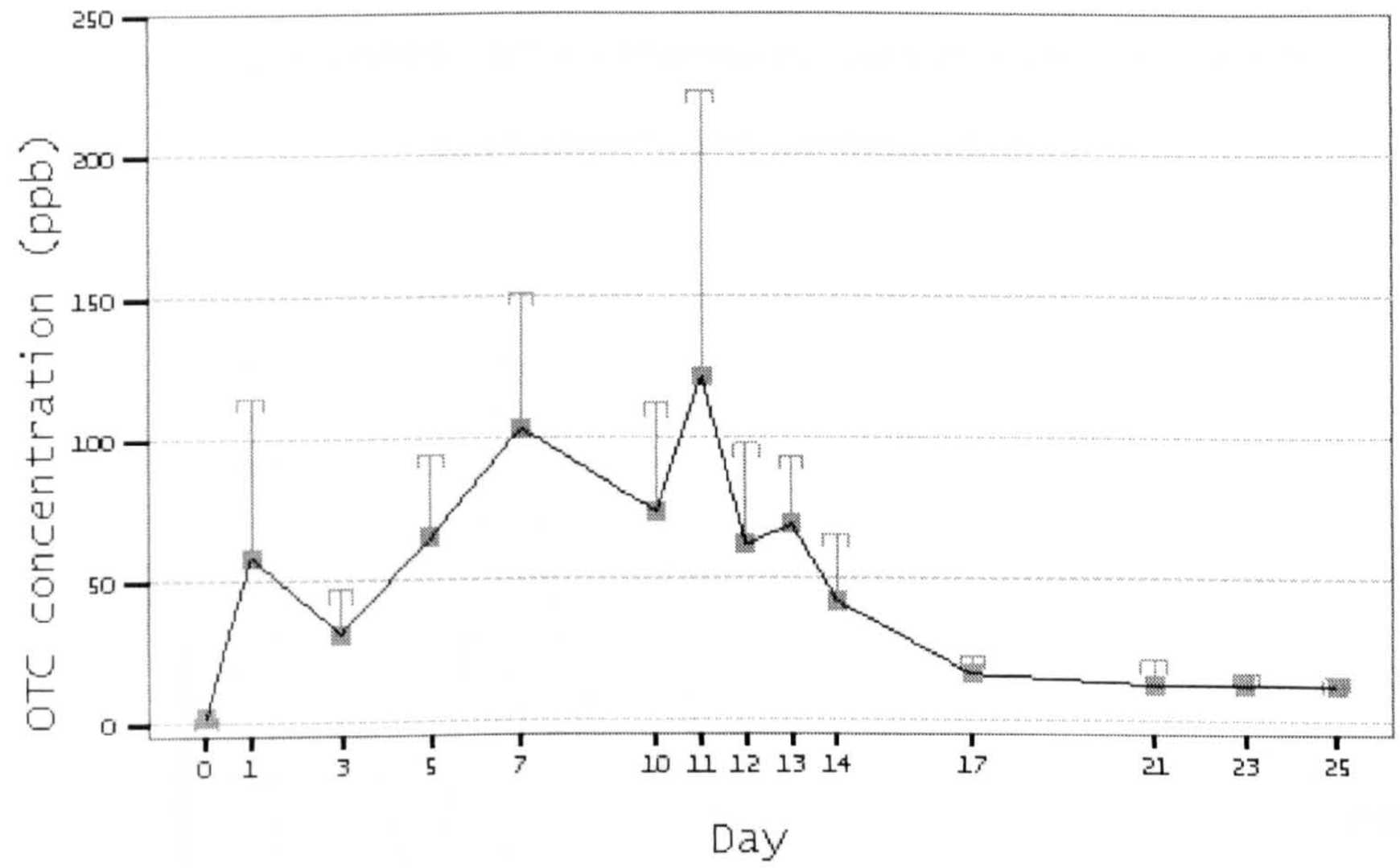
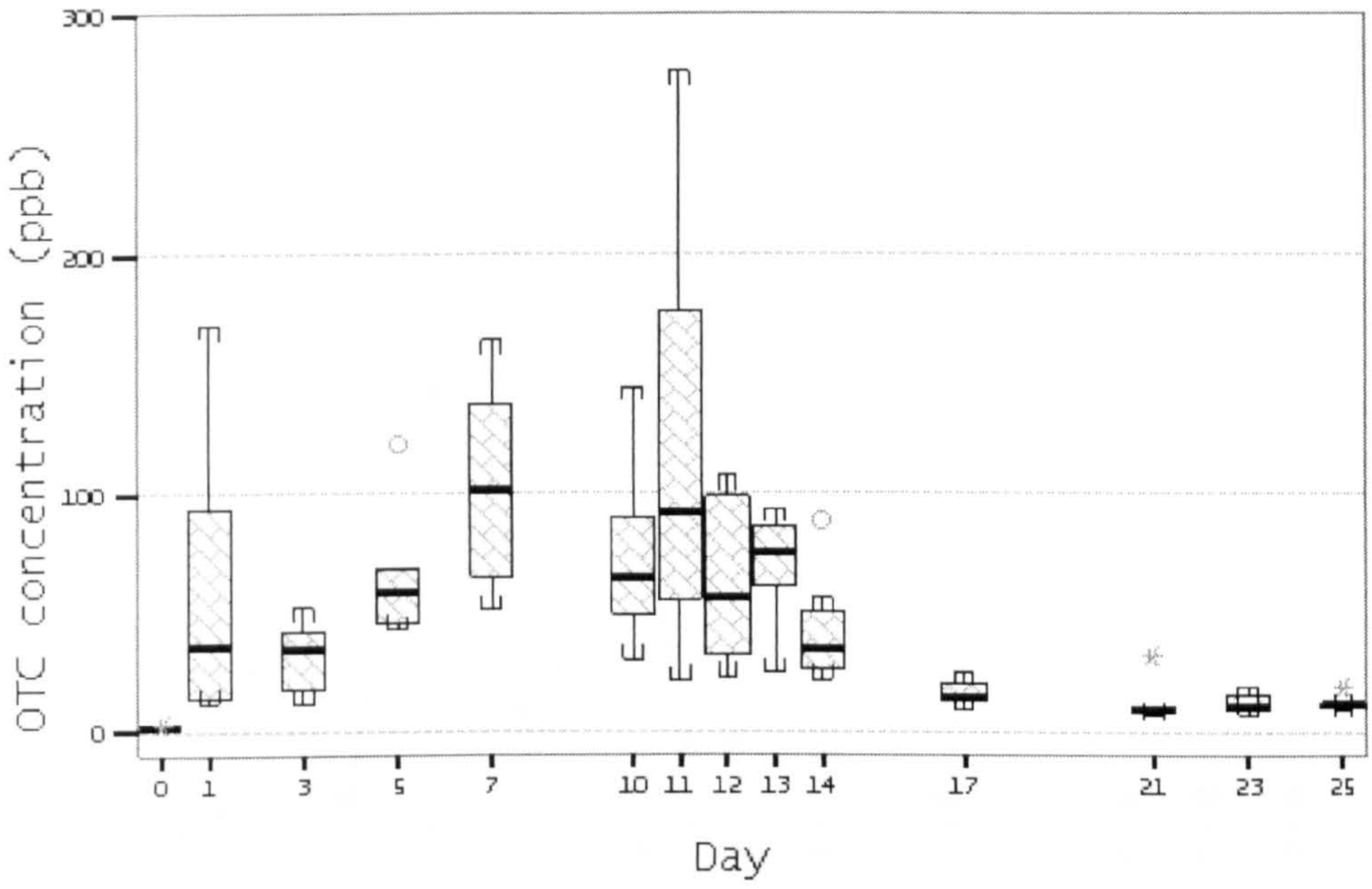


Figure 71: EXPERIMENT V – Pharmacokinetics of Oxytetracycline in the muscle of



sea bream (*Sparus aurata)* at 24 ± 1◦ C - Box plot presentation

Figure 72: EXPERIMENT V – Pharmacokinetics of Oxytetracycline in the liver of sea bream (*Sparus aurata)* at 24 ± 1◦ C

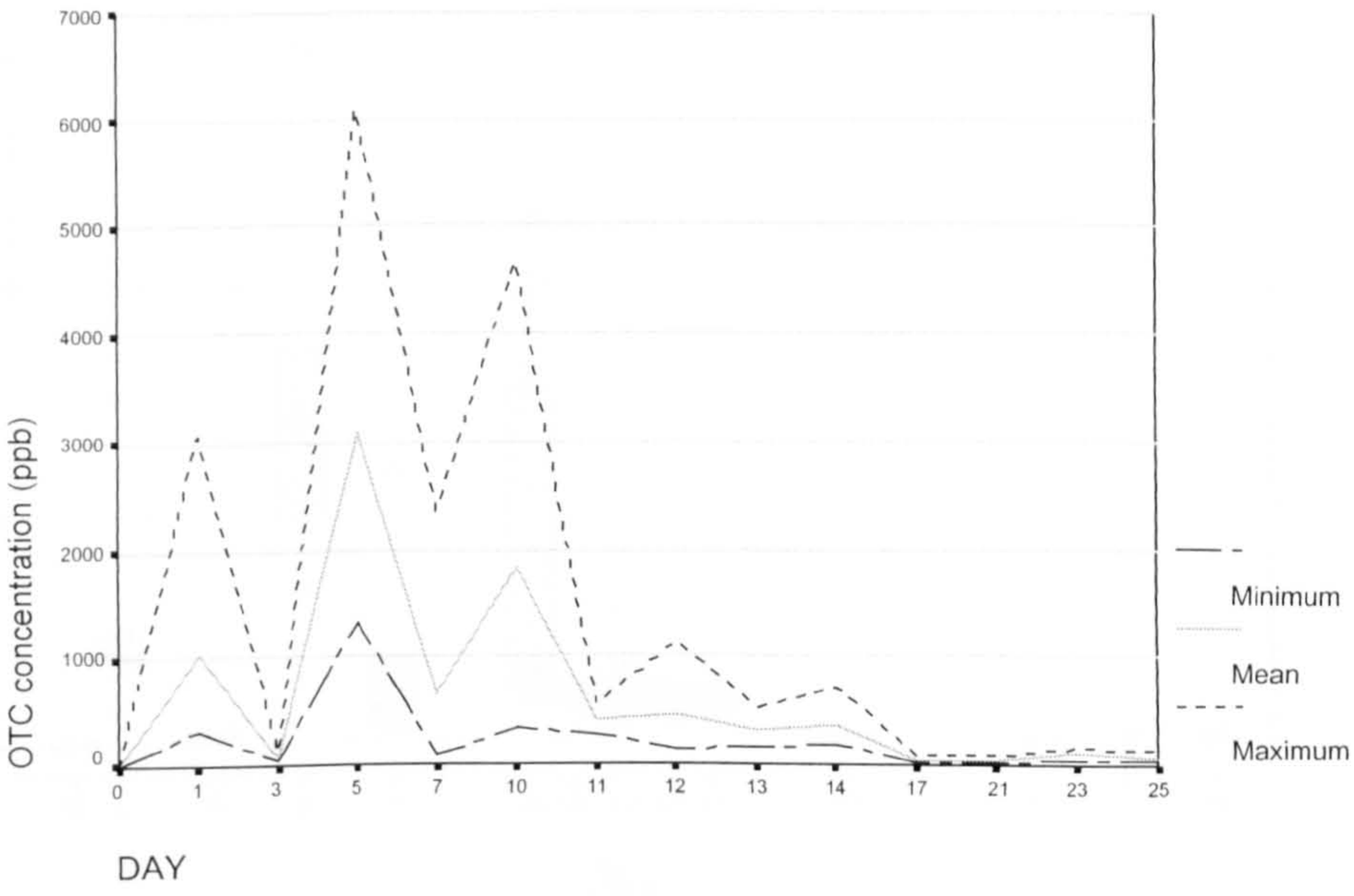
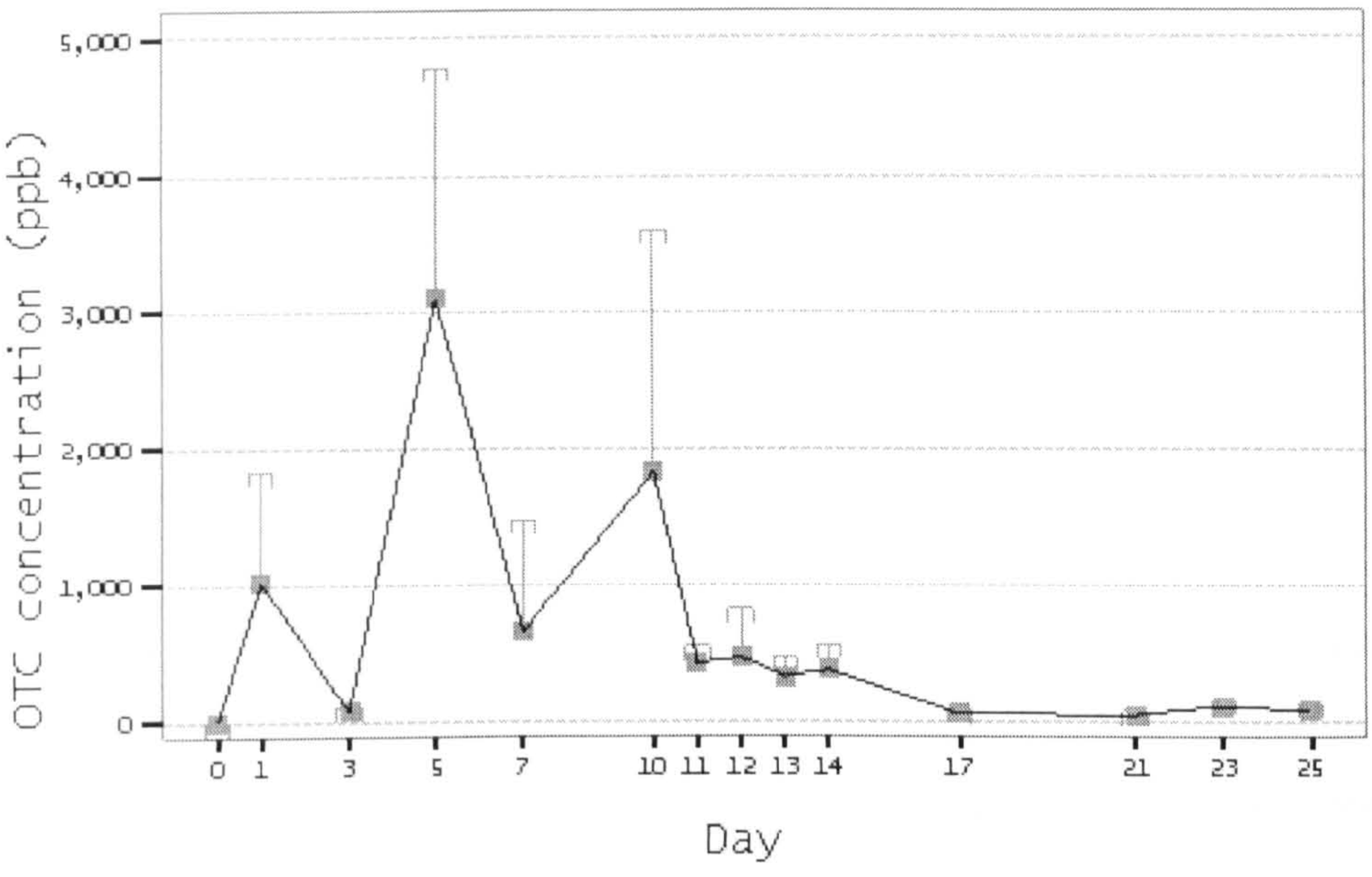
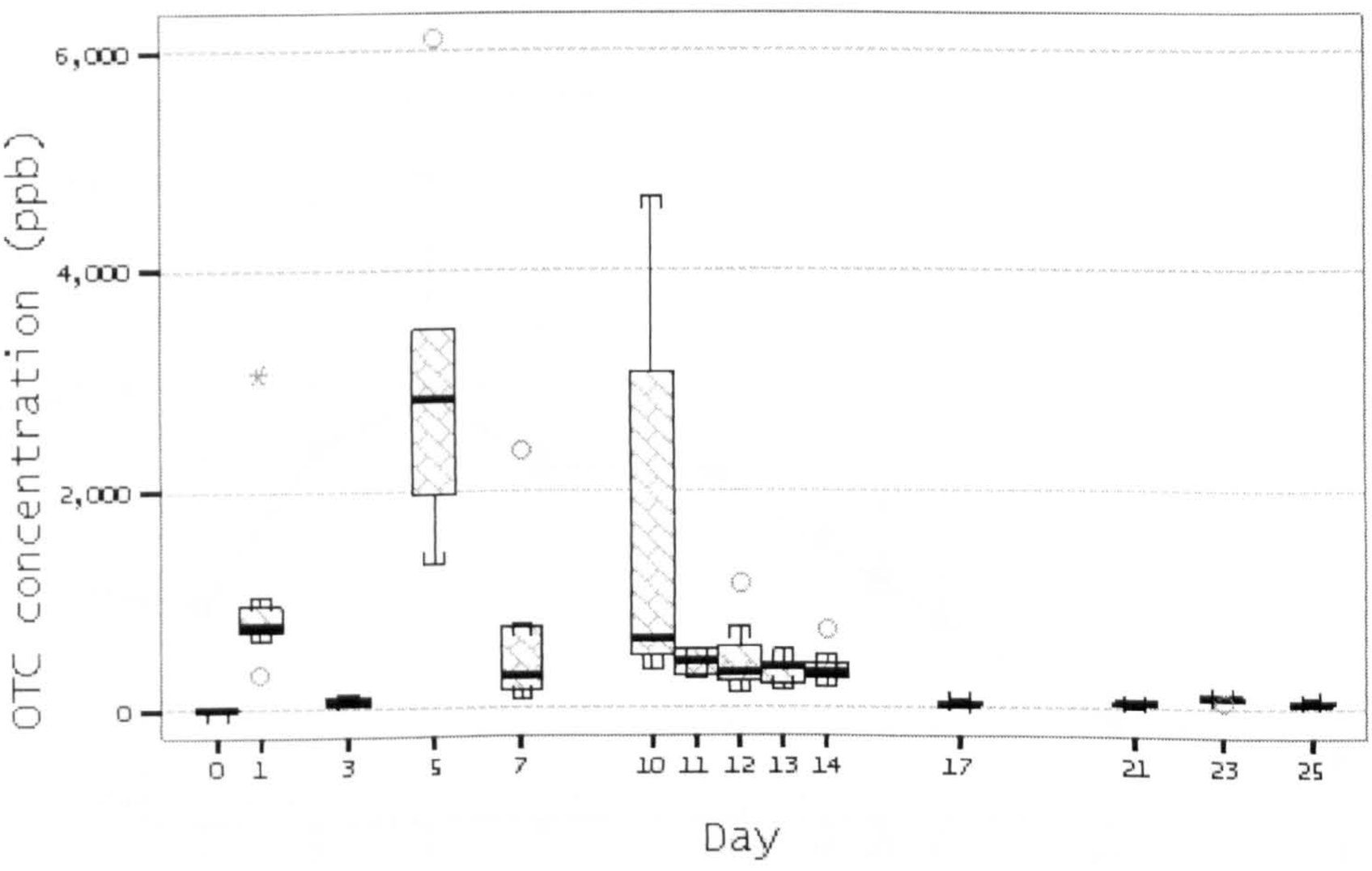


Figure 73: EXPERIMENT V – Pharmacokinetics of Oxytetracycline in the liver of sea bream,



(*Sparus aurata)* at 24 ± 1◦ C – Mean and Standard deviation

Figure 74: EXPERIMENT V – Pharmacokinetics of Oxytetracycline in the liver of sea bream,



(*Sparus aurata)* at 24 ± 1 C – Box plot presentation

Figure 75: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the serum of sea bream, (*Sparus aurata)* at 24 ± 1 C

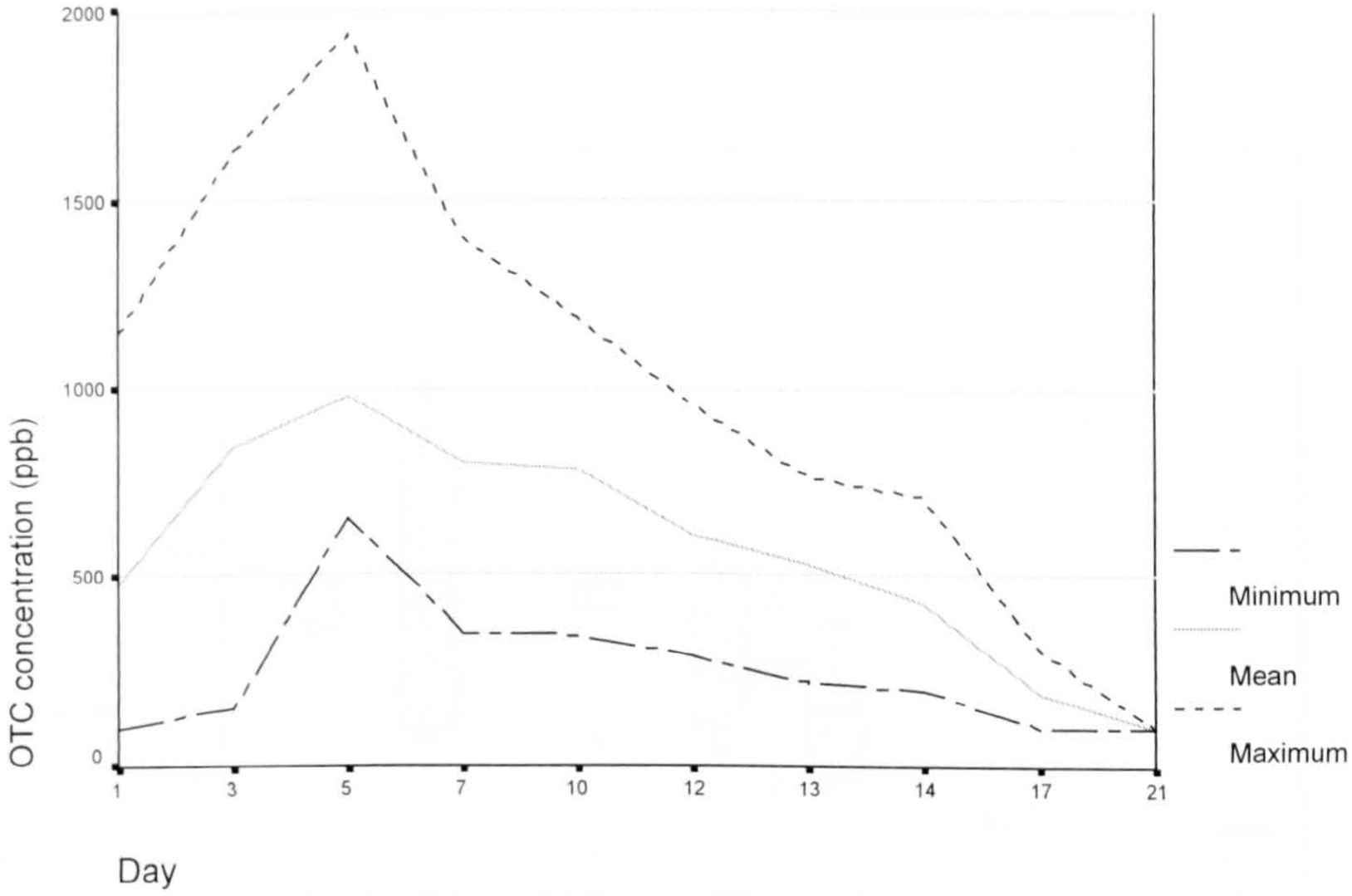


Figure 76: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the serum of sea bream (*Sparus aurata)* at 24 ± 1 C – Mean and Standard deviation

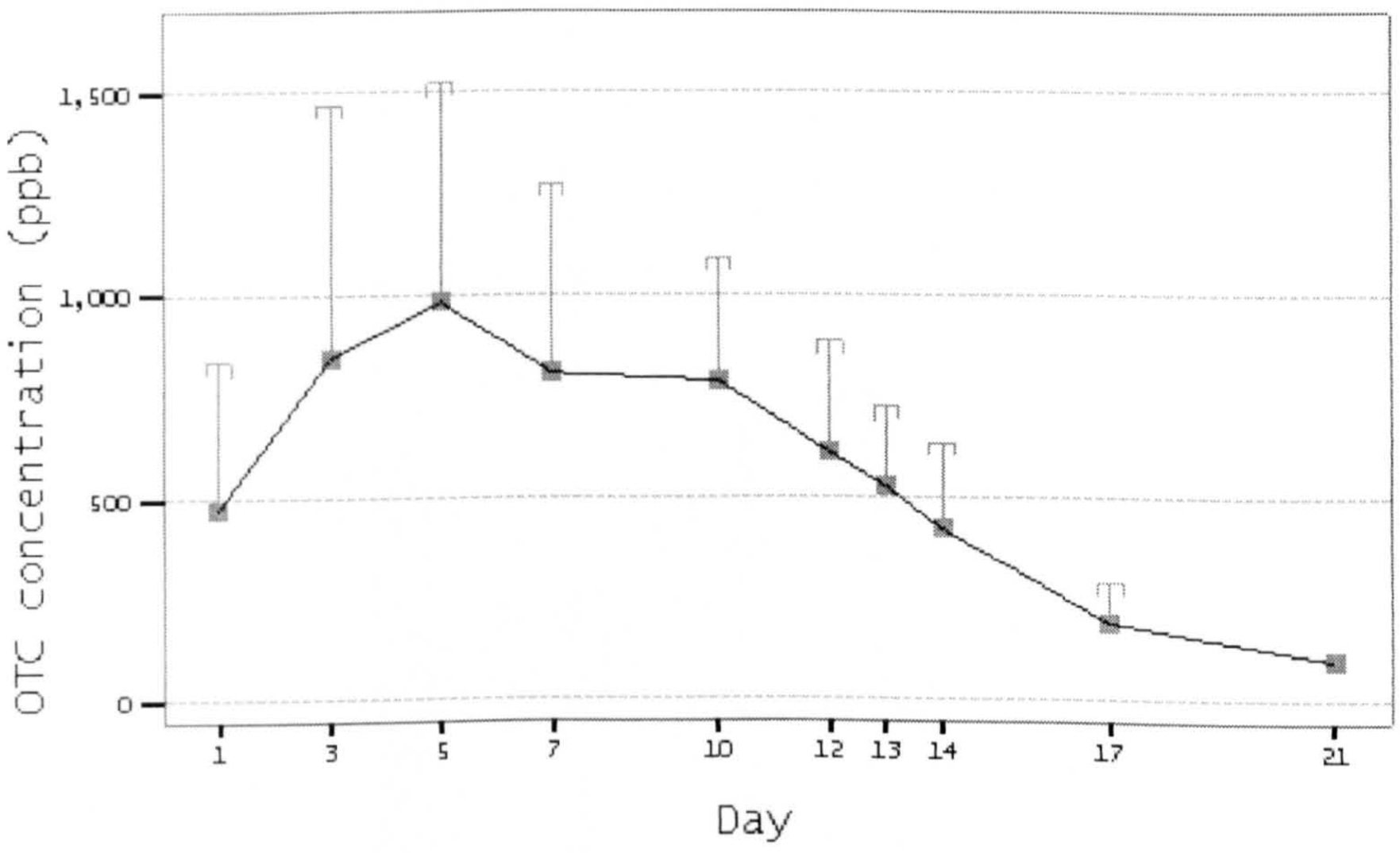
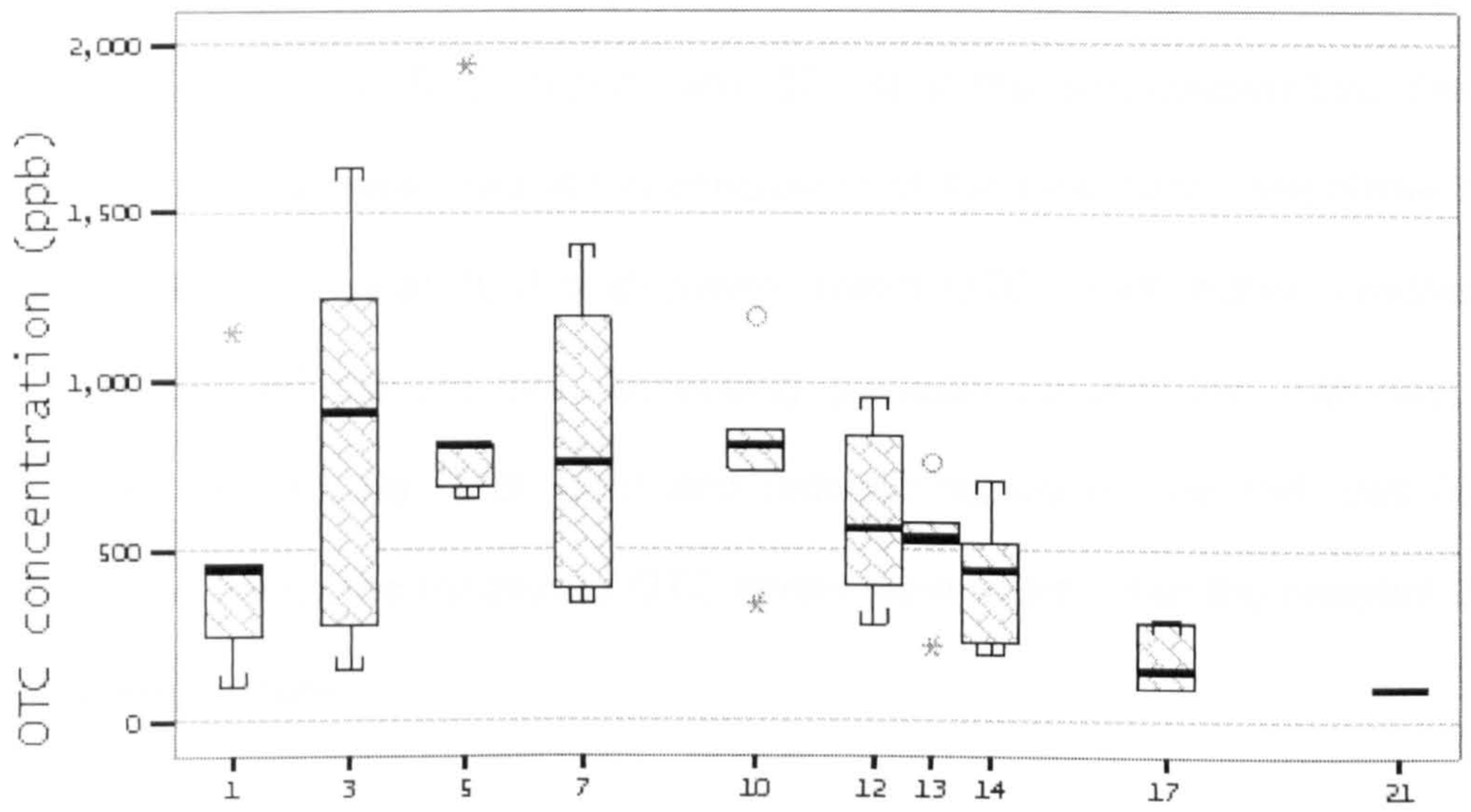


Figure 77: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the serum of sea bream (*Sparus aurata)* at 24 ± 1◦ C – Box plot presentation



3.7.1.2 Low water temperature

3.7.1.2.1 Oxytetracycline depletion in muscle

The presence of extreme and outlier values on the 3rd, 5th and 7th day and the very low concentration present on the 10th day indicated again that analysis of the mean OTC concentration achieved in the muscle is a better indicator. The biphasic elimination pattern of OTC found at the high water temperature was also recorded at the low temperature (Figures 78,79,80 and Table 57). As in the high temperature, the maximum concentration was measured at the conclusion of the treatment. Detectable drug levels were calculated even at 30 d post-dosing. Mean OTC concentration reached an early peak 440 ppb on the 3rd day decreasing gradually up until the 10th day, increased rapidly on the 11th day (280 ppb) and reduced rapidly on the 13th day (40 ppb). In several time points the measured OTC levels were higher than the relevant OTC levels at high temperature.

3.7.1.2.2 Oxytetracycline depletion in liver

The biphasic elimination pattern of OTC found at the high water temperature was also recorded at the low temperature. Mean and maximum OTC concentration in the liver increased reaching a peak on the 5th day (1848 / 5820 ppb) reducing rapidly on the 7th day and moderately up until the 10th day (Figures 81,82,83 and Table 57). A second peak was reached on the 11th day (692 / 2604 ppb) reducing sharply until the 15th day remaining low thereafter (<80 ppb).

3.7.1.2.3 Oxytetracycline depletion in Serum

Mean OTC serum concentration increased moderately up until the 5th (650 ppb), decreased on the 7th day, increased again gradually reaching a peak on the 18th day (1434 ppb) and decreased rapidly below the detection limit on the 25th day (Figures 84,85,86 and Table 57). It is characteristic that minimum concentration remained below the detection limit up until the 13th day giving a peak of 1 ppm only of the 18th day and fell below detection limit on the 25th day. It was obvious that feeding response of the fish on the 7th day of the treatment period was problematic.

3.7.1.2.4 Oxytetracycline depletion in skin

Skin samples displayed the highest OTC levels. Mean OTC concentration in the skin increased rapidly reaching a peak (1.7 ppm) on the 3rd day reduced rapidly on the 5th day and staying low until the 10th day (Figures 87,88 and Table 57). On the 11th day mean concentration reached another peak (2ppm) and reduced rapidly on the 13th day. A slight peak was evident on the 18th day and skin OTC concentration remained very low after the 20th day of the experiment.

Table 57: EXPERIMENT VI Oxytetracycline kinetics in Sea bream at low temperature after 10 day oral treatment - Statistical interpretation

Statistical Analysis *Oxytetracycline concentration - Sea Bream Muscle - Low Temperature*								
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 13
Samples		8	5	9	7	8	8	8
Minimum concentration(ppb)		4,06	58,11	18,37	53,64	12,21	10,53	4,53
Mean concentration(ppb)		10,68	439,34	126,33	164,78	34,55	279,67	39,22
Maximum concentration(ppb)		22,97	1391,48	359,37	569,68	69,83	970,27	79,73
Standard deviation		7,79	561,48	113,18	182,47	18,10	331,73	26,44
25% percentile		5,05	67,46	40,54	71,09	23,92	77,28	19,81
Median		7,39	167,86	87,03	93,30	29,19	119,91	30,35
75% Percentile		19,68	946,95	199,44	169,48	47,22	503,59	66,48

	Day 15	Day 18	Day 20	Day 25	Day 30	Day 40		
Samples	8	8	8	8	8	8		
Minimum concentration(ppb)	8,15	4,73	4,66	6,47	4,22	6,76		
Mean concentration(ppb)	17,48	9,37	8,48	13,43	7,77	15,40		
Maximum concentration(ppb)	34,12	16,99	15,44	28,73	16,22	25,04		
Standard deviation	11,24	3,92	3,45	7,69	4,69	6,46		
25% Percentile	8,52	6,02	6,23	7,12	4,42	10,40		
Median	10,96	9,15	7,29	11,81	5,90	14,60		
75% Percentile	29,30	11,73	10,55	18,74	12,27	22,19		

Oxytetracycline concentration - Sea Bream Liver - Low Temperature								
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 13
Samples		8	7	8	8	8	8	8
Minimum concentration(ppb)		6,71	31,57	178,67	282,32	154,46	84,01	11,85
Mean concentration(ppb)		99,06	1467,62	1848,41	624,02	416,68	691,61	275,64
Maximum concentration(ppb)		466,75	3611,25	5820,37	1073,03	949,20	2604,29	737,55
Standard deviation		158,34	1447,79	2116,10	270,93	300,37	810,88	214,72
25% percentile		7,03	182,53	309,79	377,05	204,54	225,81	133,85
Median		39,58	1161,99	645,48	631,00	255,63	450,76	247,79
75% Percentile		143,19	3327,36	3556,93	852,42	711,52	758,84	335,68

	Day 15	Day 18	Day 20	Day 25	Day 30	Day 40		
Samples	7	7	8	8	8	8		
Minimum concentration(ppb)	6,56	12,34	7,29	48,12	6,01	83,34		
Mean concentration(ppb)	62,29	79,00	26,43	175,02	12,52	187,06		
Maximum concentration(ppb)	143,77	171,87	72,13	284,40	25,25	271,86		
Standard deviation	53,59	60,56	21,31	82,63	6,09	63,58		
25% Percentile	12,23	28,91	13,76	103,39	8,71	152,41		
Median	41,81	57,90	19,16	167,20	10,77	169,97		
75% Percentile	109,15	146,00	38,09	252,55	15,94	252,83		

Oxytetracycline concentration - Sea Bream Serum (BIOASSAY) - Low Temperature								
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 13
Samples	5		7	9	7		8	5
Minimum concentration(ppb)	100,00		100,00	100,00	100,00		100,00	100,00
Mean concentration(ppb)	100,00		519,00	656,78	300,28		688,25	1299,60
Maximum concentration(ppb)	100,00		1408,00	2172,00	470,00		1947,00	3746,00
Standard deviation	0,00		502,10	669,66	121,44		614,96	1426,61
25% percentile			100,00	100,00	258,00		159,75	312,50
Median			470,00	422,00	273,00		561,50	1011,00
75% Percentile			958,00	984,50	422,00		1038,25	2431,00

	Day 15	Day 18	Day 20	Day 25	Day 30	Day 40		
Samples		7		6				
Minimum concentration(ppb)		1011,00		100,00				
Mean concentration(ppb)		1434,71		100,00				
Maximum concentration(ppb)		2172,00		100,00				
Standard deviation		399,31		0,00				
25% Percentile		1128,00						
Median		1329,00						
75% Percentile		1744,00						

Figure 78: EXPERIMENT VI – Pharmacokinetics of Oxytetracycline in the muscle of sea bream (*Sparus aurata)* at 18 ± 1◦ C

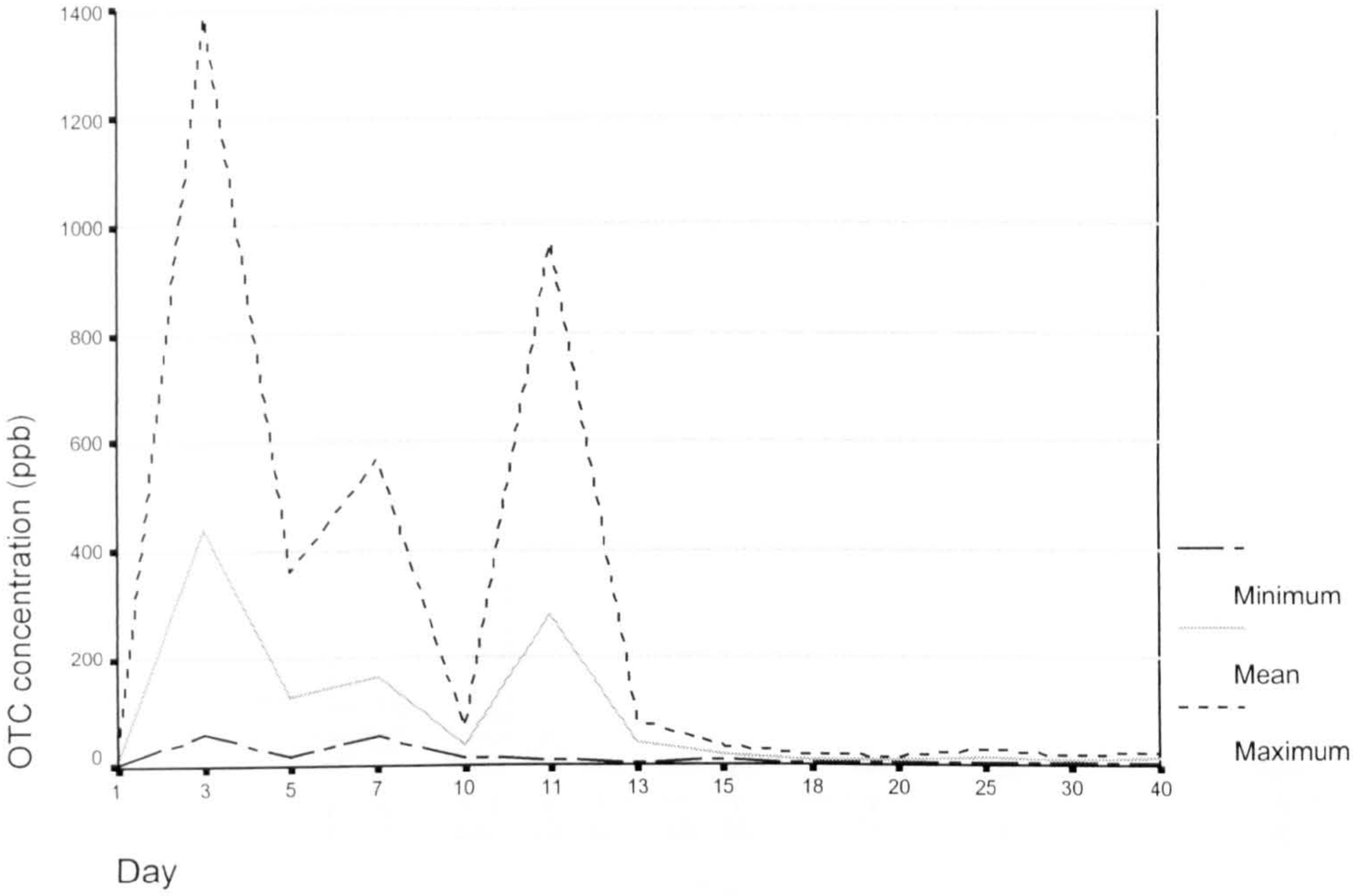


Figure 79: EXPERIMENT VI – Pharmacokinetics of Oxytetracycline in the muscle of sea bream (*Sparus aurata)* at 18 ± 1◦ C - Mean and Standard deviation

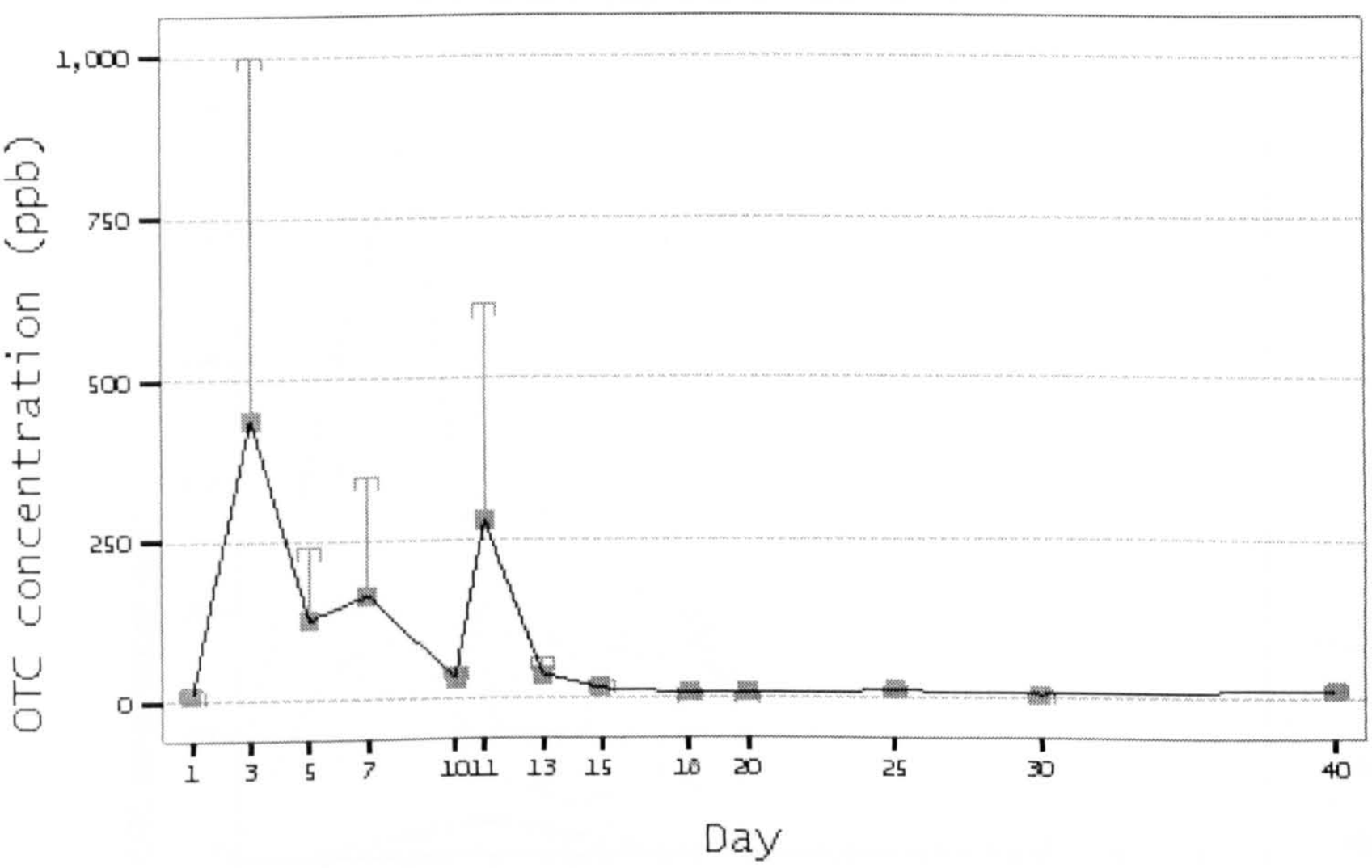
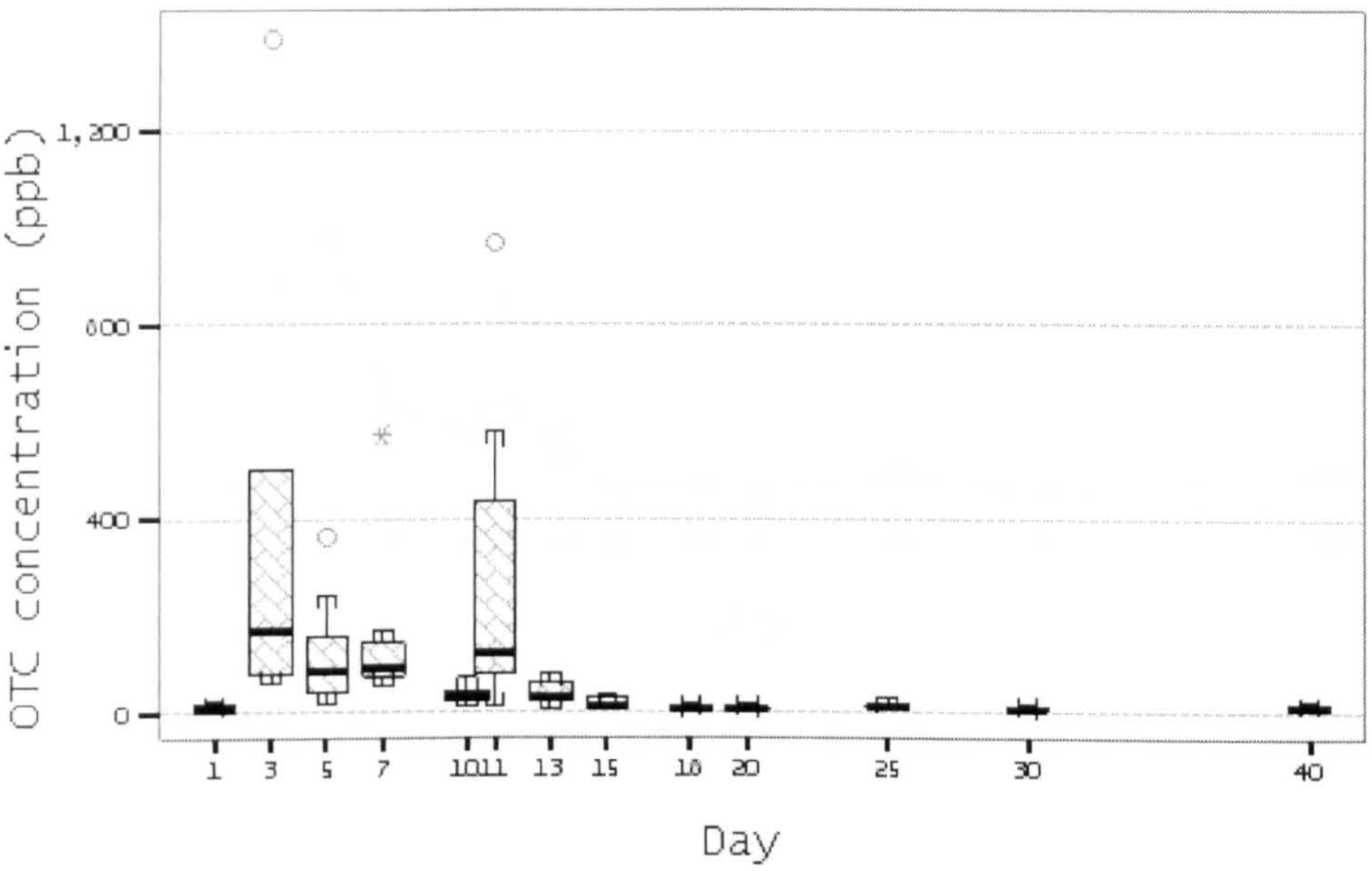


Figure 80: EXPERIMENT VI – Pharmacokinetics of Oxytetracycline in the muscle of sea bream



(*Sparus aurata)* at 18 ± 1◦ C - Box Plot presentation

Figure 81: EXPERIMENT VI – Pharmacokinetics of Oxytetracycline in the liver of sea bream (*Sparus aurata)* at 18 ± 1◦ C

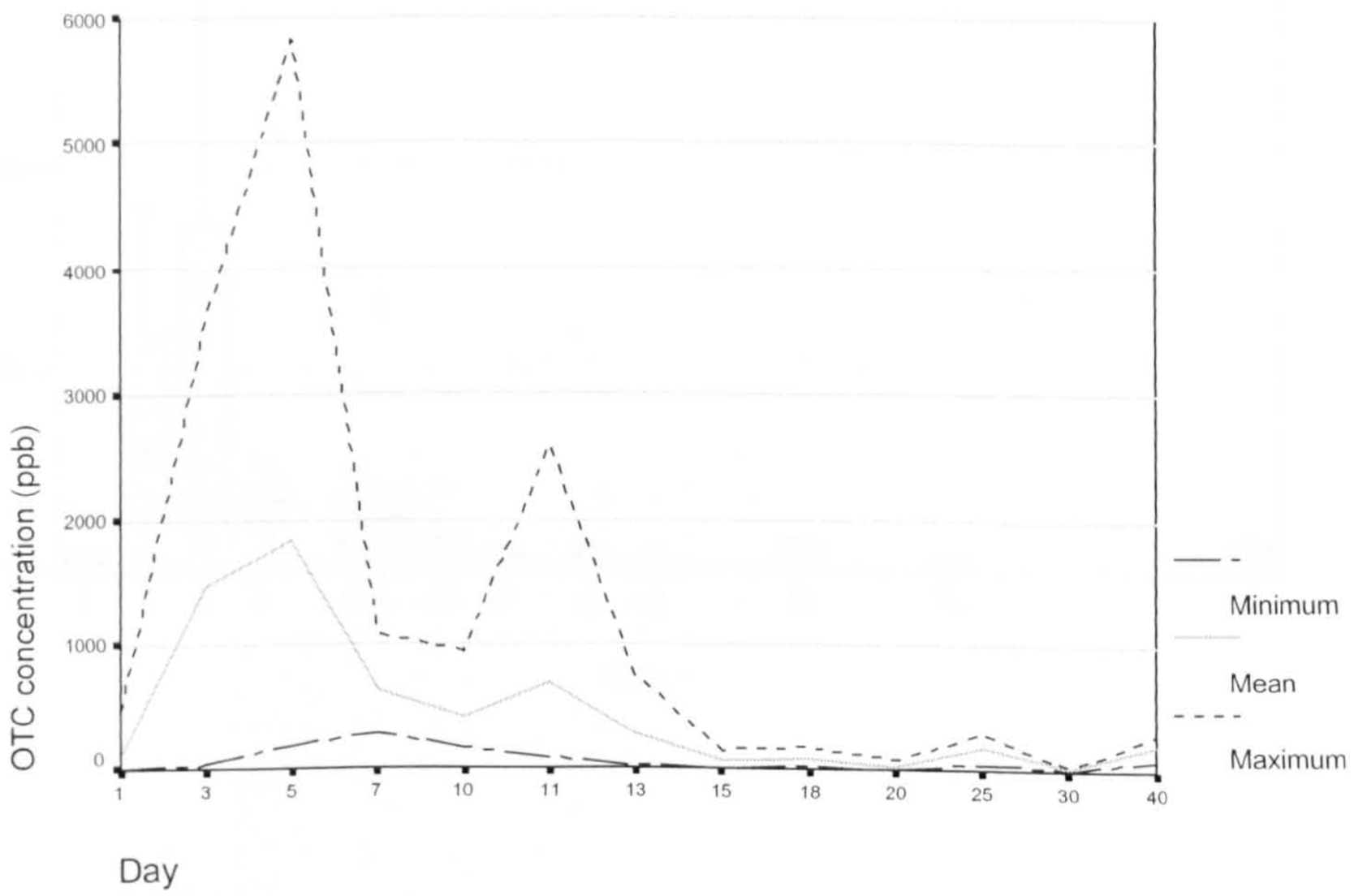
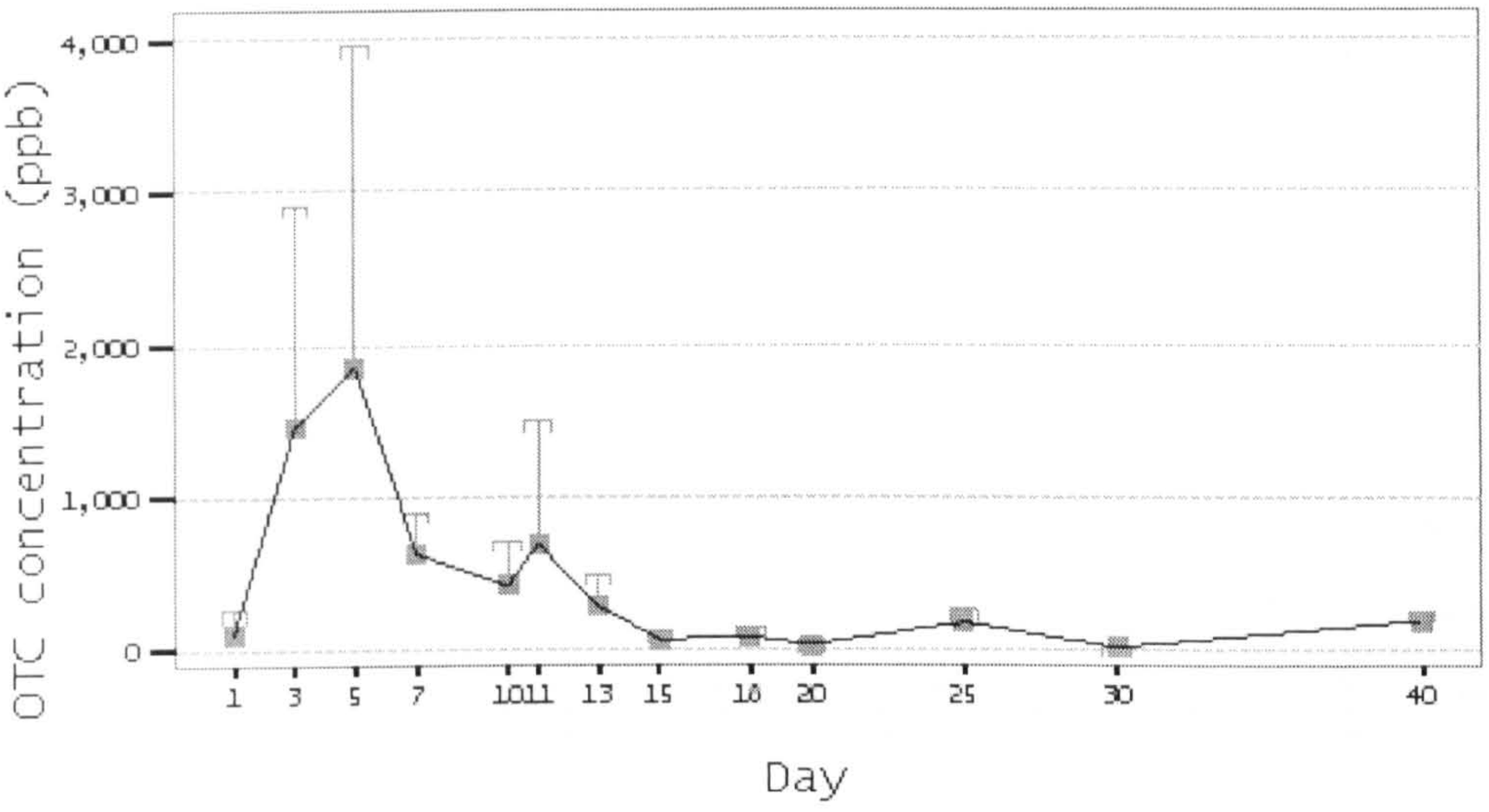


Figure 82: EXPERIMENT VI – Pharmacokinetics of Oxytetracycline in the liver of



sea bream (*Sparus aurata)* at 18 ± 1◦ C - Mean and Standard deviation

Figure 83: EXPERIMENT VI – Pharmacokinetics of Oxytetracycline in the liver of

sea bream (*Sparus aurata)* at 18 ± 1◦ C - Box Plot presentation

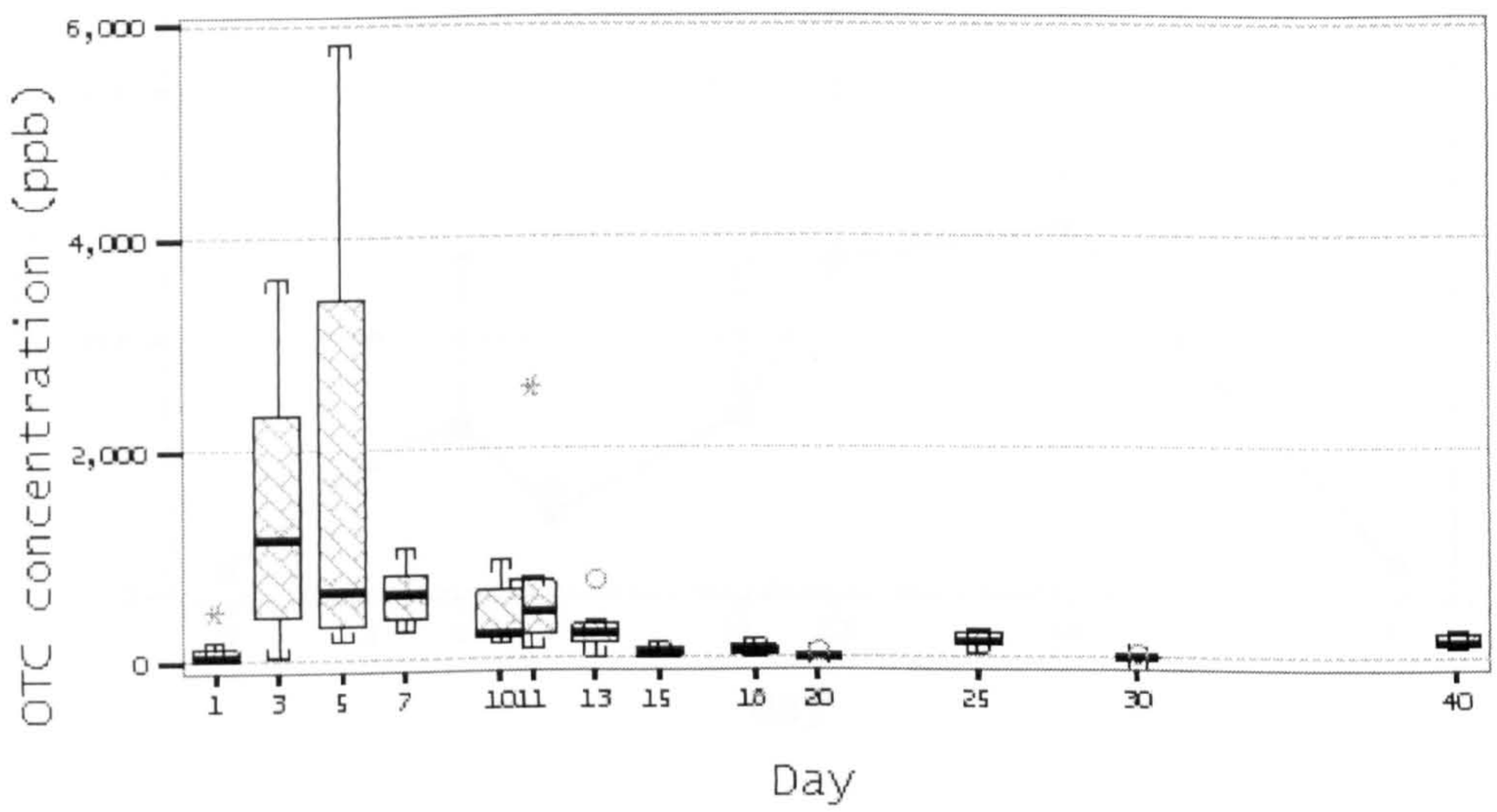


Figure 84: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the serum of sea bream, (*Sparus aurata*) at 18 ± 1◦ C

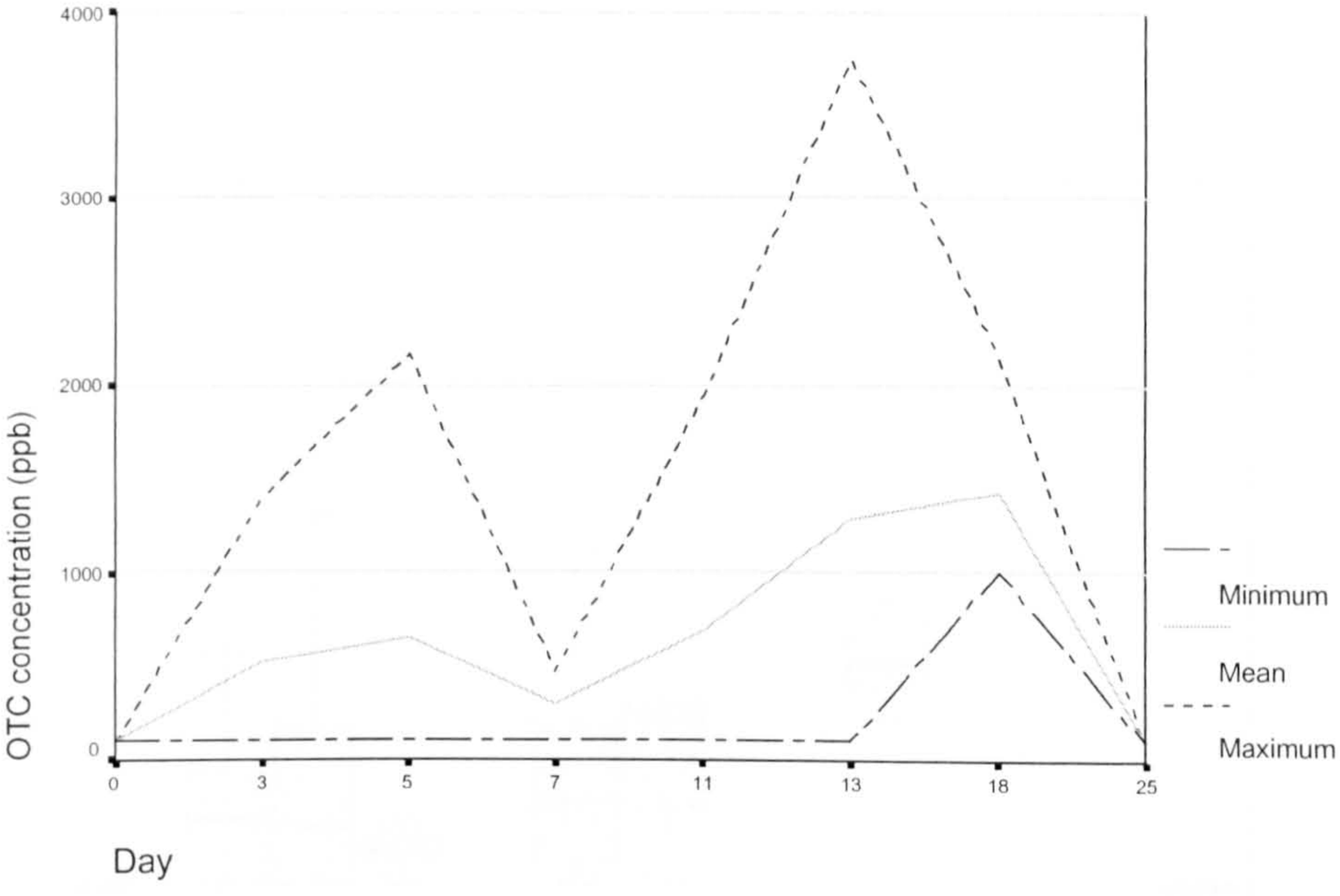


Figure 85: EXPERIMENT VI – Pharmacokinetics of Oxytetracycline in the serum of sea bream (*Sparus aurata)* at 18 ± 1◦ C - Mean and Standard deviatio

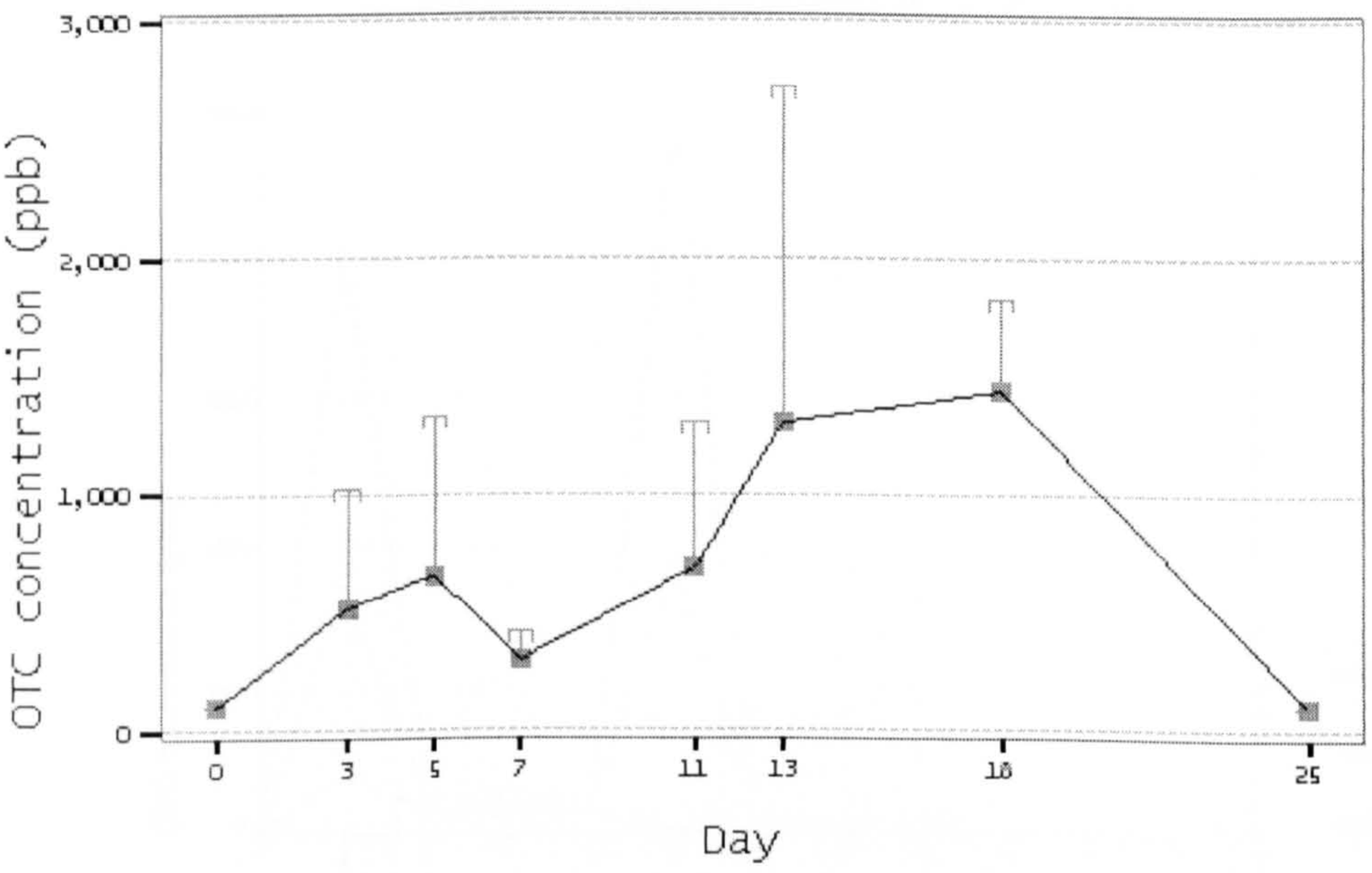


Figure 86: EXPERIMENT VI – Pharmacokinetics of Oxytetracycline in the serum of sea bream (*Sparus aurata)* at 18 ± 1◦ C - Box Plot presentation

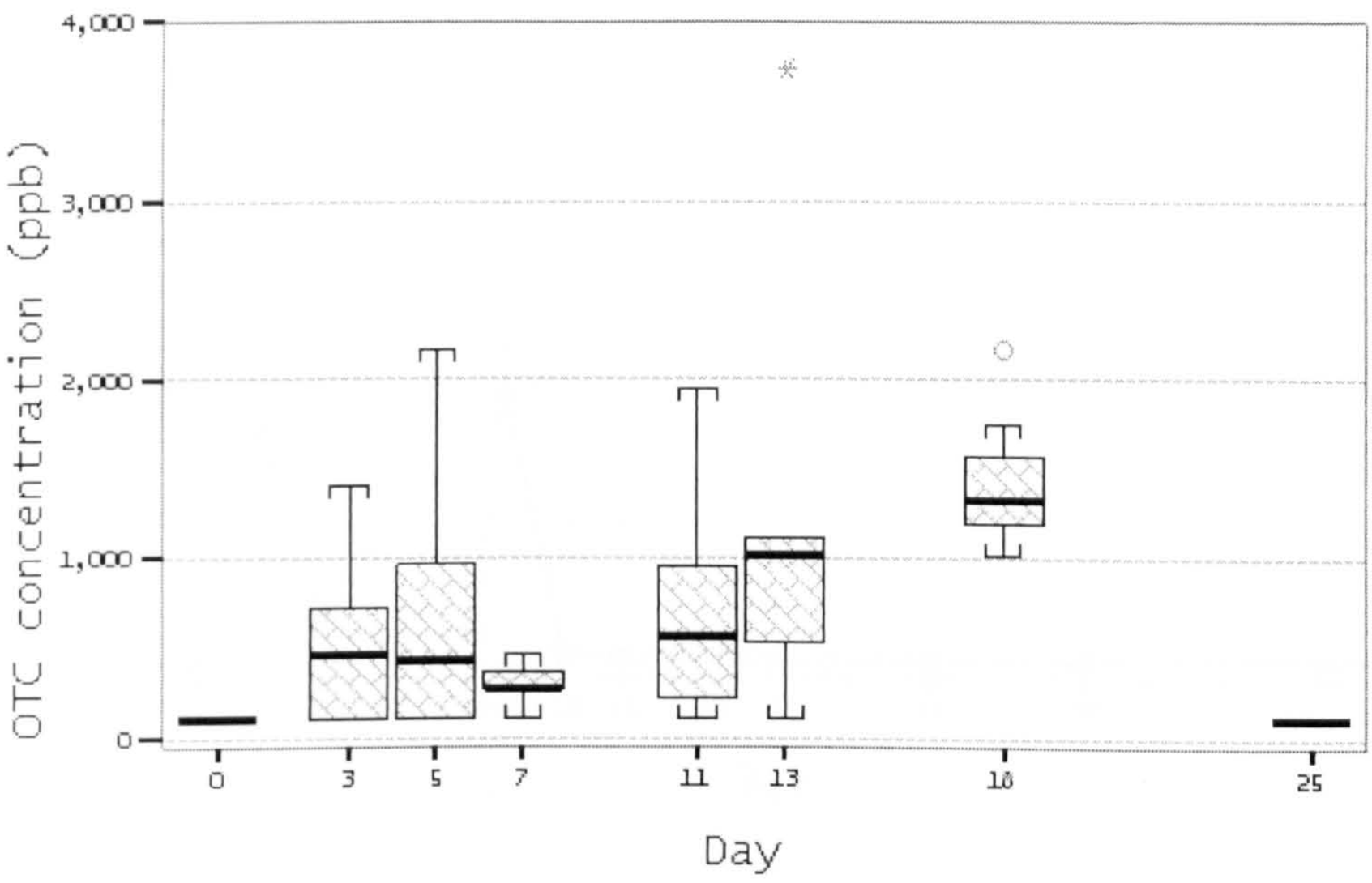


Figure 87: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the skin of sea bream, (*Sparus aurata)* at 18 ± 1◦ C

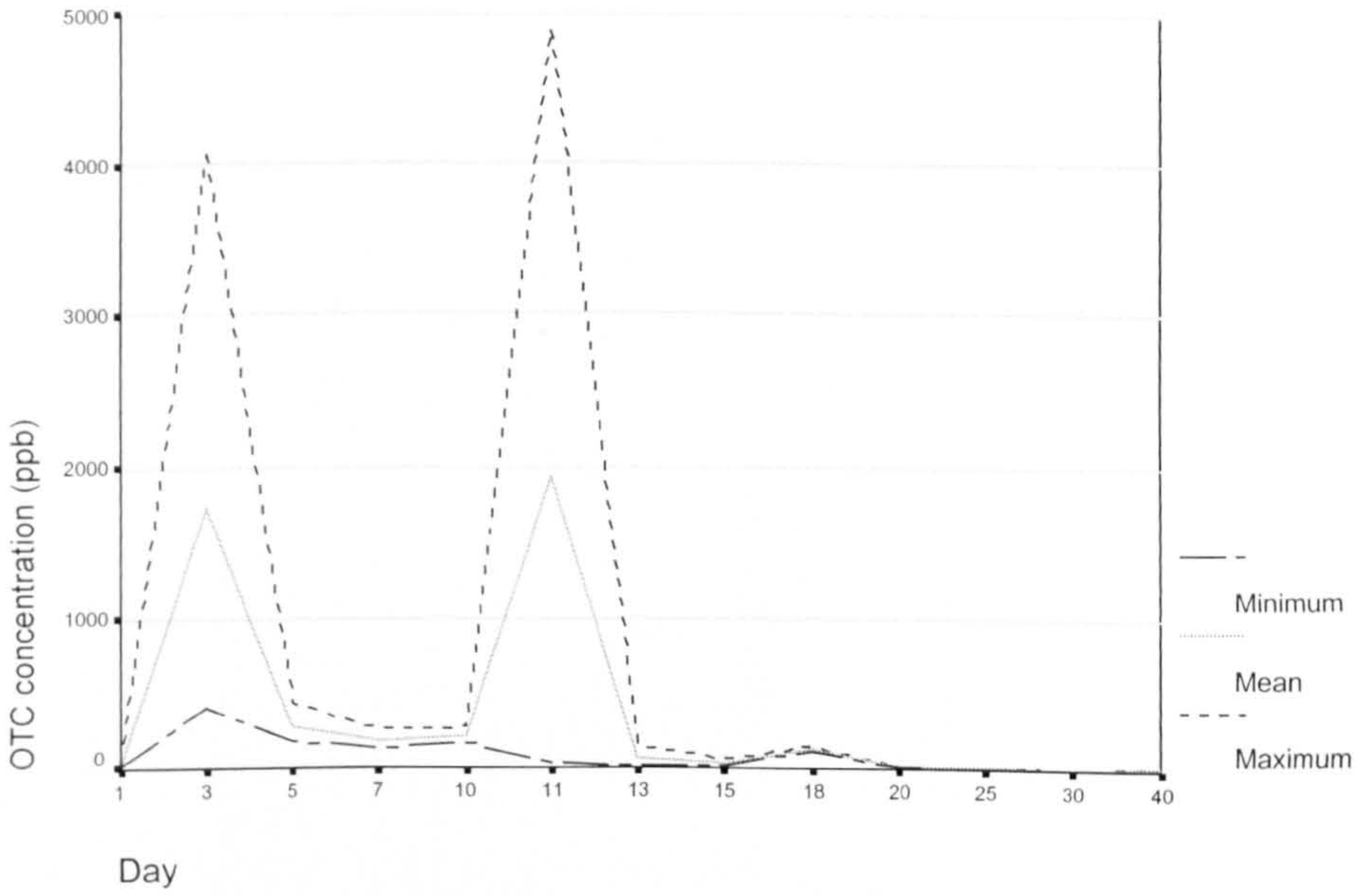
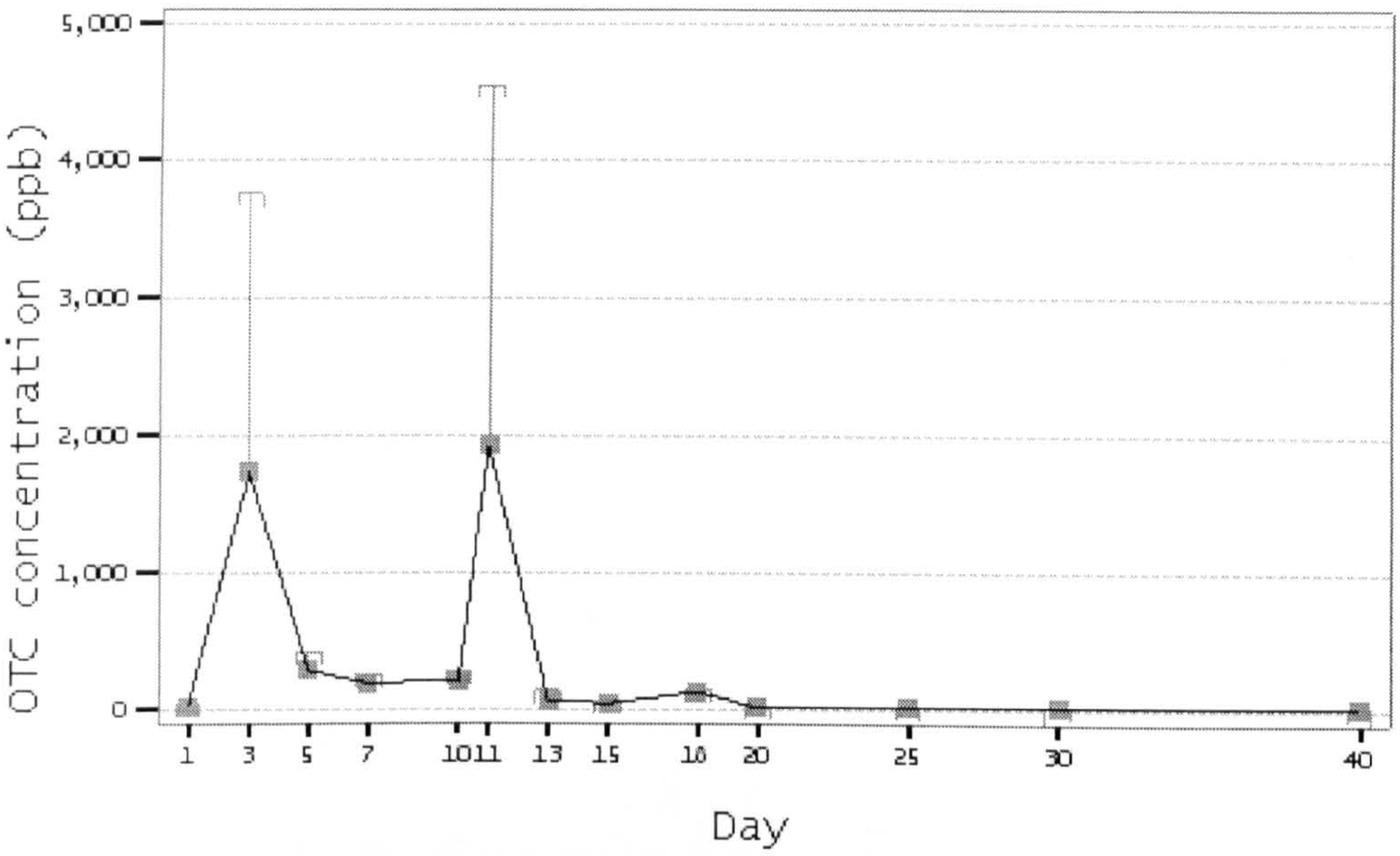


Figure 88: EXPERIMENT VI – Pharmacokinetics of Oxytetracycline in the skin of



sea bream (*Sparus aurata)* at 18 ± 1◦ C - Mean and Standard deviation

3.7.2 Comparisons

3.7.1.2 Muscle versus liver versus serum Depletion

Figure 89: Comparison of OTC mean concentration in the muscle, liver and serum of sea bream at high temperature

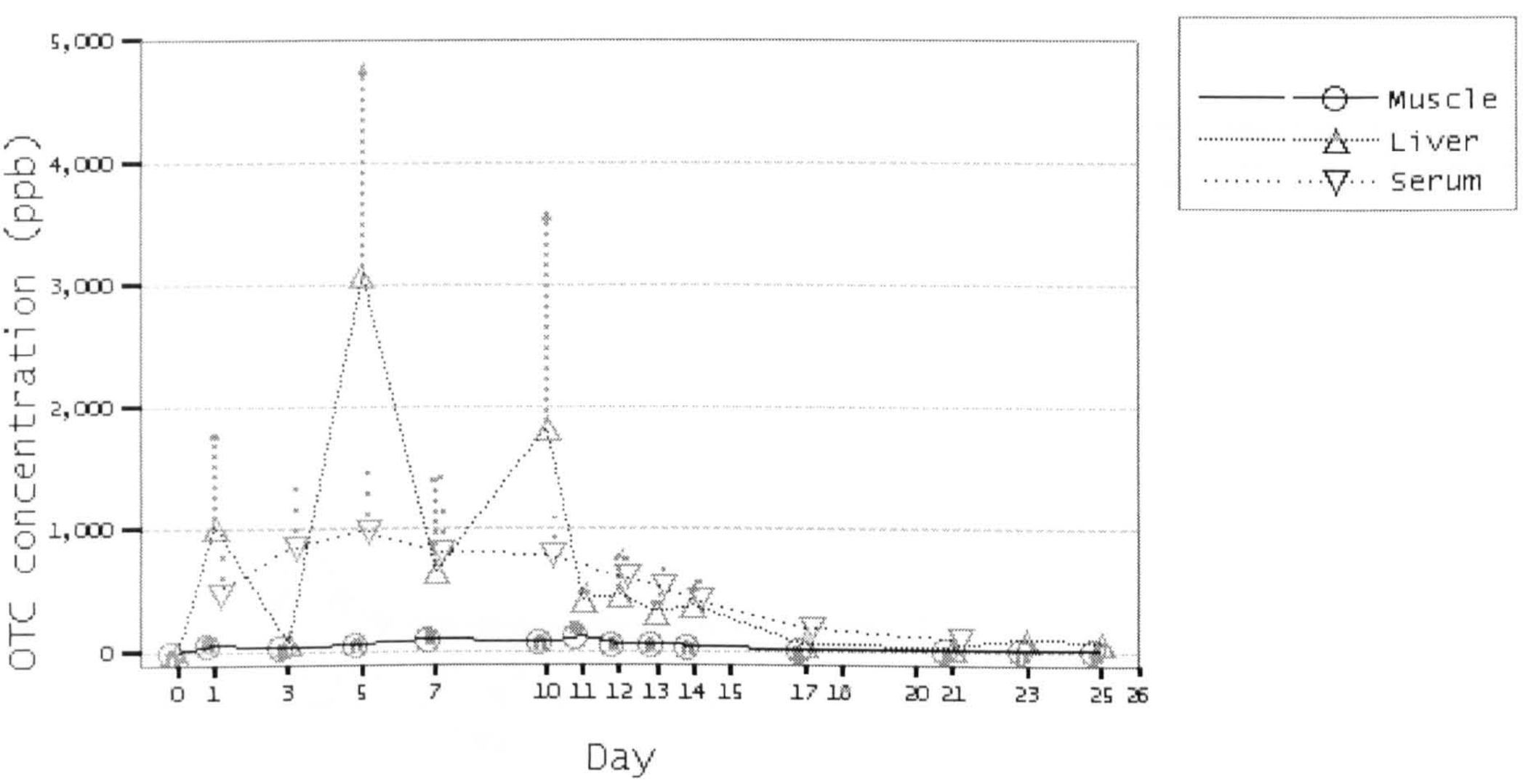
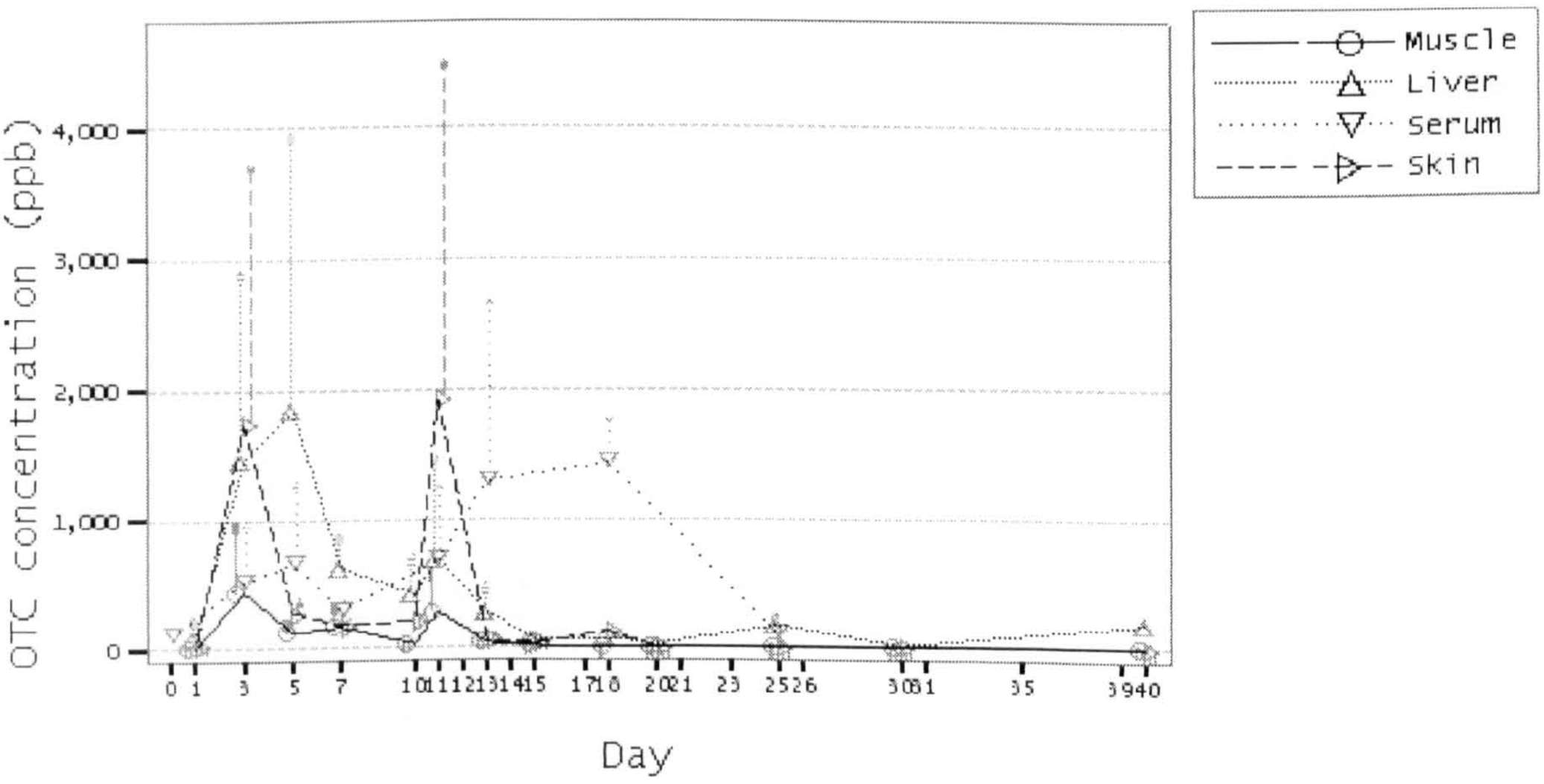


Figure 90: Comparison of OTC mean concentration in the muscle, liver, serum and skin of sea bream at low temperature



3.7.1.2 Depletion at High versus Low temperature

Figure 91: Comparison of OTC mean concentration in the muscle of sea bream at high and low temperature

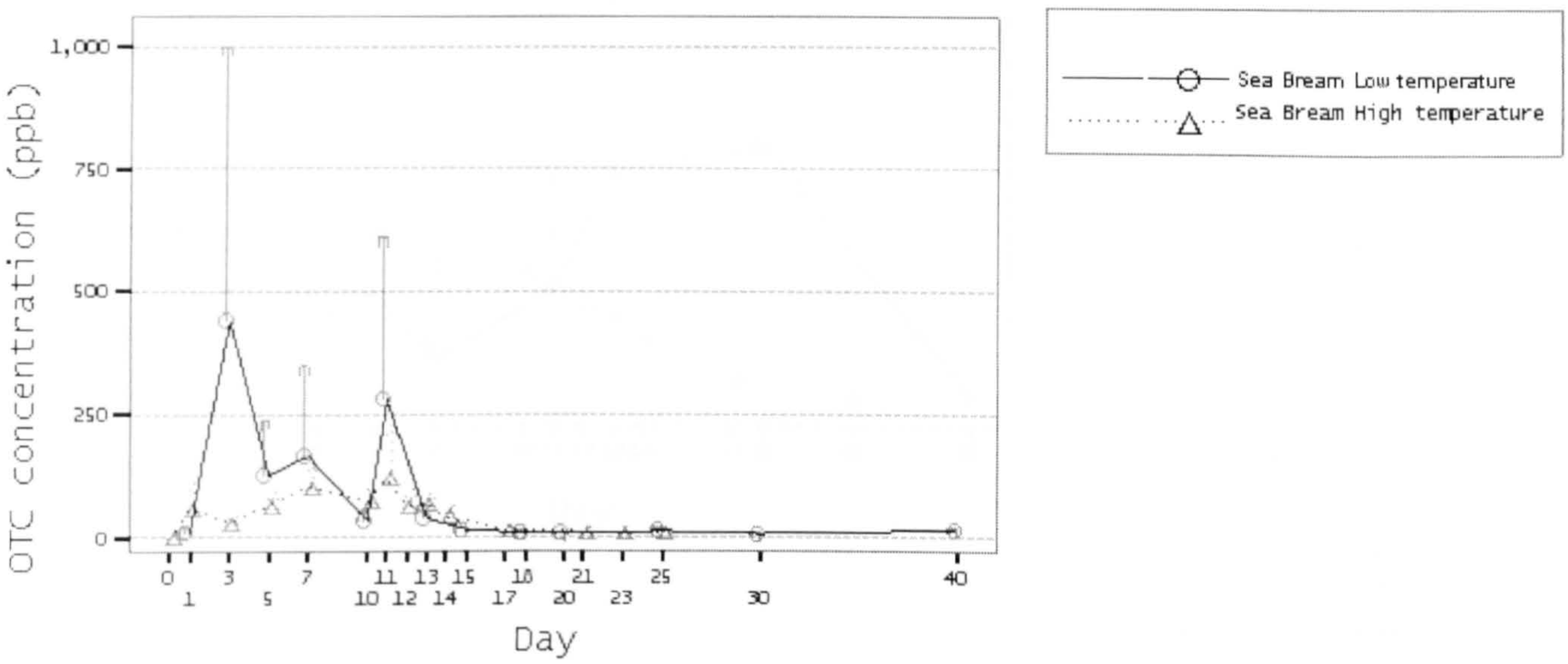


Figure 92: Comparison of OTC mean concentration in the liver of sea bream at high and low temperature

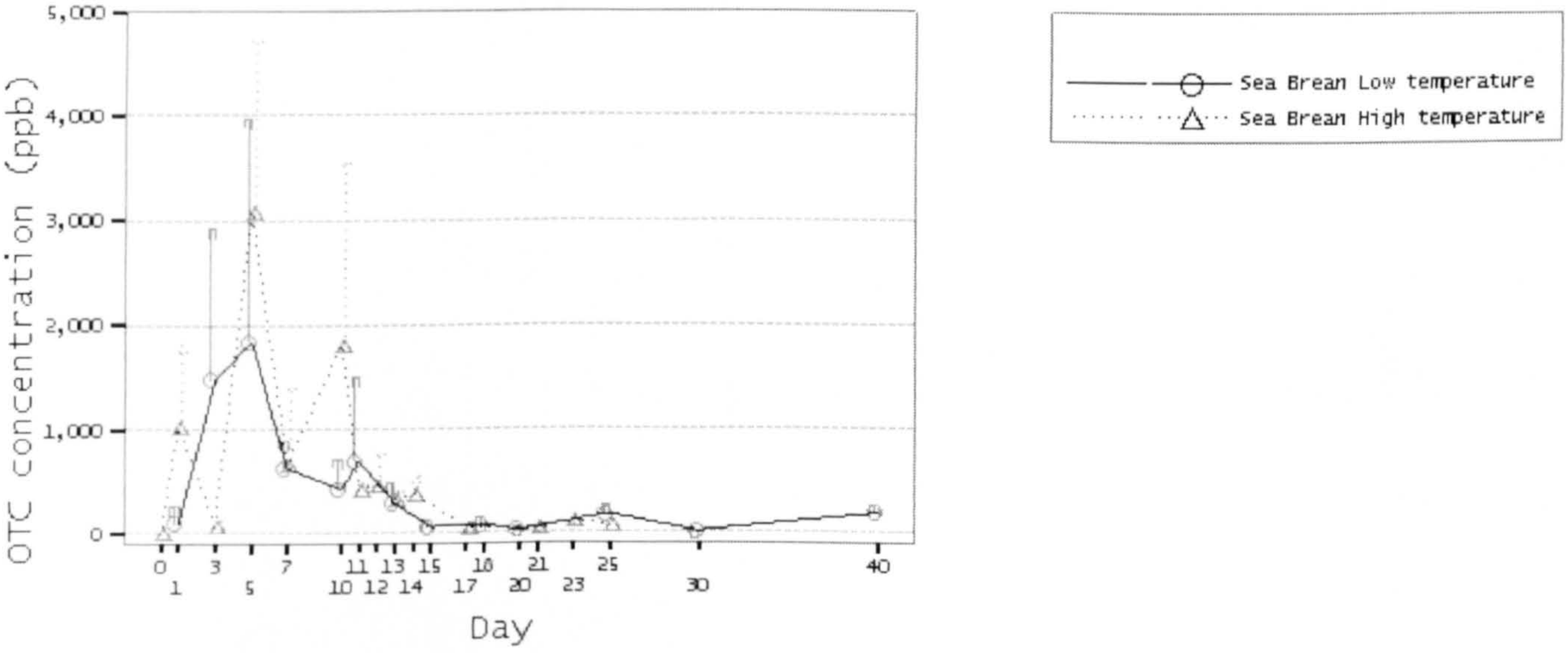
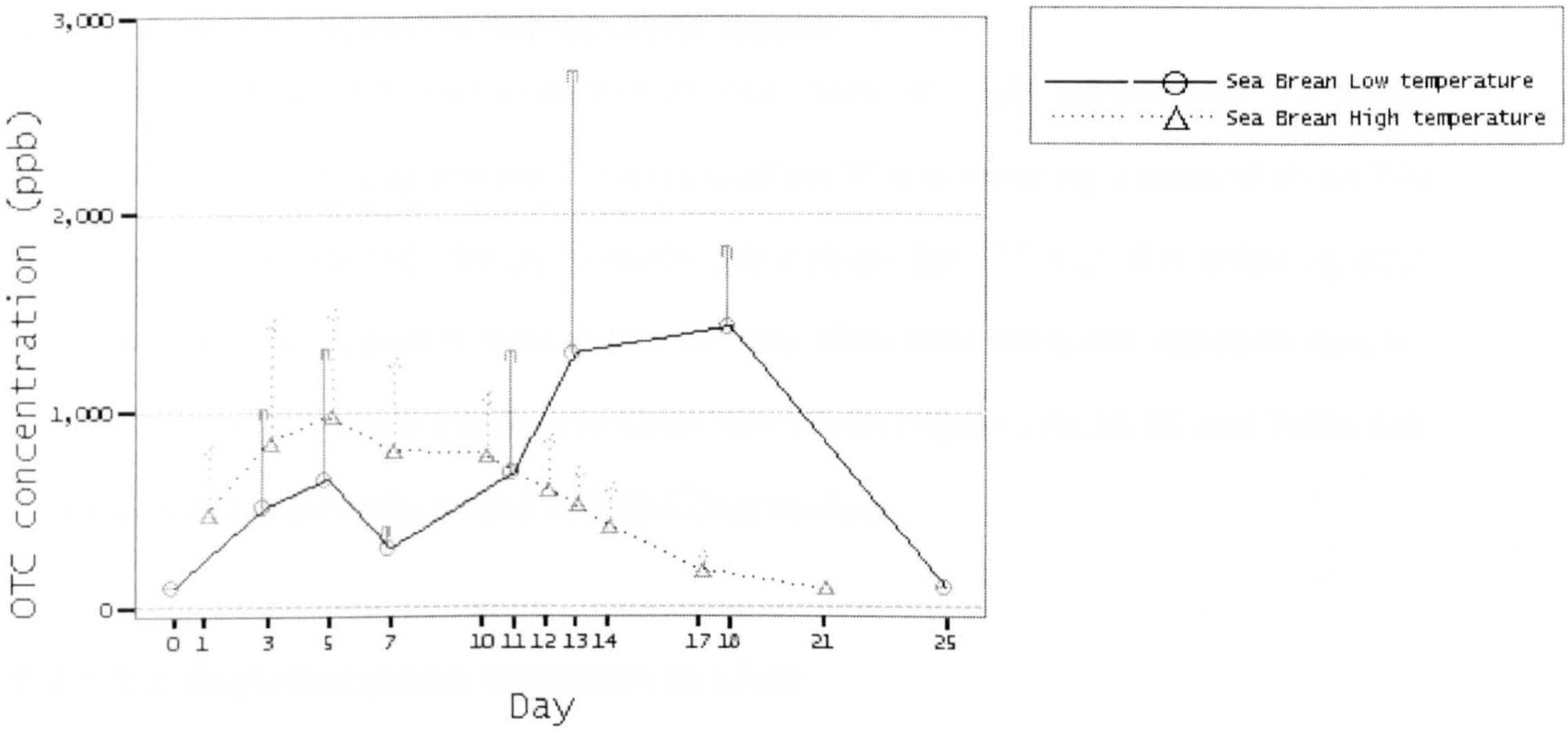


Figure 93: Comparison of OTC mean concentration in the serum of sea bream at high and low temperature



3.8 Oxytetracycline Kinetics in Sea Bass (*Dicentrarchus labrax*)

3.8.1.2 High Water Temperature

3.8.1.2.1 Oxytetracycline depletion in Muscle

Oxytetracycline muscle concentration in sea bass at high temperature increased moderately on the 1st day and then sharply until the 5th day reaching a peak of about 700 ppb while it was reduced sharply to reach 300 ppb on the 11th day. The following days another peak was apparent around the 13th day (350 ppb) while the concentration fell sharply on the 21st day reaching consumer safe levels (Figures 94,95,96 and Table 58). The estimated withdrawal period was 264 Degree-days

3.8.1.2.2 Oxytetracycline depletion in Liver

In the liver of sea bass (if extreme and outlier values are excluded), OTC reached a peak concentration around 8 ppm on the 3rd day reducing rapidly until the 11th day (1ppm), while it was increased slightly again on the 13th day (1.6 ppm) to fall gradually below consumer safe levels on the 25th day (Table 58). The estimated withdrawal period was 15 X 24 = 360 Degree-days. In the presentation of OTC mean and median concentration in the muscle and liver (Figures 97,98 and 99) of sea bass at 24◦ C indicated a three-step depletion process on the 3rd, 7th and 13th day of the experiment.

3.8.1.2.3 Oxytetracycline depletion in Serum

Mean and maximum oxytetracycline concentration in the serum increased moderately up until the first day and rapidly thereafter to reach peak concentrations of 6.25 ppm (mean) and 12.4 ppm maximum on the 10th day of the experiment (final day of the oral treatment). OTC concentration decreased rapidly until the 12th day and gradually thereafter but remained high even on the 21st day (maximum concentration: 1ppm, mean concentration: 758 ppb). Figures 100,101 and 102 as well as Table 58 indicated High standard deviation was evident between the 3rd and the 11th day and Minimum OTC serum concentration remained below the method detection limit up until the 10th day reaching a peak on the 12th day (5.2 ppm) and was reduced gradually thereafter. The estimated withdrawal period in the serum of sea bass at that temperature was over 264 degree-days.

Table 58: EXPERIMENT VII Oxytetracycline kinetics in Sea bass at high temperature after 10 day oral treatment - Statistical interpretation

Statistical Analysis *Oxytetracycline acid concentration - Sea Bass Muscle - High Temperature*								
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 12
Samples	4	7	8	7	5	8	8	6
Minimum concentration(ppb)	3,47	7,59	27,88	23,28	24,27	7,26	10,37	77,69
Mean concentration(ppb)	3,47	23,97	317,18	275,96	420,87	148,24	152,24	204,21
Maximum concentration(ppb)	3,47	51,69	574,87	686,05	658,60	391,11	310,28	378,92
Standard deviation	0,00	15,82	189,96	265,62	284,92	159,36	95,72	114,31
25% percentile		8,49	125,20	96,08	122,10	16,90	79,58	117,89
Median		26,94	334,24	192,10	546,99	85,59	145,04	170,29
75% Percentile		31,59	473,68	622,49	656,59	332,77	230,40	317,32

	Day 13	Day 14	Day 17	Day 21	Day 23	Day 25		
Samples	8	8	8	8	7	6		
Minimum concentration(ppb)	104,39	17,81	8,21	20,18	9,28	16,86		
Mean concentration(ppb)	247,74	203,00	97,66	37,94	24,57	27,87		
Maximum concentration(ppb)	355,44	93,00	309,33	68,41	36,33	42,08		
Standard deviation	83,56	328,48	91,13	16,93	10,34	9,53		
25% Percentile	193,81	140,24	50,37	23,21	12,69	20,10		
Median	241,85	229,56	74,20	35,67	29,16	26,32		
75% Percentile	333,44	246,66	108,98	52,21	33,24	36,36		

Oxytetracyline concentration - Sea Bass Liver - High Temperature								
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 12
Samples	8	6	5	8	8	7	8	8
Minimum concentration(ppb)	3,47	6,18	333,19	24,70	22,76	26,94	10,36	484,86
Mean concentration(ppb)	6,51	96,26	6668,32	987,80	1434,37	1210,79	630,20	746,84
Maximum concentration(ppb)	19,94	224,64	19410,16	2824,06	3572,68	4182,27	1909,09	1012,91
Standard deviation	6,08	99,49	7666,44	878,17	1210,19	1648,52	626,75	179,35
25% percentile	3,47	15,60	1521,92	231,31	231,35	66,61	131,60	598,78
Median	3,47	55,27	2803,08	886,25	1403,19	384,67	468,33	711,37
75% Percentile	9,35	219,27	13747,36	1276,69	2282,94	2910,91	1008,83	907,60

Sea Bass Liver - High Temperature								
	Day 13	Day 14	Day 17	Day 21	Day 23	Day 25		
Samples	8	8	6	8	8	6		
Minimum concentration(ppb)	373,36	43,55	115,17	41,42	31,23	27,41		
Mean concentration(ppb)	1010,62	838,14	250,10	72,32	173,19	269,95		
Maximum concentration(ppb)	1620,16	2683,30	430,84	118,54	307,34	1253,23		
Standard deviation	487,28	821,91	114,36	27,25	99,53	483,36		
25% Percentile	489,64	416,34	159,78	46,34	89,81	42,10		
Median	1031,03	507,37	240,54	67,18	173,15	76,04		
75% Percentile	1498,75	1105,73	331,86	94,52	280,36	417,40		

Oxolinic acid concentration - Sea Bass Serum (BIOASSAY) - High Temperature								
		Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 12
Samples		5	5	4	6	7	6	6
Minimum concentration(ppb)		100,00	100,00	100,00	100,00	100,00	1480,00	4066,00
Mean concentration(ppb)		435,20	2132,40	3952,50	4578,33	6274,28	5049,17	4674,33
Maximum concentration(ppb)		851,00	3161,00	6460,00	10470,00	12440,00	9729,00	5190,00
Standard deviation		329,01	1240,36	2719,38	4368,35	4445,51	3489,35	448,55
25% percentile		100,00	971,00	1162,50	100,00	1190,00	2335,00	4234,00
Median		524,00	2632,00	4625,00	4270,00	7620,00	3750,00	4725,00
75% Percentile		726,00	3044,00	6070,00	8812,50	9460,00	9156,75	5085,00

Sea Bass Serum (BIOASSAY) - High Temperature								
	Day 13	Day 14	Day 17	Day 21	Day 23	Day 25		
Samples	5	6	5	5				
Minimum concentration(ppb)	1010,00	2224,00	100,00	570,00				
Mean concentration(ppb)	3258,00	3484,83	937,40	758,00				
Maximum concentration(ppb)	4530,00	4658,00	1890,00	1011,00				
Standard deviation	1453,95	918,37	684,05	163,68				
25% Percentile	1855,00	2543,50	298,50	620,50				
Median	3640,00	3598,00	1010,00	769,00				
75% Percentile	4470,00	4300,25	1540,00	890,00				

Figure 94: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the muscle of sea bass (*Dicentrarchus labrax)* at 24 ± 1◦ C

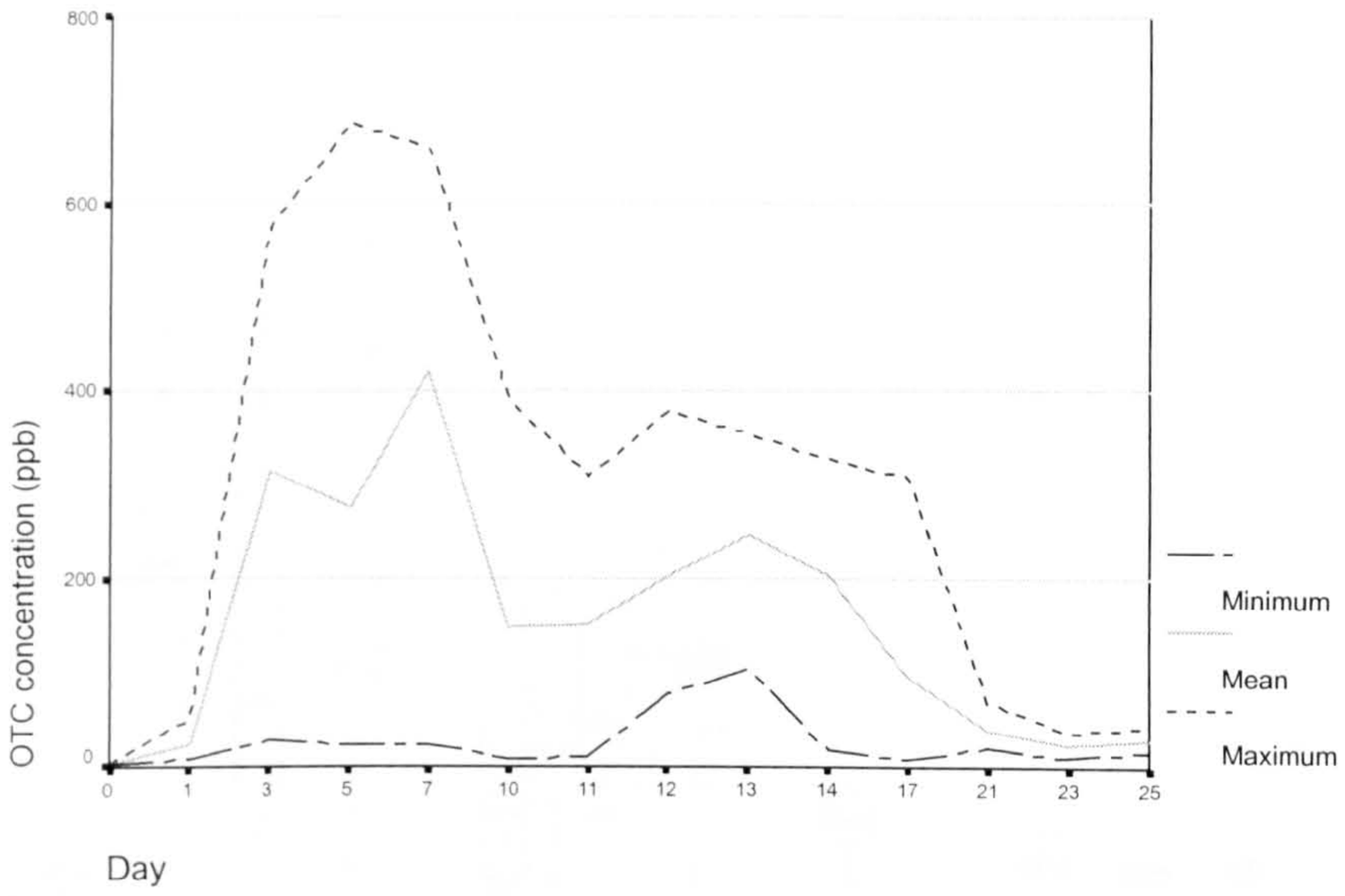


Figure 95: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the muscle of sea bass (*Dicentrarchus labrax)* at 24 ± 1◦ C - Mean and Standard deviation

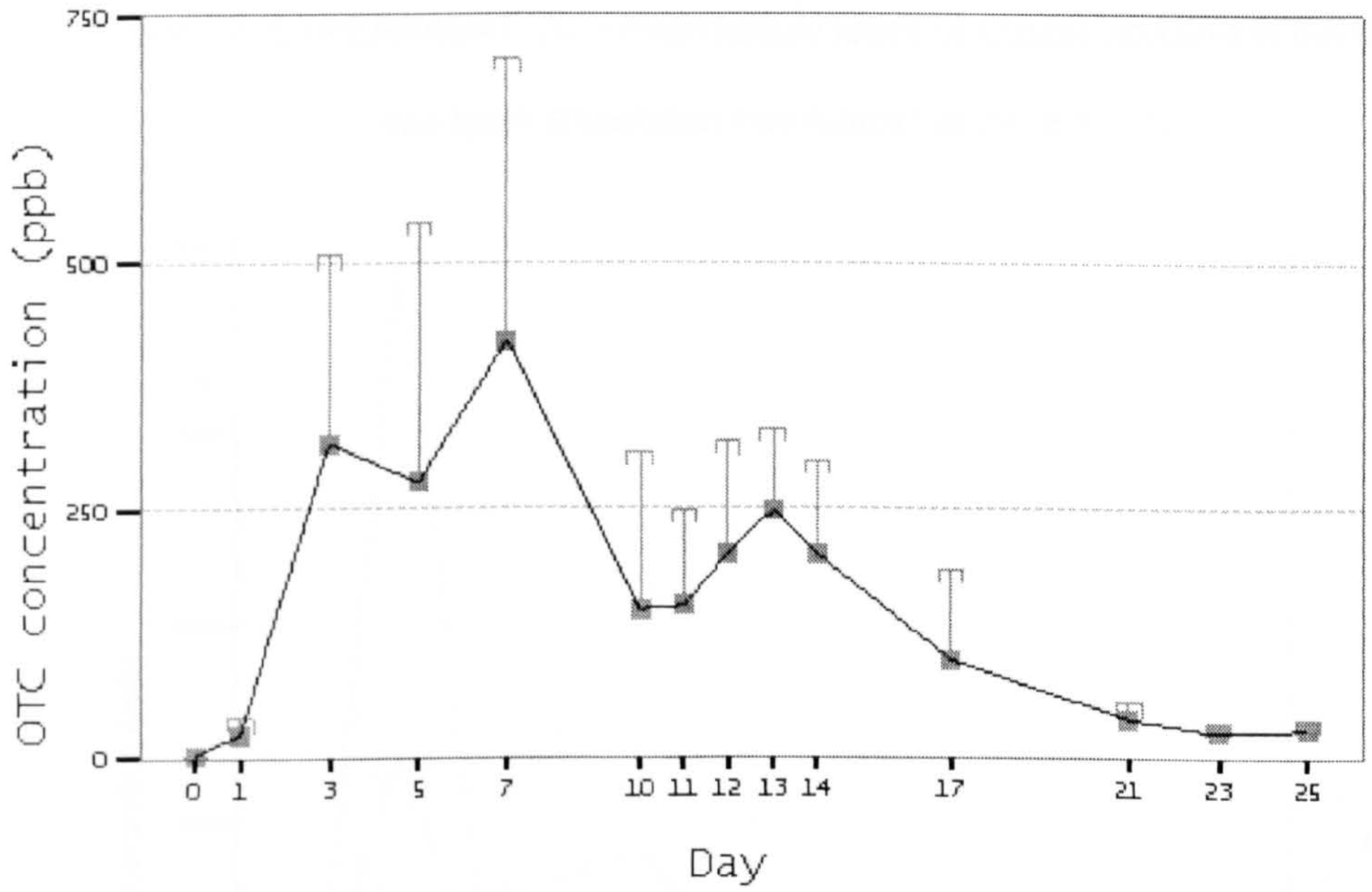
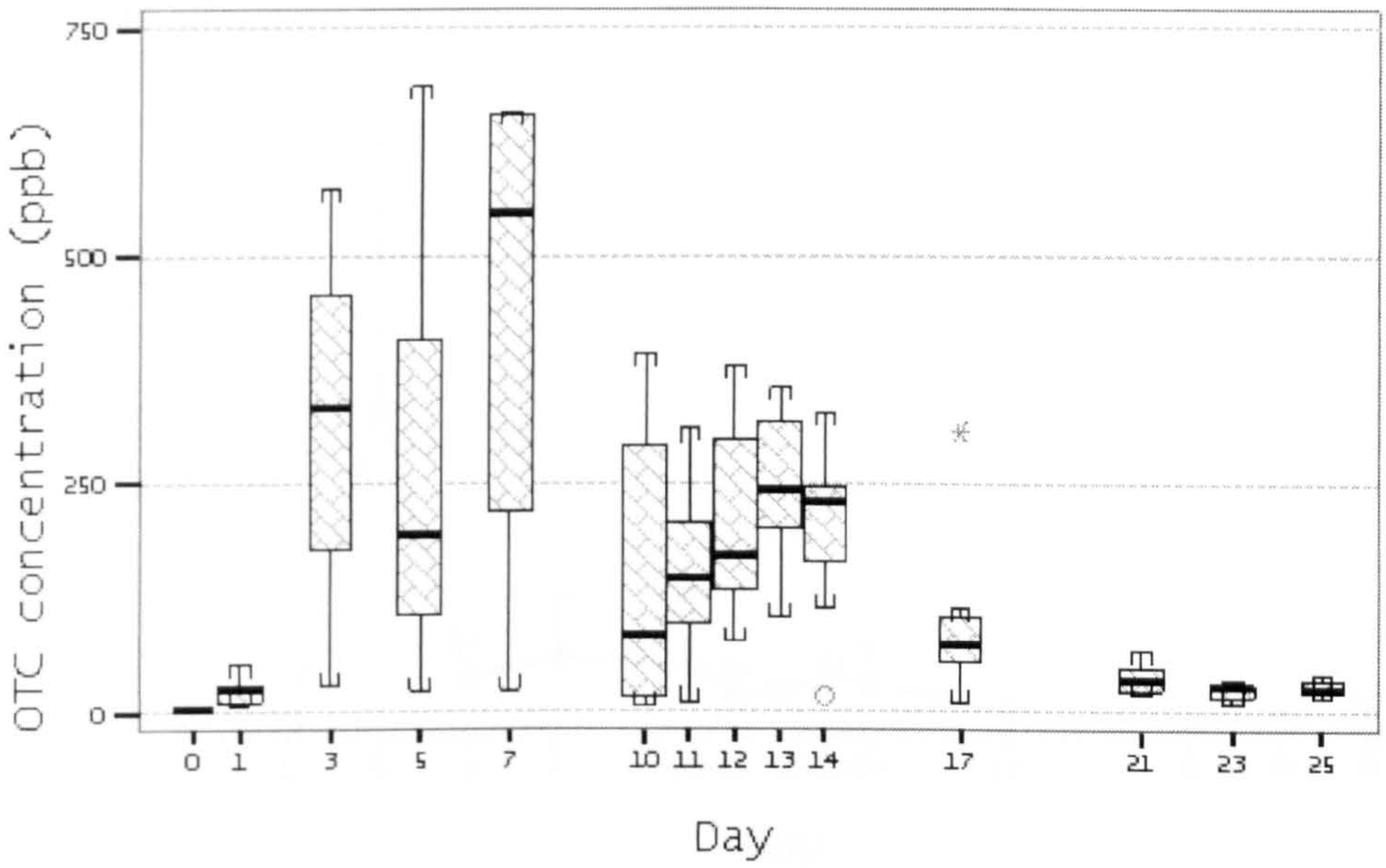


Figure 96: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the muscle of



sea bass (*Dicentrarchus labrax)* at 24 ± 1◦ C - Box plot presentation

Figure 97: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the liver of

sea bass (*Dicentrarchus labrax)* at 24 ± 1◦ C

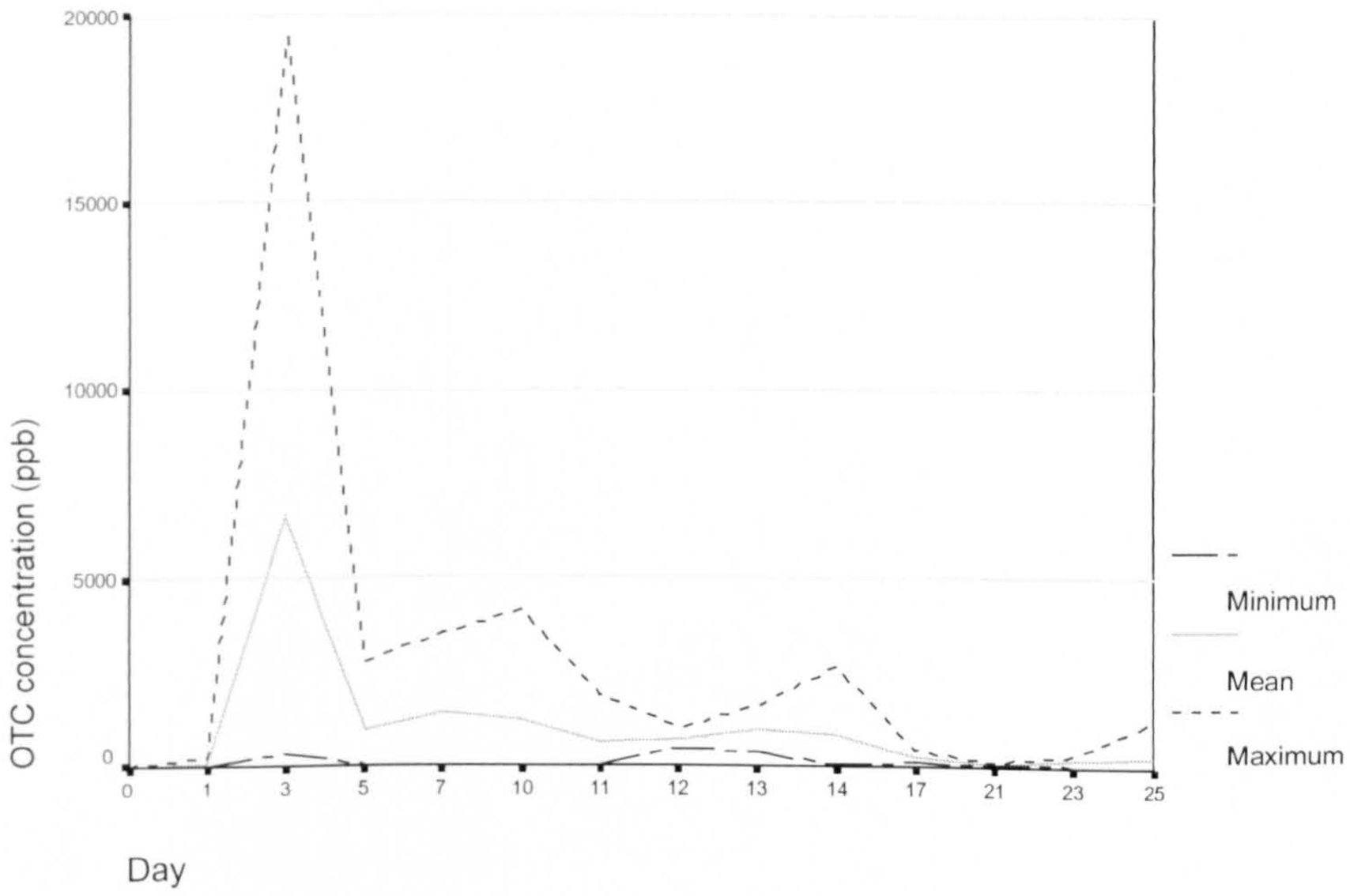
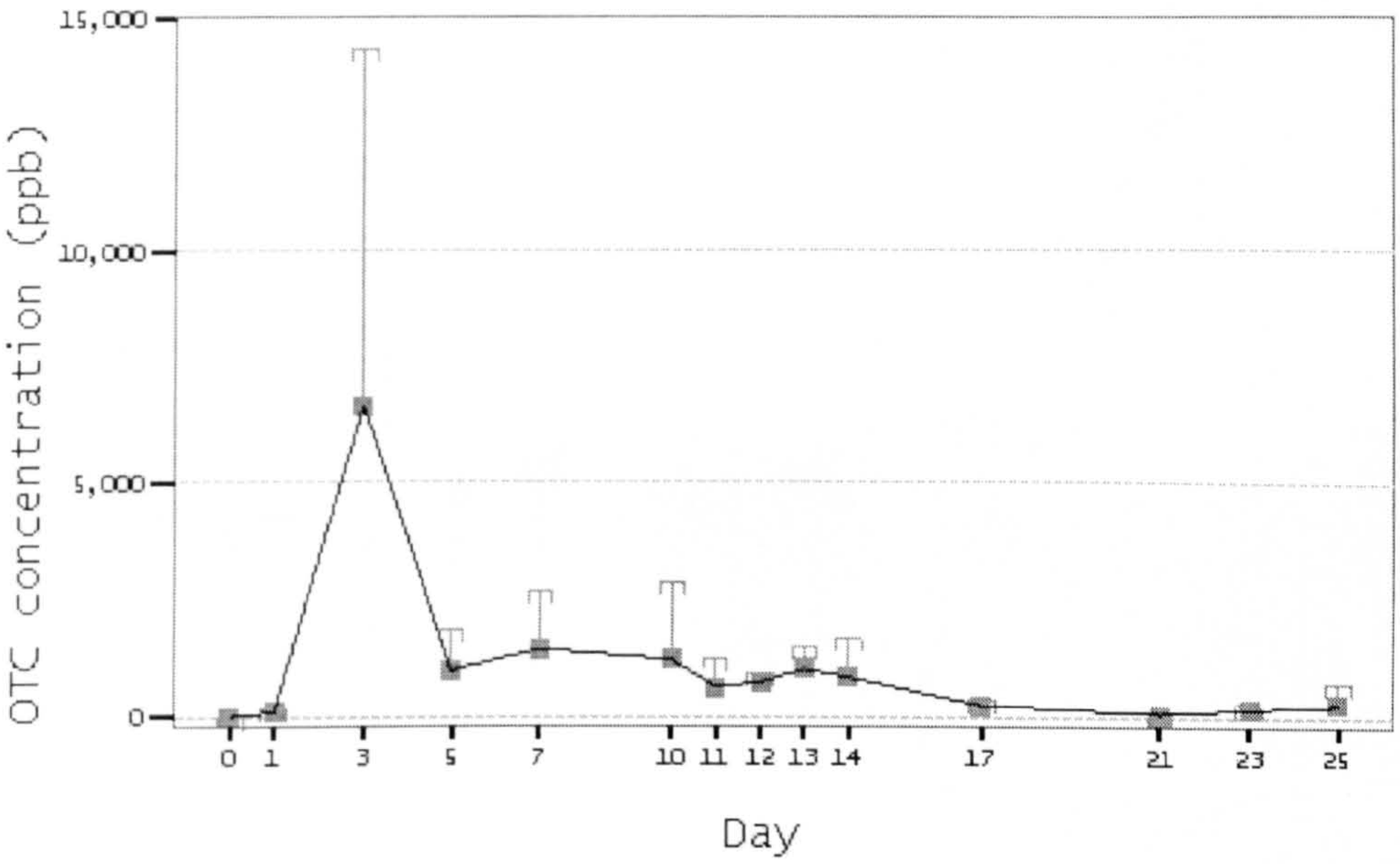


Figure 98: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the liver of



sea bass (*Dicentrarchus labrax)* at 24 ± 1◦ C – Mean and Standard deviation

Figure 99: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the liver of sea bass

(*Dicentrarchus labrax)* at 24 ± 1◦ C – Box plot presentation

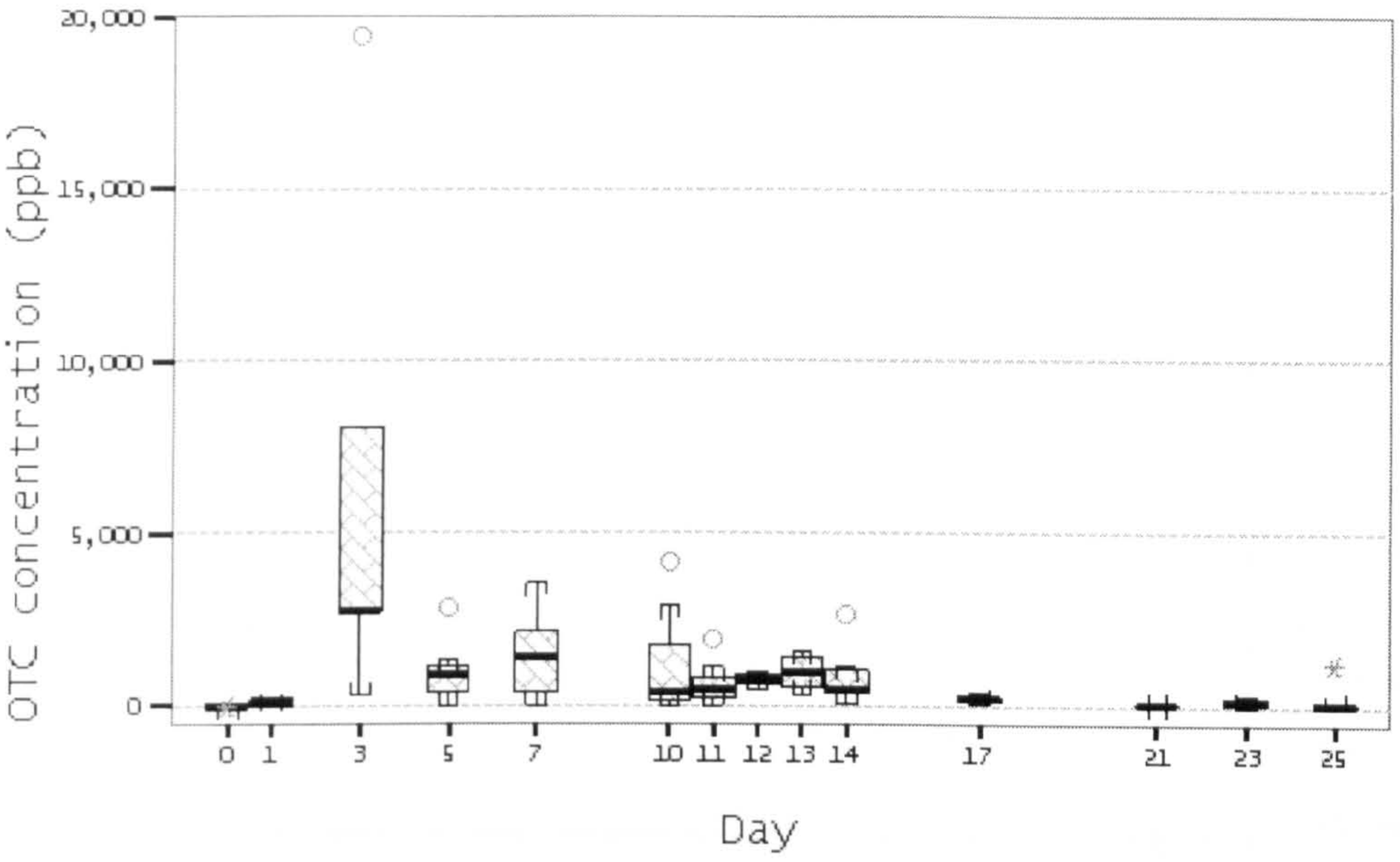

OTC concentration (ppb)
20,000
15,000
10,000
5,000
0
0 1 3 5 7 10 11 12 13 14 17 21 23 25
Day

Figure 100: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the serum of sea bass (*Dicentrarchus labrax)* at 24 ± 1◦ C

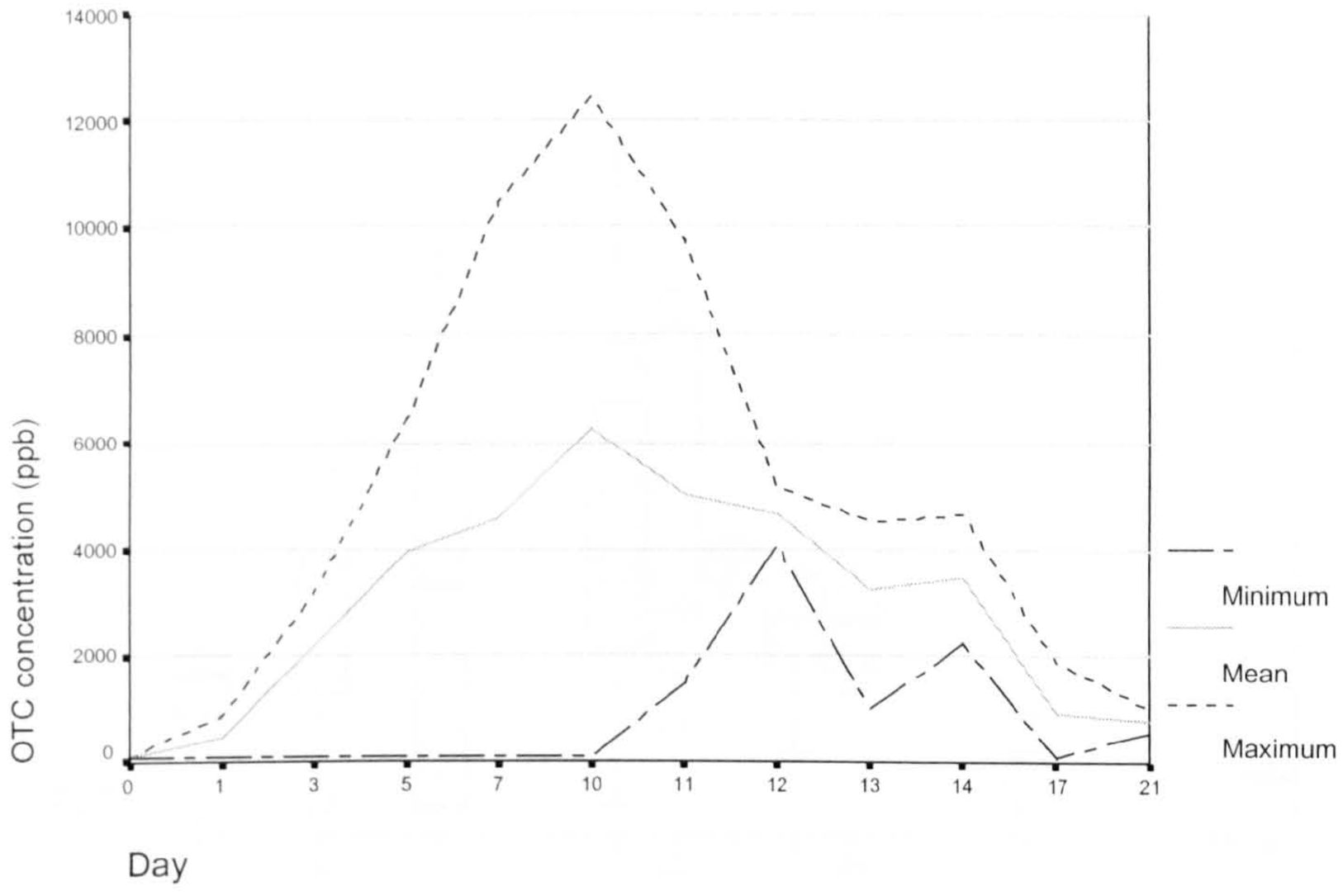


Figure 101: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the serum of sea bass (*Dicentrarchus labrax)* at 24 ± 1◦ C – Mean and Standard deviation

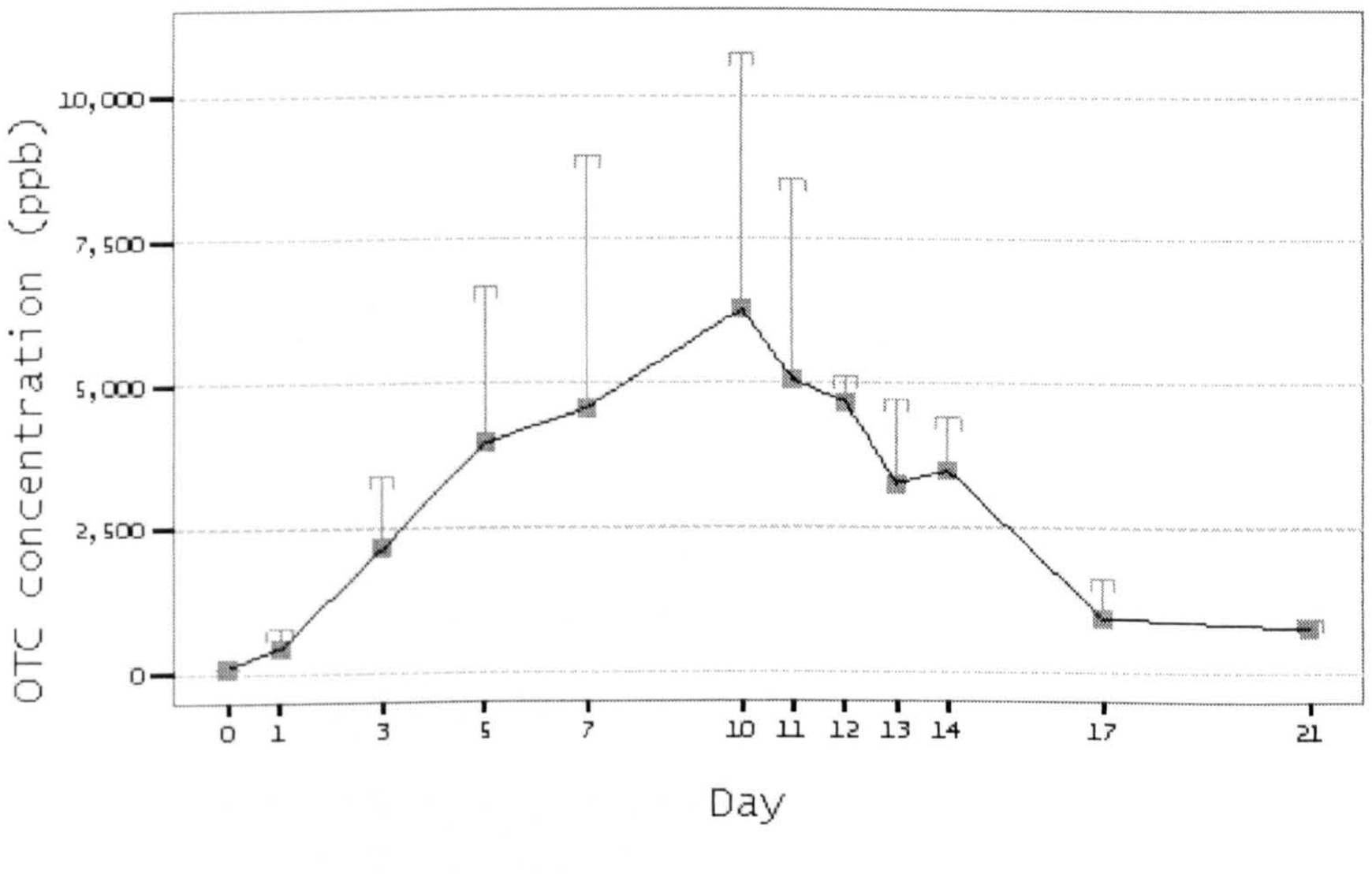
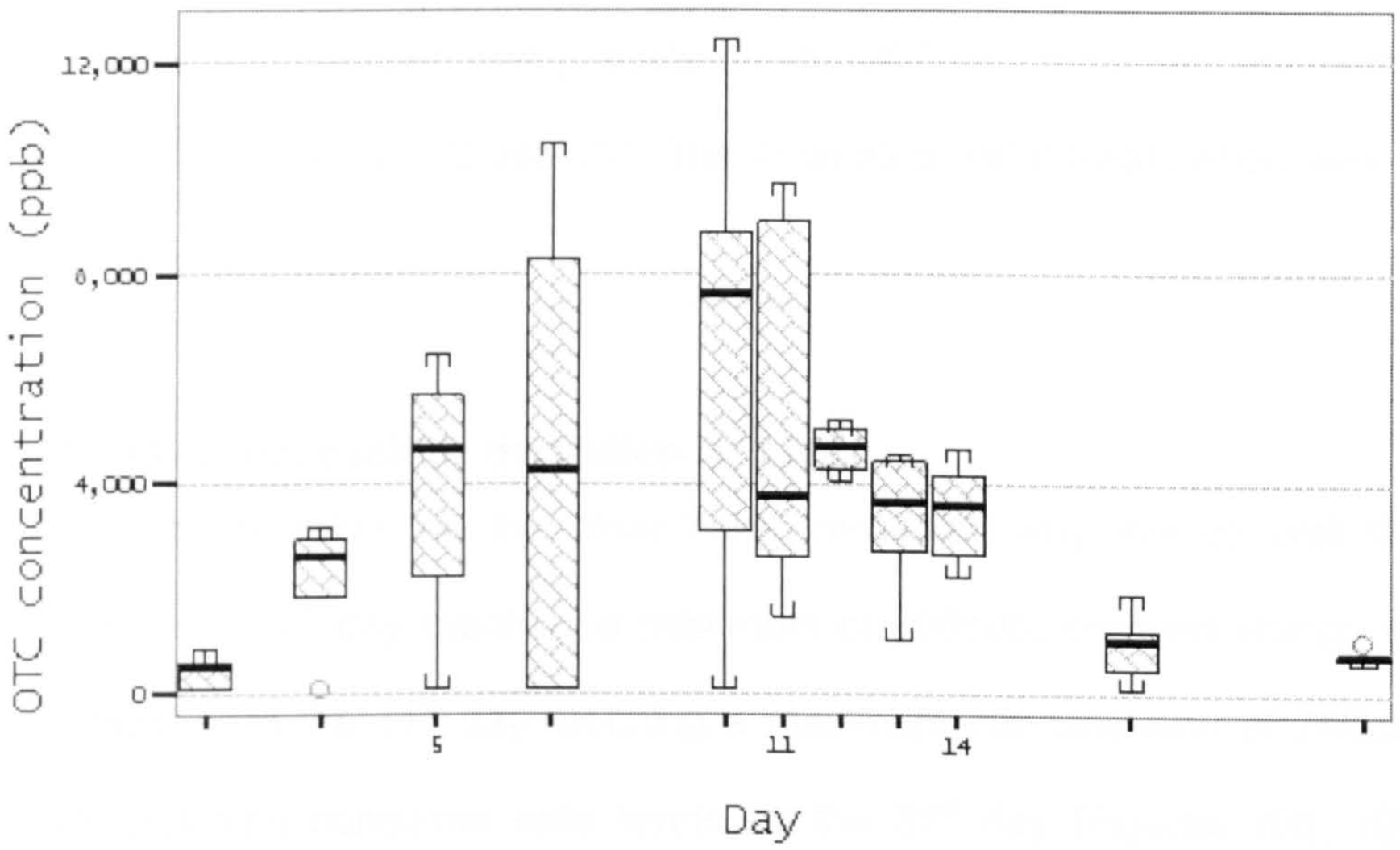


Figure 102: EXPERIMENT VII – Pharmacokinetics of Oxytetracycline in the serum of



sea bass (*Dicentrarchus labrax)* at 24 ± 1◦ C – Box plot presentation

3.8.1.2 Low water temperature

3.8.1.2.1 Oxytetracycline depletion in Muscle

Analysis of muscle OTC concentration (if extreme and outlier values are excluded) increased gradually to reach maximum (about 400ppb) on the 10th day decreasing gradually below consumer safety levels on the 20th and remained very low thereafter (Figures 103,104,105 and Table 59). The estimated withdrawal period was 10 X 14 = 140 Degree-days.

3.8.1.2.2 Oxytetracycline depletion in Liver

OTC liver concentration on the other hand, remained very low up until the 5th day, increased on the 7th day reaching a maximum of 600ppb, dropped sharply on the 10th day, increased on the 11th day reaching a maximum concentration of 740ppb and fell gradually reaching consumer safe levels on the 31st day (Figures 106, 107,108 and Table 59). The estimated withdrawal period was 21 X 14 = 294 Degree-days.

3.8.1.2.3 Oxytetracycline depletion in Serum

Mean and maximum OTC concentration (Figures 109, 110,111 and Table 59) in the serum increased moderately until the 5th day (740ppb/2173 ppb). Maximum concentration reached a peak on the 7th day (6.13 ppm) and was reduced up until the 15th day (4.6 ppm), then sharply until the 18th day (1.4 ppm) and moderately until the 40th day reaching the detection limit of the method. Mean OTC serum concentration reached a plato between the 7th and 10th day (2.7 ppm) reduced sharply on the 11th day (1.37 ppm) and gradually thereafter. It is important to indicate that minimum concentration remained below the detection limit of the bioassay method throughout the experimental period. The estimated withdrawal period was 30 X 14 = 420 Degree-days.

Table 59: EXPERIMENT VIII Oxytetracycline kinetics in Sea bass at low temperature after 10 day oral treatment - Statistical interpretation

Statistical Analysis *Oxytetracycline concentration - Sea Bass Muscle - Low Temperature*								
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 13
Samples	4			7	7	8	7	9
Minimum concentration(ppb)	7,27			6,20	4,45	14,23	4,52	6,75
Mean concentration(ppb)	17,7			28,80	161,35	155,60	93,45	76,21
Maximum concentration(ppb)	28,51			110,34	431,19	397,73	342,80	311,60
Standard deviation	10,2			36,30	139,93	154,19	124,61	102,54
25% percentile	8,18			13,44	35,03	28,90	5,50	7,97
Median	17,52			16,49	153,10	91,15	36,50	20,22
75% Percentile	27,4			22,28	214,36	329,29	173,92	131,84

	Day 15	Day 18	Day 20	Day 23	Day 26	Day 31	Day 35	Day 39
Samples	8	9	6	2	8	8	6	6
Minimum concentration(ppb)	4047	4,71	7,20	24,29	3,47	3,89	4,13	3,47
Mean concentration(ppb)	25,31	42,92	49,96	47,40	33,04	7,35	13,64	8,81
Maximum concentration(ppb)	84,88	153,29	91,91	70,42	81,74	1,79	23,81	23,32
Standard deviation	35,94	53,12	36,20	32,54	31,29	3,27	9,60	8,24
25% Percentile	5,35	7,04	9,91	24,39	5,02	4,01	4,13	3,52
Median	6,53	11,22	56,84	47,40	26,07	7,49	13,28	4,14
75% Percentile	63,39	86,29	80,09		62,94	9,84	23,34	16,50

Oxytetracycline concentration - Sea Bass Liver - Low Temperature								
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 13
Samples	6			8	5	8	8	9
Minimum concentration(ppb)	5,99			4,56	10,54	3,47	6,33	4,87
Mean concentration(ppb)	7,63			31,60	243,54	53,56	269,29	174,49
Maximum concentration(ppb)	10,42			167,35	605,84	351,57	735,92	676,83
Standard deviation	1,59			55,77	244,75	120,66	258,41	257,48
25% percentile	6,6			6,03	15,68	5,23	10,76	7,56
Median	7,02			9,99	283,2	10,59	276,80	11,20
75% Percentile	8,99			30,05	451,57	24,08	431,42	379,76

Sea Bream Liver - Low Temperature								
	Day 15	Day 18	Day 20	Day 23	Day 26	Day 31	Day 35	Day 39
Samples	7	7	8		8	6	6	6
Minimum concentration(ppb)	7,48	14,35	7,45		3,47	3,47	4,94	4,52
Mean concentration(ppb)	344,86	137,8	103,89		48,6	16,60	58,58	29,29
Maximum concentration(ppb)	1267,47	282	210,42		148,06	54,80	120,94	100,36
Standard deviation	432,78	105,35	77,34		56,29	20,04	37,58	40,24
25% Percentile	15,18	15,83	30,11		4,61	3,47	33,67	4,56
Median	253,88	152,96	92,44		23,22	8,09	58,68	5,43
75% Percentile	396,07	247,58	190,39		104,6	29,94	78,97	66,66

Oxytetracycline concentration - Sea Bass Serum (BIOASSAY) - LowTemperature								
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 10	Day 11	Day 13
Samples		5	5	5	5	5	5	5
Minimum concentration(ppb)		100,00	100,00	100,00	100,00	100,00	100,00	100,00
Mean concentration(ppb)		370,28	1084,62	739,91	2786,42	2711,10	1376,73	1363,44
Maximum concentration(ppb)		1129,00	2173,00	2173,00	6128,00	2540,26	4184,00	4929,00
Standard deviation		446,26	893,28	852,74	2335,76	5806,00	1723,63	2033,81
25% percentile		100,00	273,01	100,00	833,16	100,00	100,00	100,00
Median		100,00	858,73	555,48	2173,33	3365,33	654,32	618,88
75% Percentile		775,70	2009,18	1472,04	5046,23	4995,09	3014,66	2999,15

Sea Bass Serum (BIOASSAY) - Low Temperature								
	Day 15	Day 18	Day 20	Day 40				
Samples	5	5	5	5				
Minimum concentration(ppb)	100	100	100,00	100,00				
Mean concentration(ppb)	1165,65	976,7	732,94	100,00				
Maximum concentration(ppb)	4670	1.404	1069,00	100,00				
Standard deviation	1986,1	532,79	413,92	0,00				
25% Percentile	100	479,36	312,70					
Median	100	1191,52	958,31					
75% Percentile	2764,14	1366,62	1040,49					

Figure 103: EXPERIMENT VIII – Pharmacokinetics of Oxytetracycline in the muscle of sea bass (*Dicentrarchus labrax)* at 14 ± 1◦ C

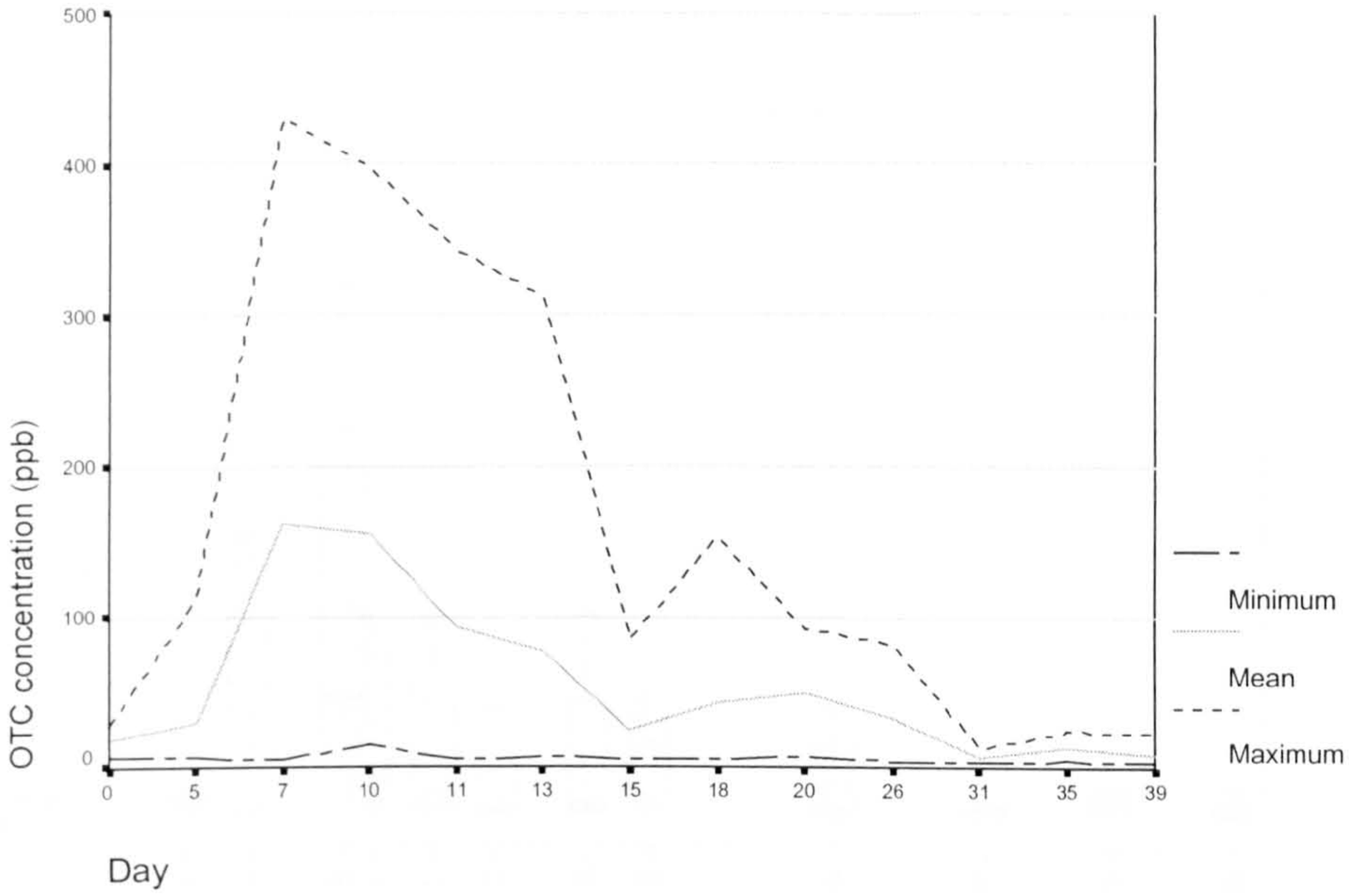


Figure 104: EXPERIMENT VIII – Pharmacokinetics of Oxytetracycline in the muscle of sea bass (*Dicentrarchus labrax)* at 14 ± 1◦ C–Mean and Standard deviation

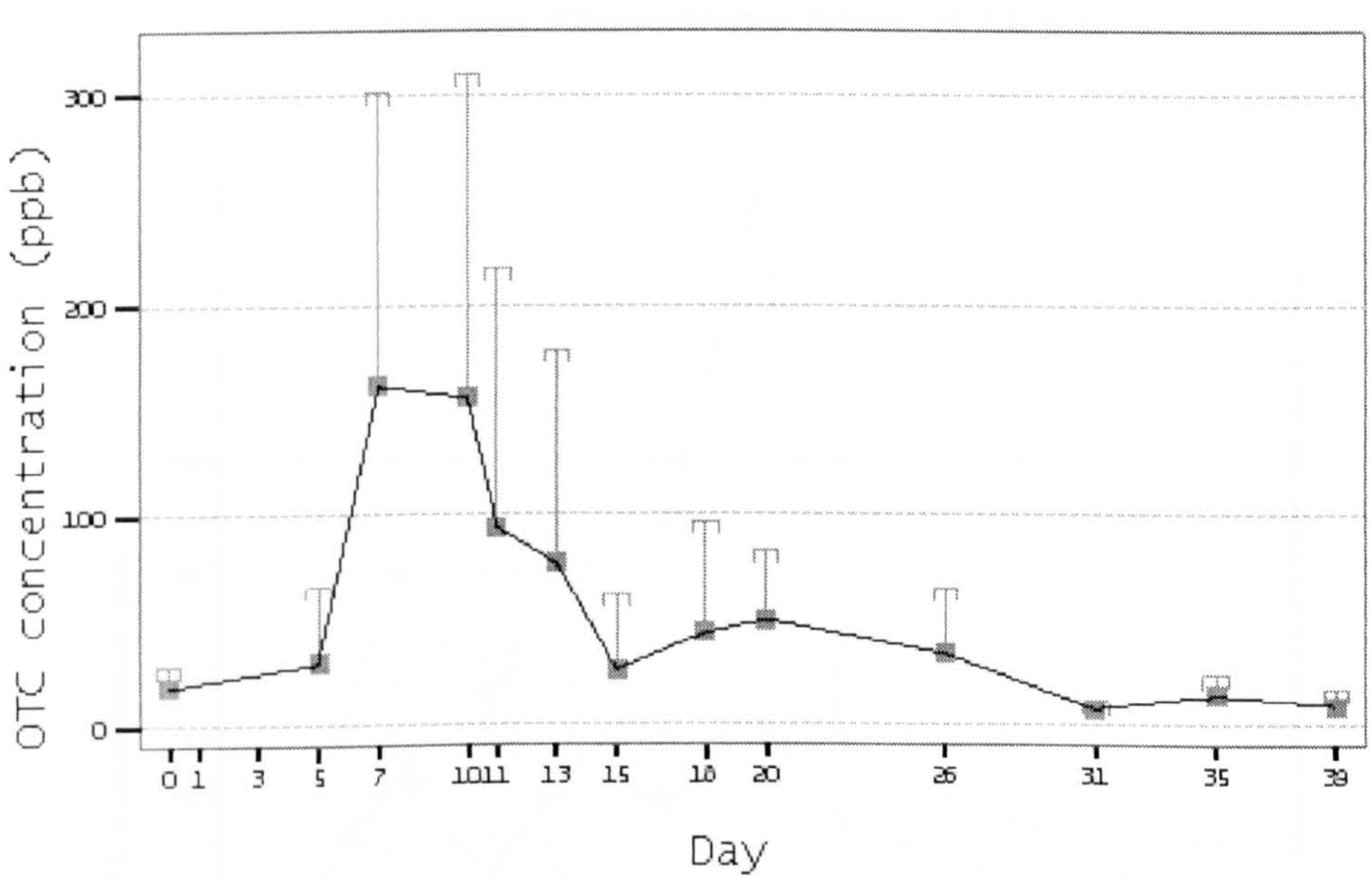
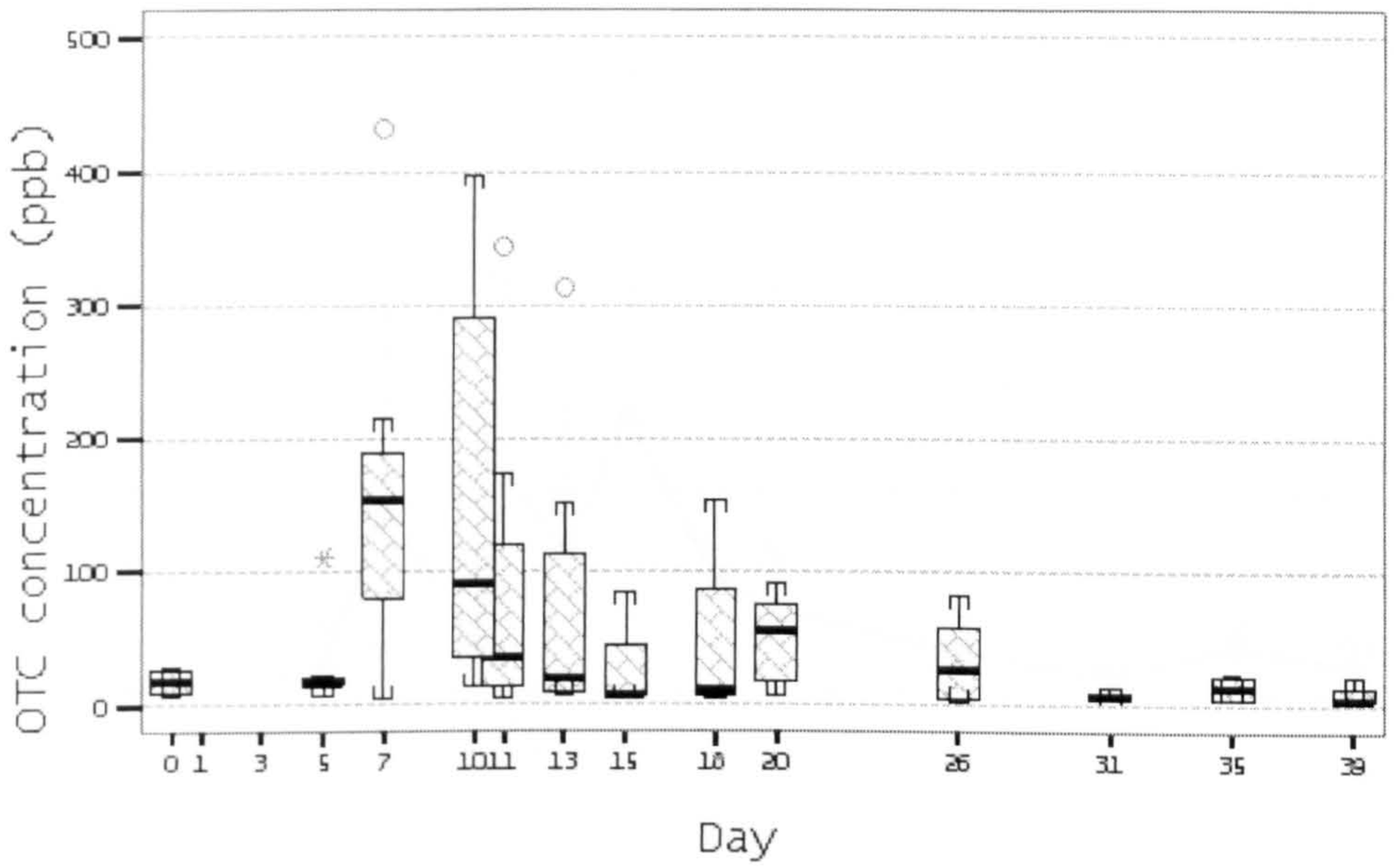


Figure 105: EXPERIMENT VIII – Pharmacokinetics of Oxytetracycline in the muscle of



sea bass (*Dicentrarchus labrax)* at 14 ± 1◦ C – Box plot presentation

Figure 106: EXPERIMENT VIII – Pharmacokinetics of Oxytetracycline in the liver of sea bass (*Dicentrarchus labrax)* at 14 ± 1◦ C

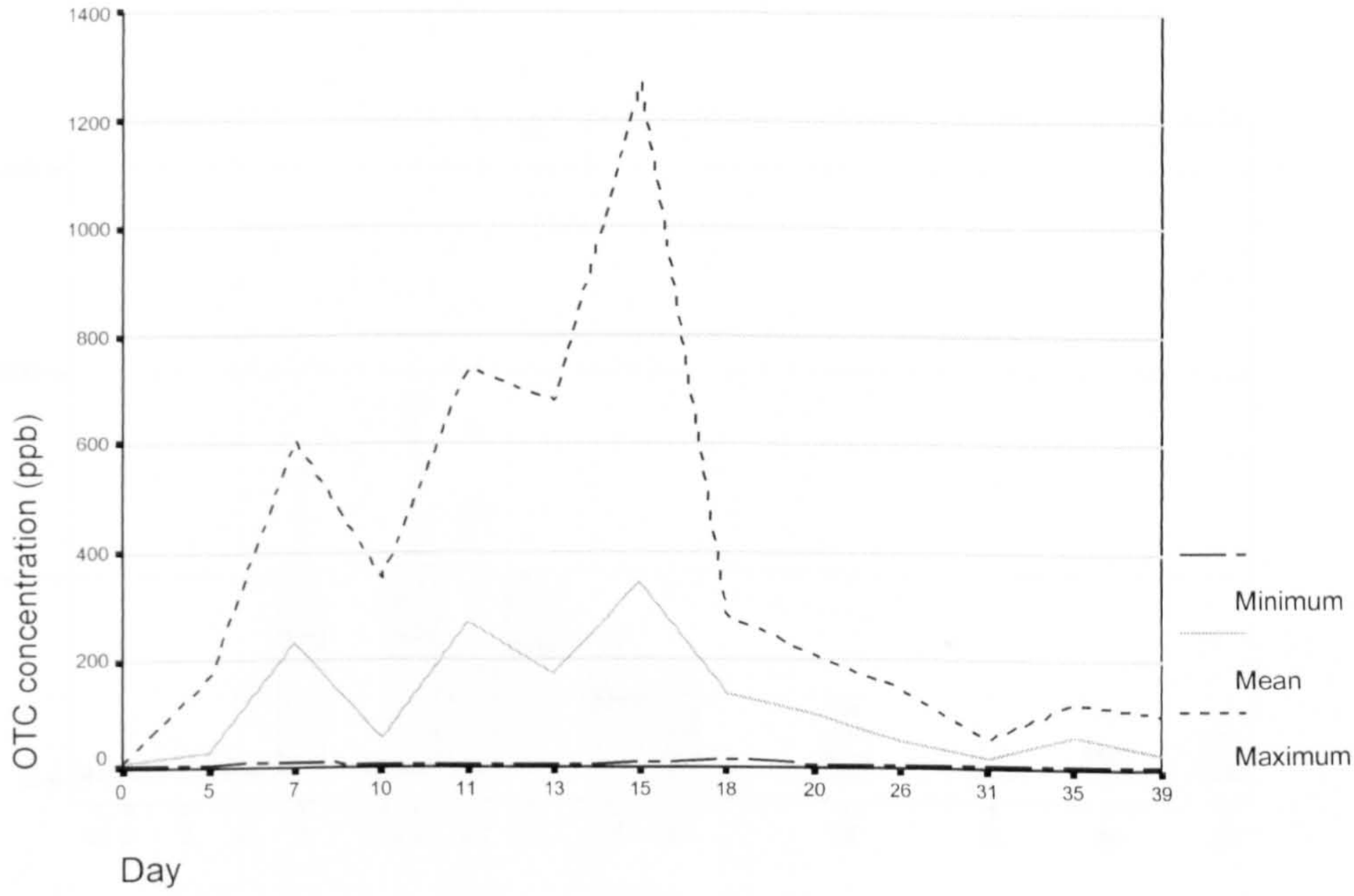
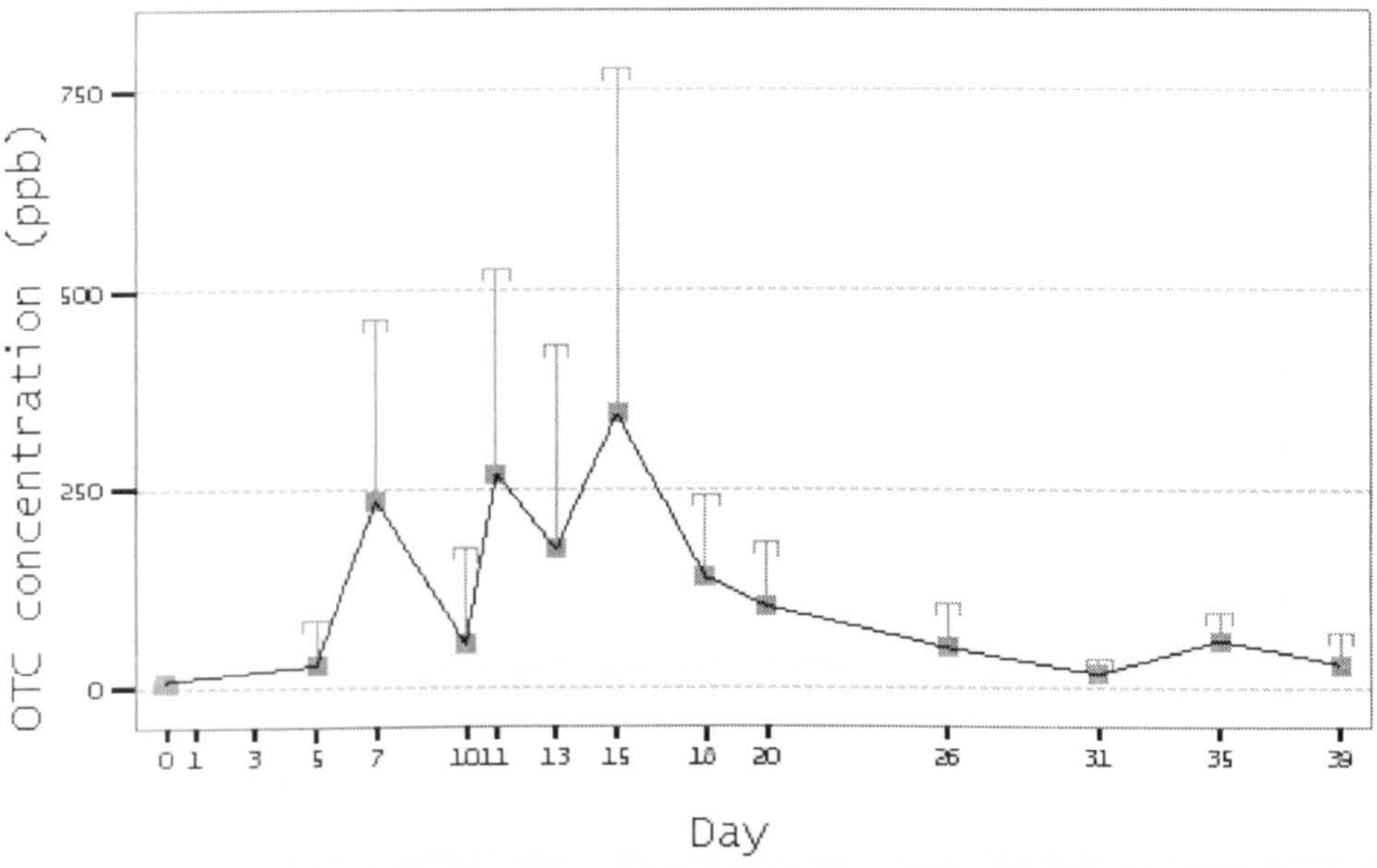


Figure 107: EXPERIMENT VIII – Pharmacokinetics of Oxytetracycline in the liver of



sea bass (*Dicentrarchus labrax)* at 14 ± 1◦ C – Mean and Standard deviation

Figure 108: EXPERIMENT VIII – Pharmacokinetics of Oxytetracycline in the liver of sea bass (*Dicentrarchus labrax)* at 14 ± 1◦ C – Box plot presentation

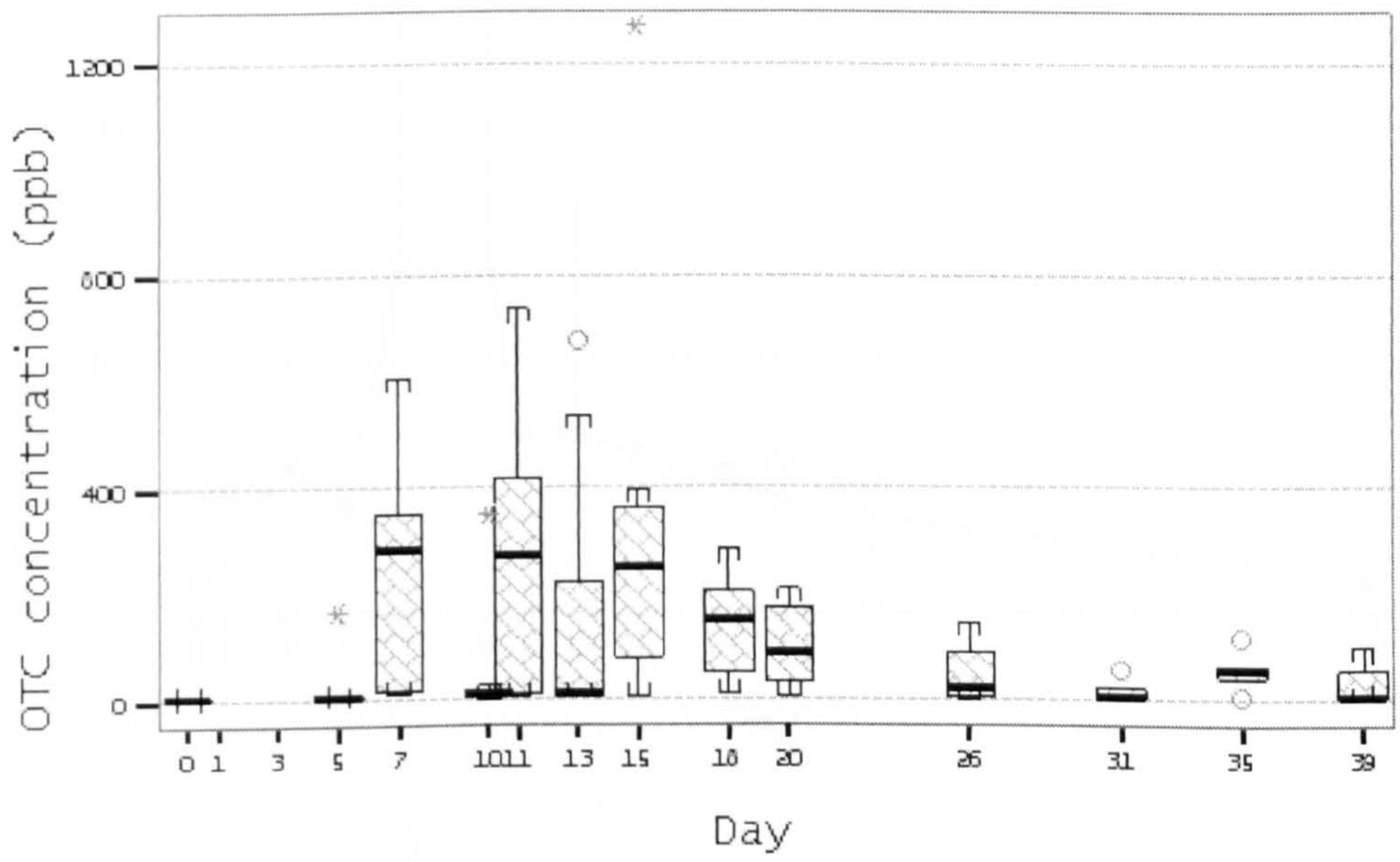


Figure 109: EXPERIMENT VIII – Pharmacokinetics of Oxytetracycline in the serum of sea bass (*Dicentrarchus labrax)* at 14 ± 1◦ C

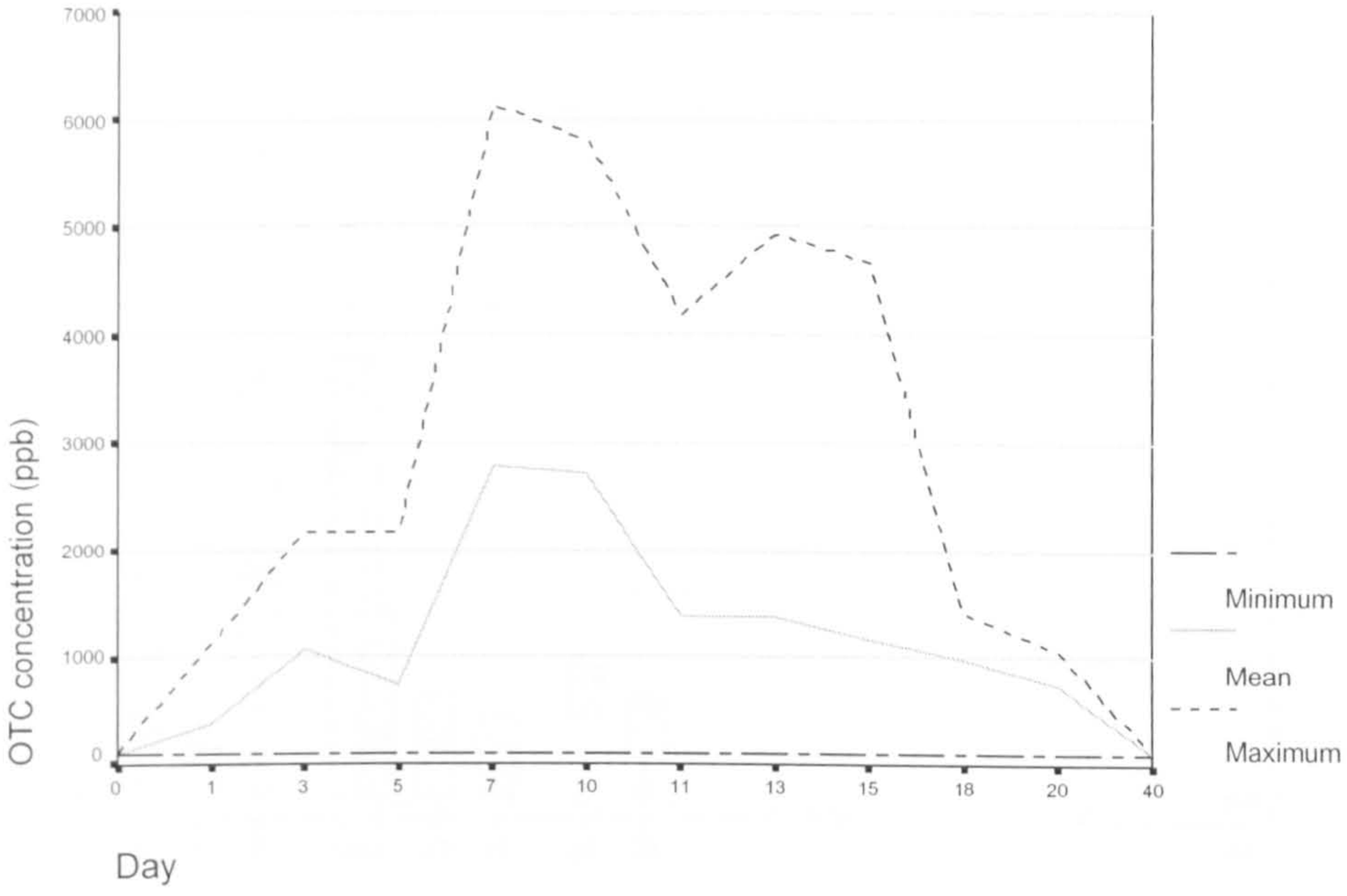


Figure 110: EXPERIMENT VIII – Pharmacokinetics of Oxytetracycline in the serum of sea bass (*Dicentrarchus labrax)* at 14 ± 1◦ C – Mean and Standard deviation

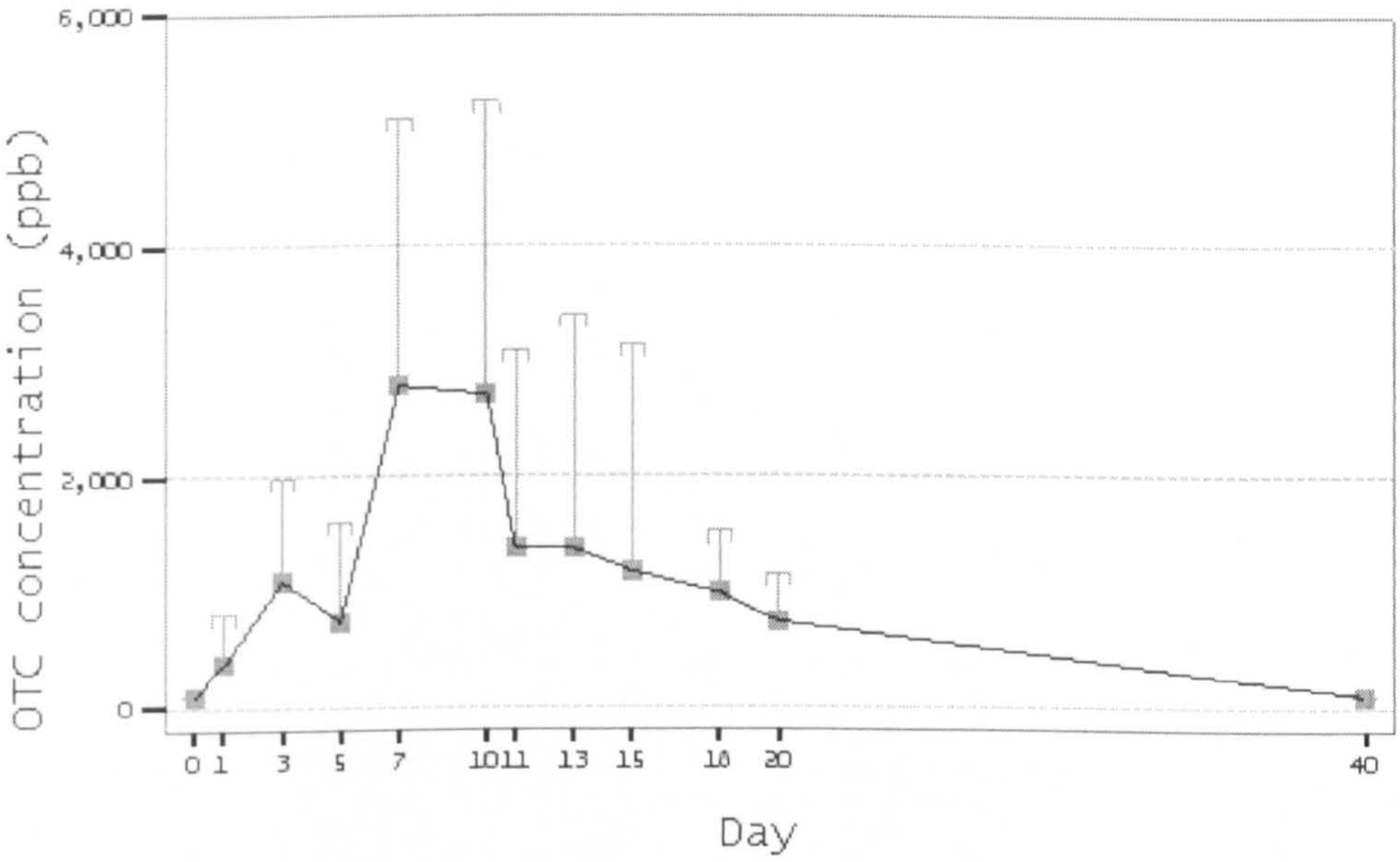
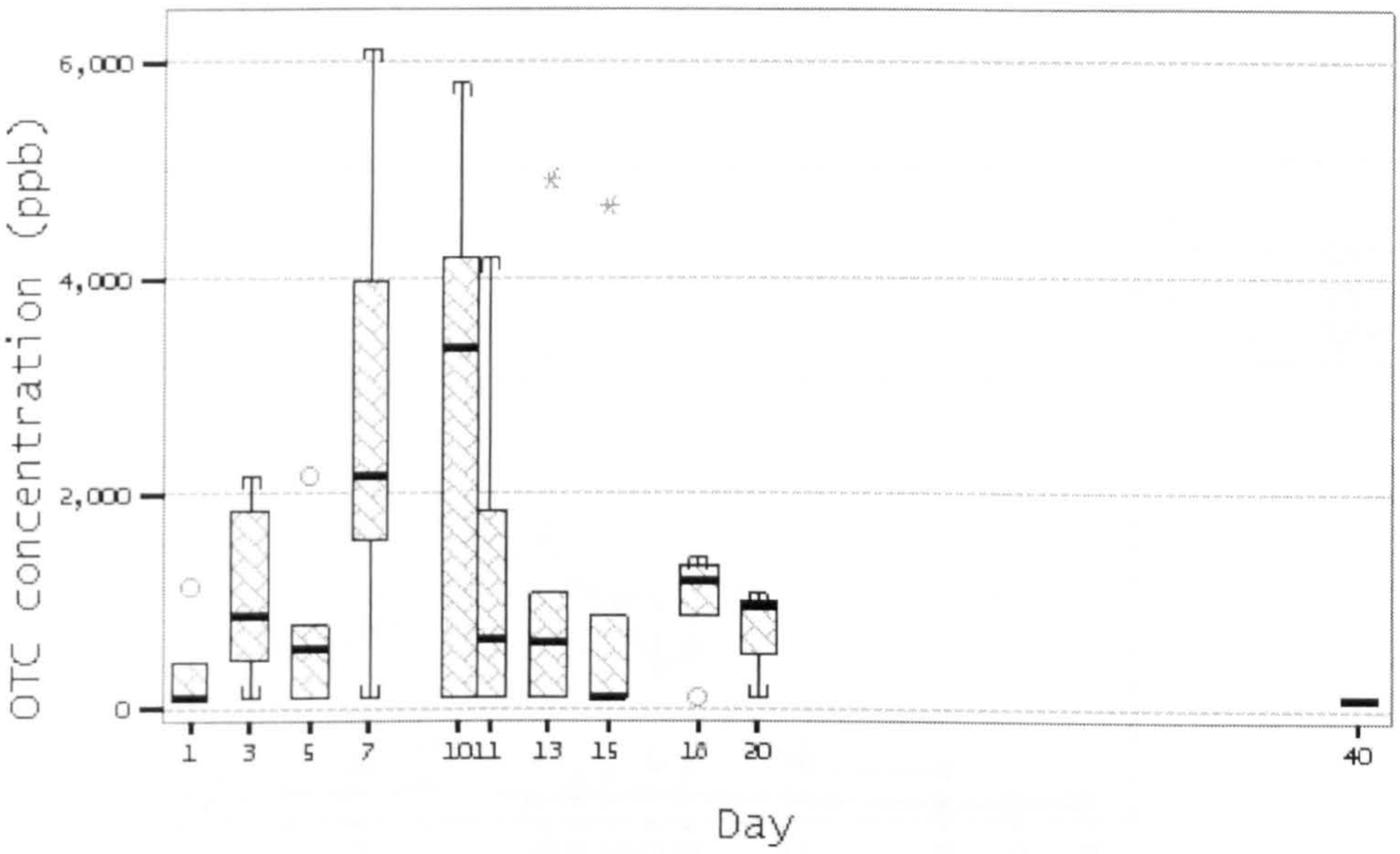


Figure 111: EXPERIMENT VIII – Pharmacokinetics of Oxytetracycline in the serum of



sea bass (*Dicentrarchus labrax)* at 14 ± 1◦ C – Box plot presentation

3.8.2 Comparisons

3.8.2.2 Muscle versus Liver versus Serum Depletion

Figure 112: Comparison of OTC mean concentration in the muscle and liver and serum of sea bass at high temperature

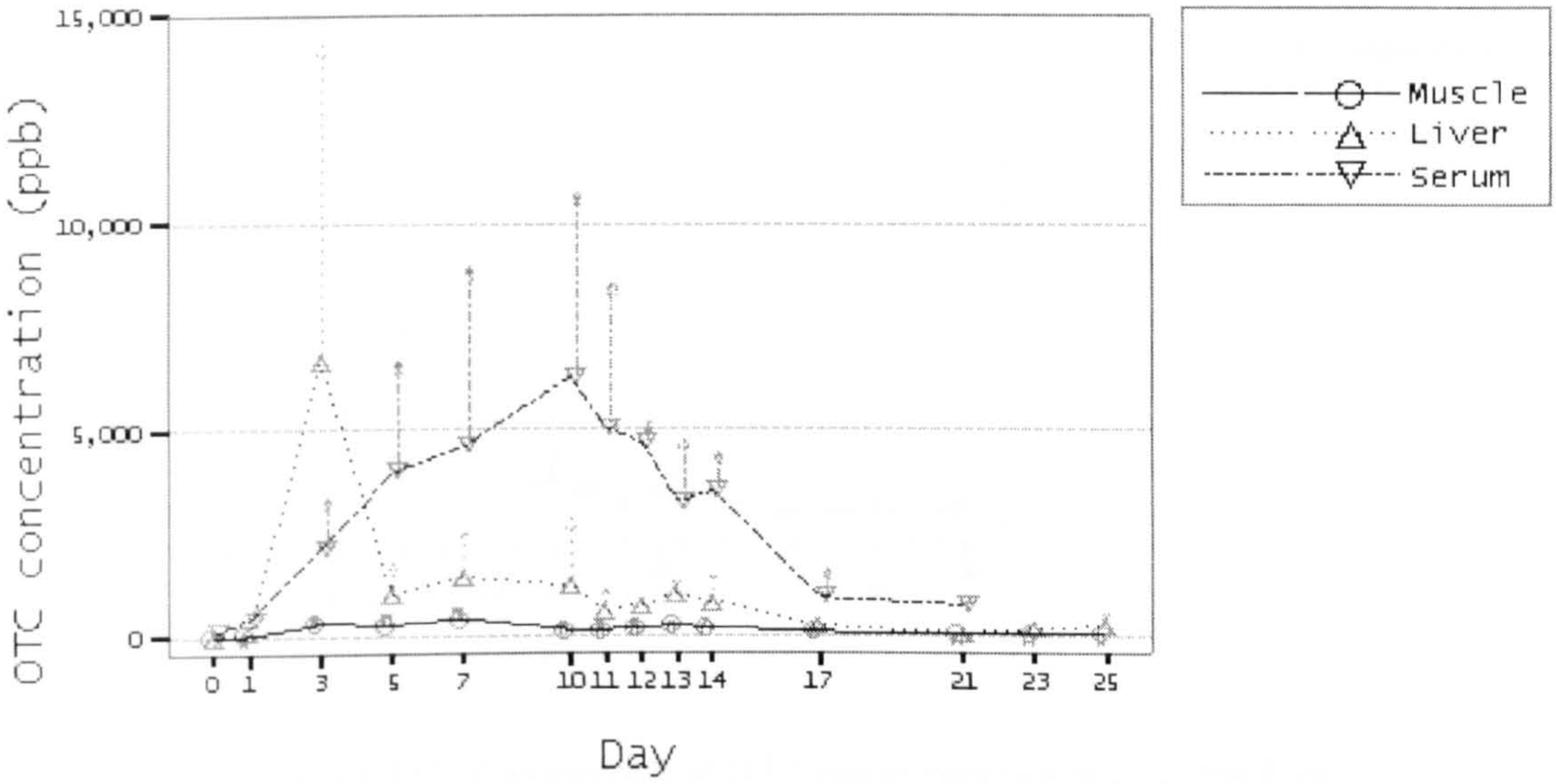
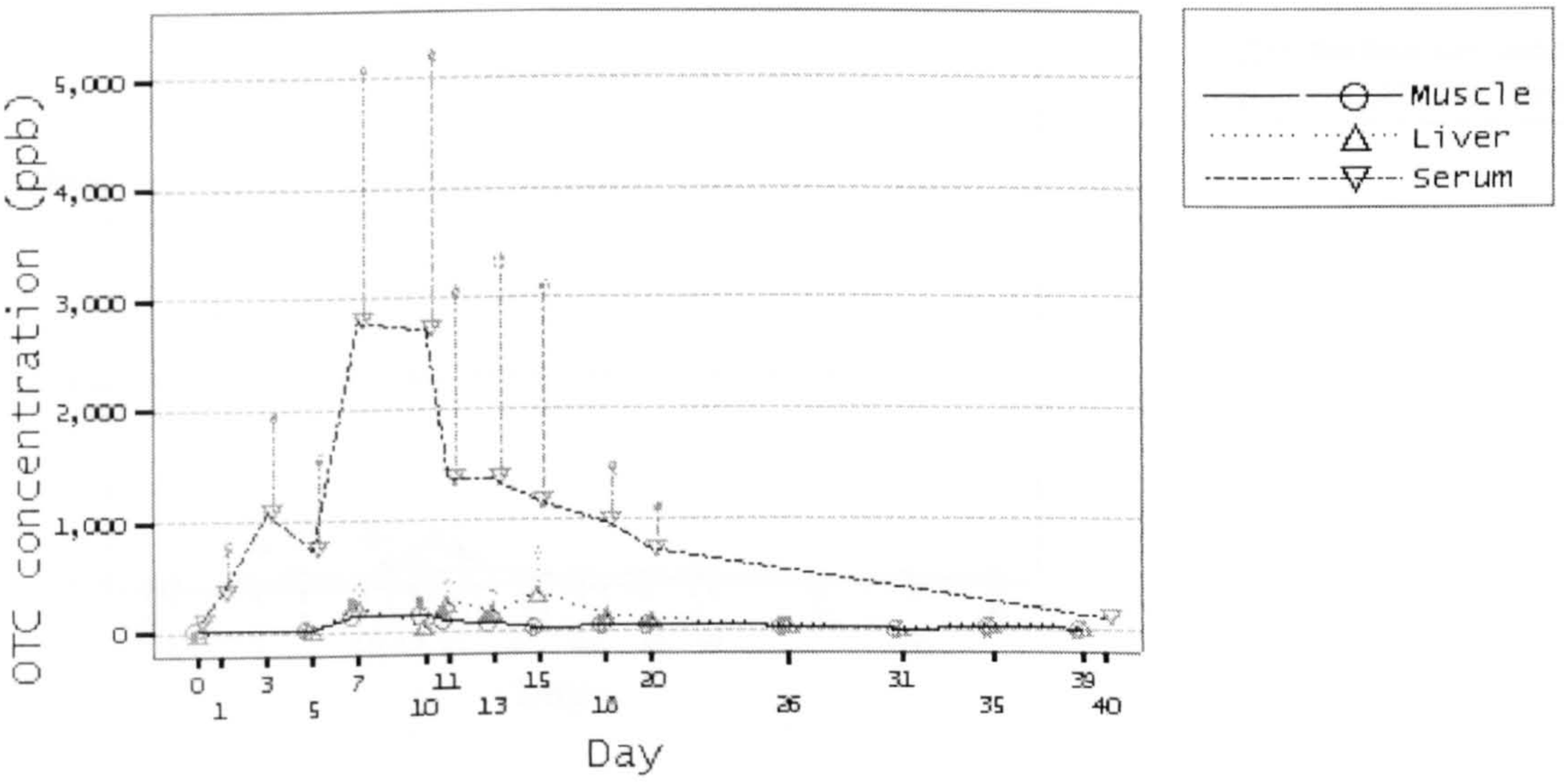


Figure 113: Comparison of OTC mean concentration in the muscle, liver and serum of sea bass at low temperature



3.8.2.2 Depletion at High versus Low temperatures

Figure 114: Comparison of OTC mean concentration in the muscle of sea bass at low and high temperature

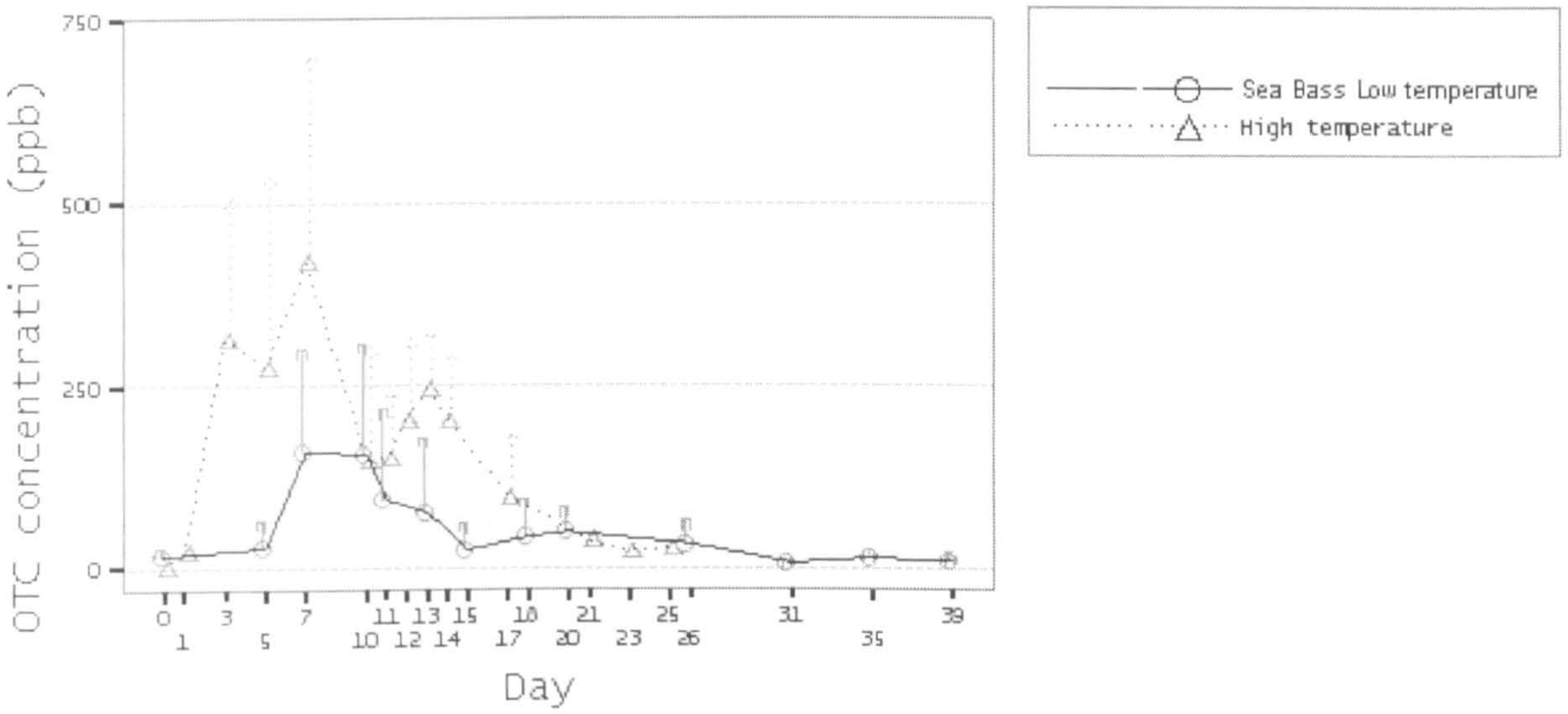


Figure 115: Comparison of OTC mean concentration in the liver of sea bass at low and high temperature

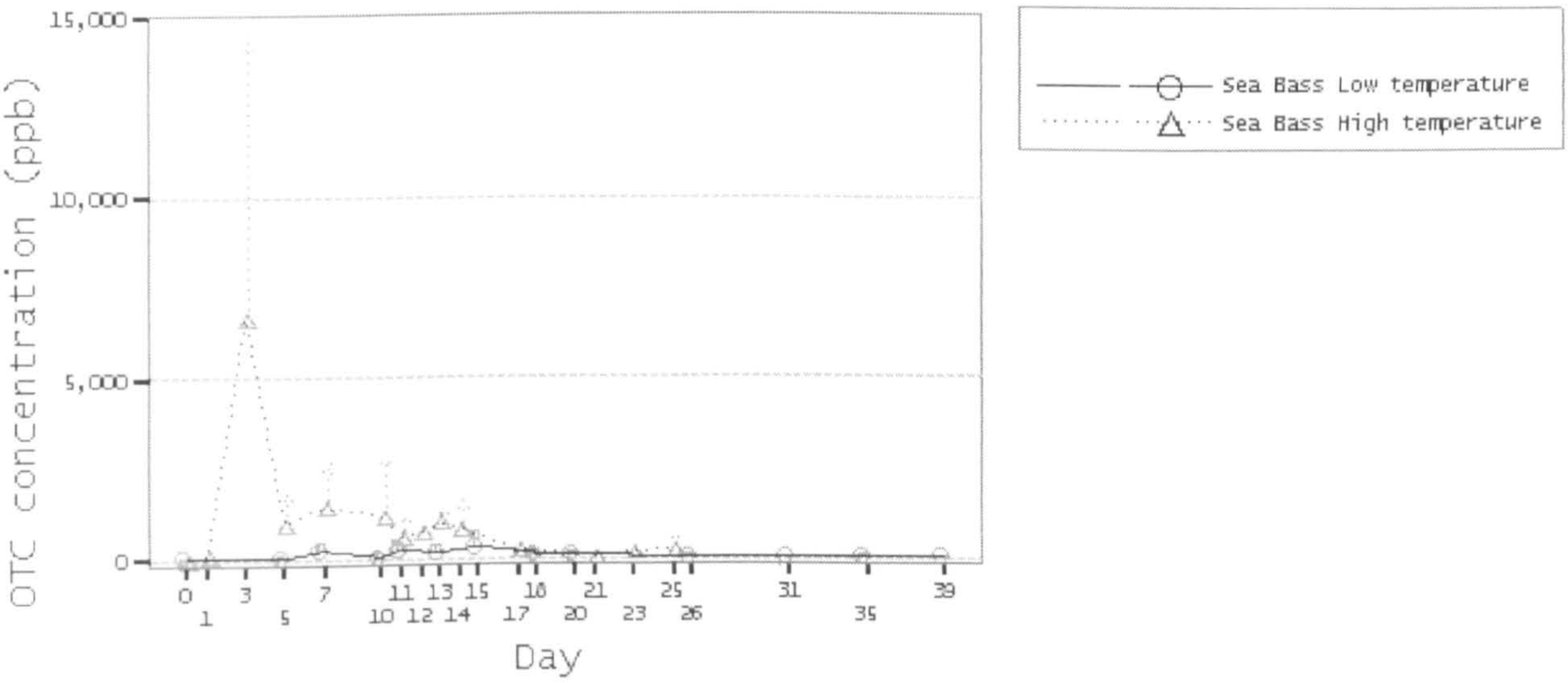
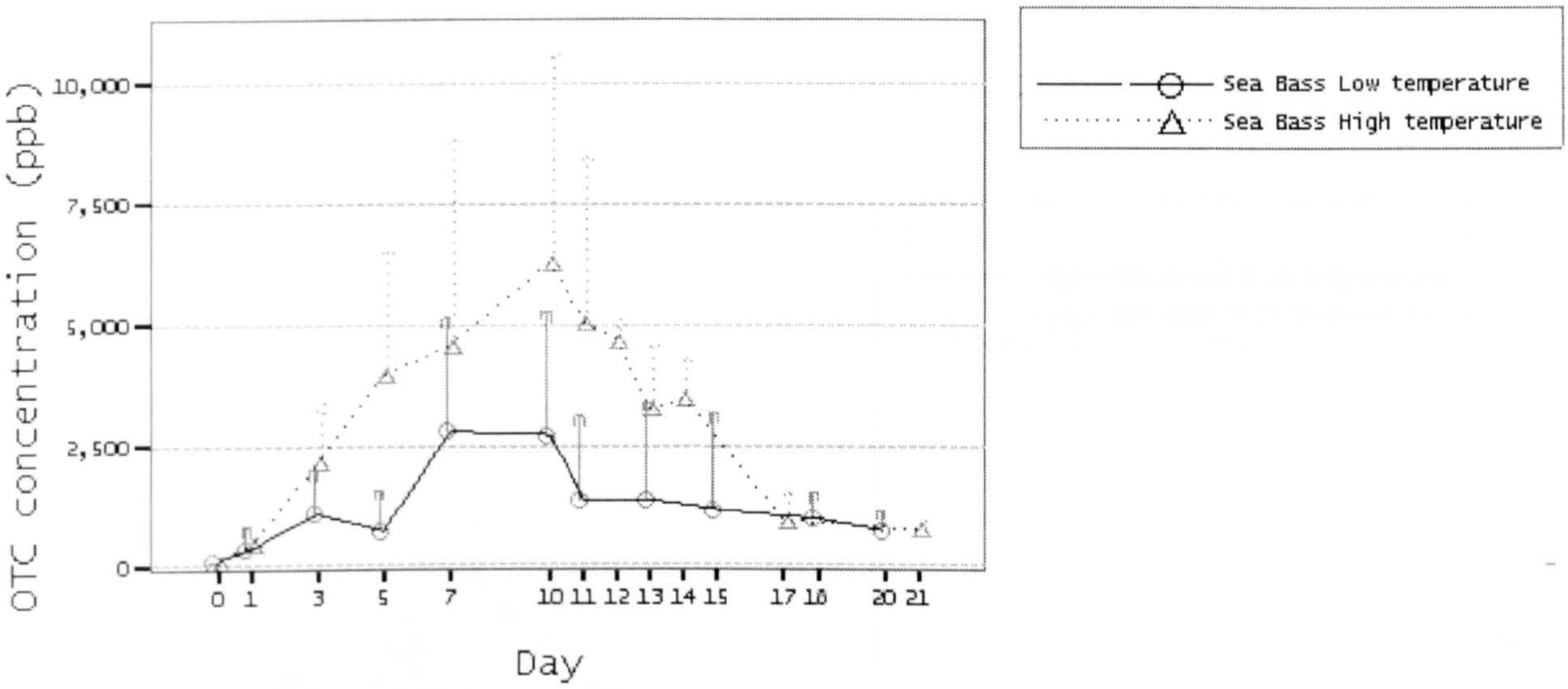


Figure 116: Comparison of OTC mean concentration in the serum of sea bass at low and high temperature



3.9 Oxytetracycline Species Comparisons

Figure 117: Comparison of OTC mean concentration in the muscle of sea bream and sea bass at high temperature

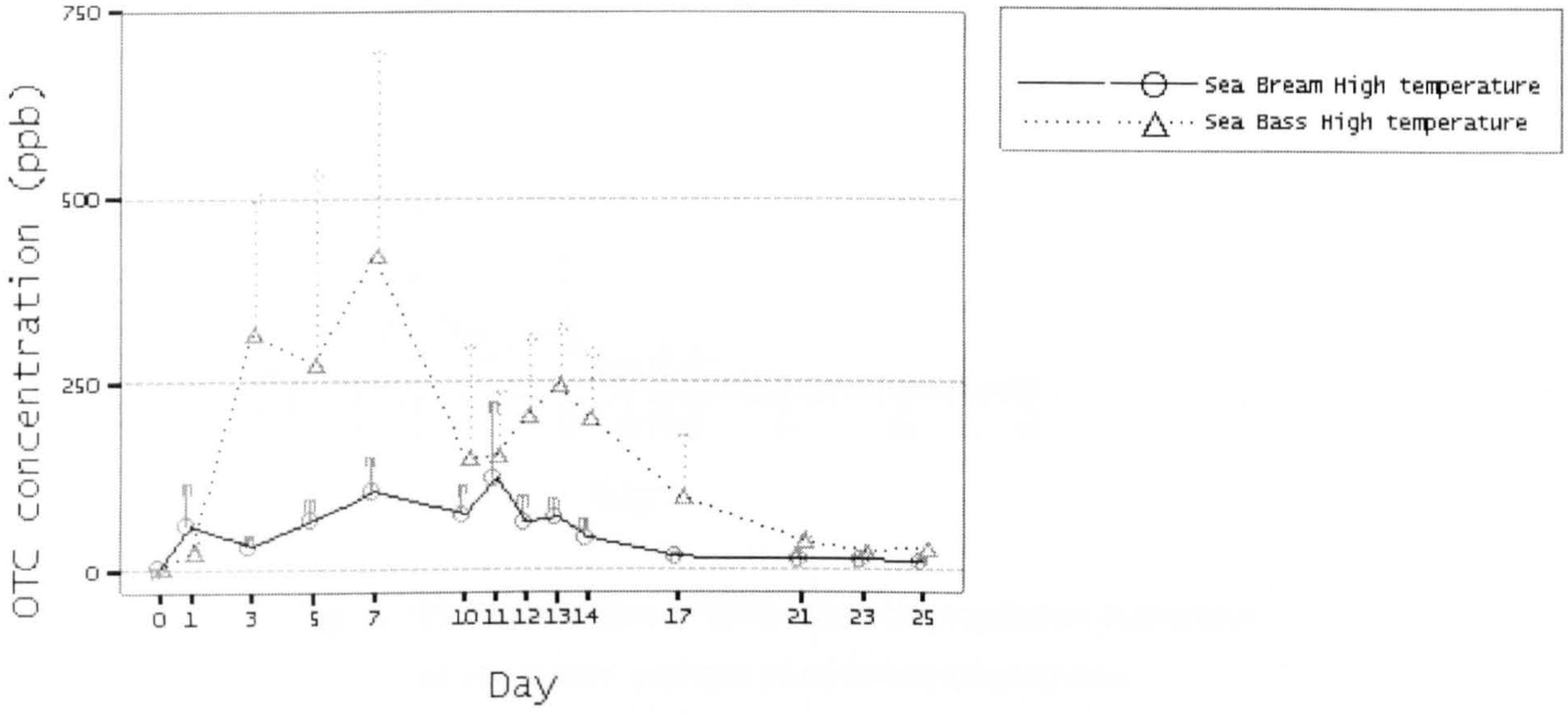


Figure 118: Comparison of OTC mean concentration in the muscle of sea bream and sea bass at low temperature

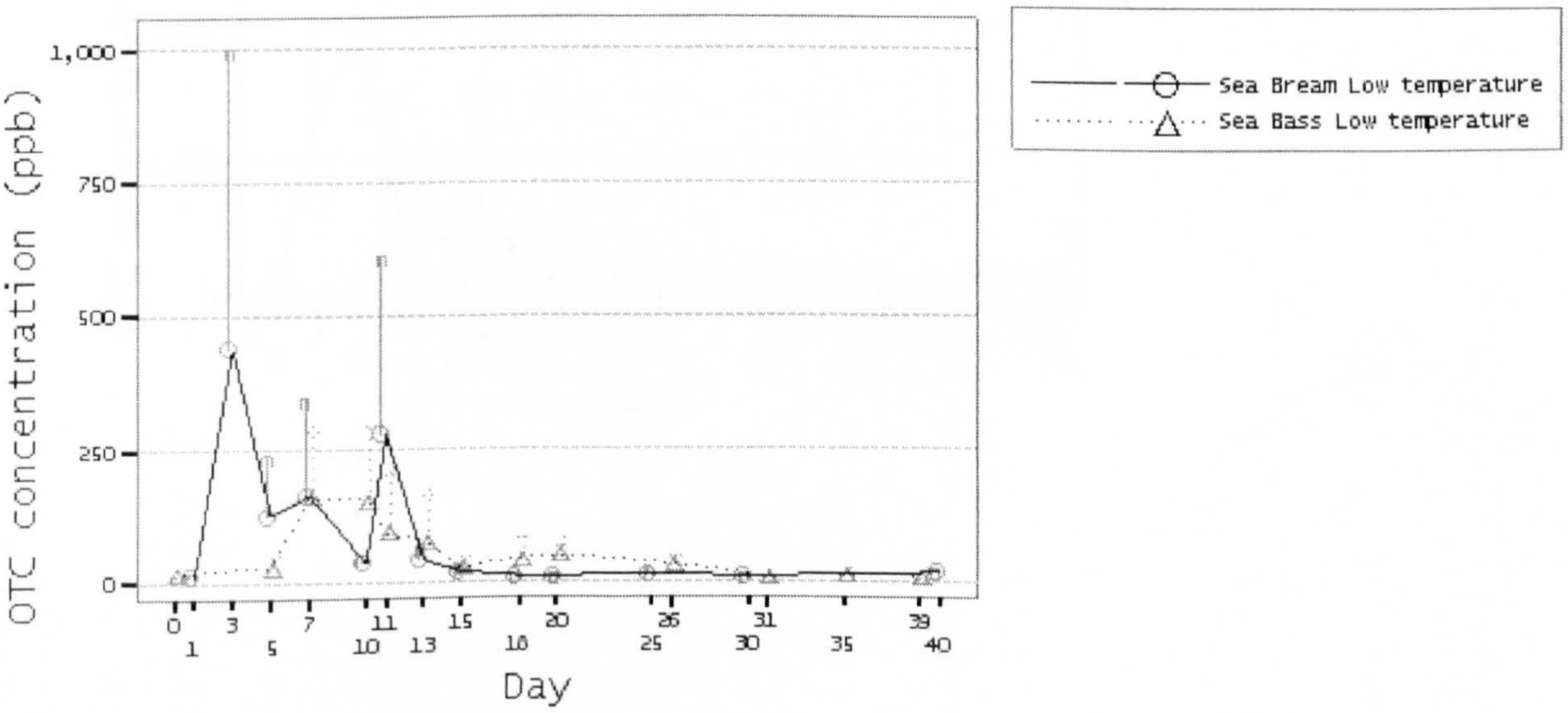


Figure 119: Comparison of OTC mean concentration in the liver of sea bream and sea bass at high temperature

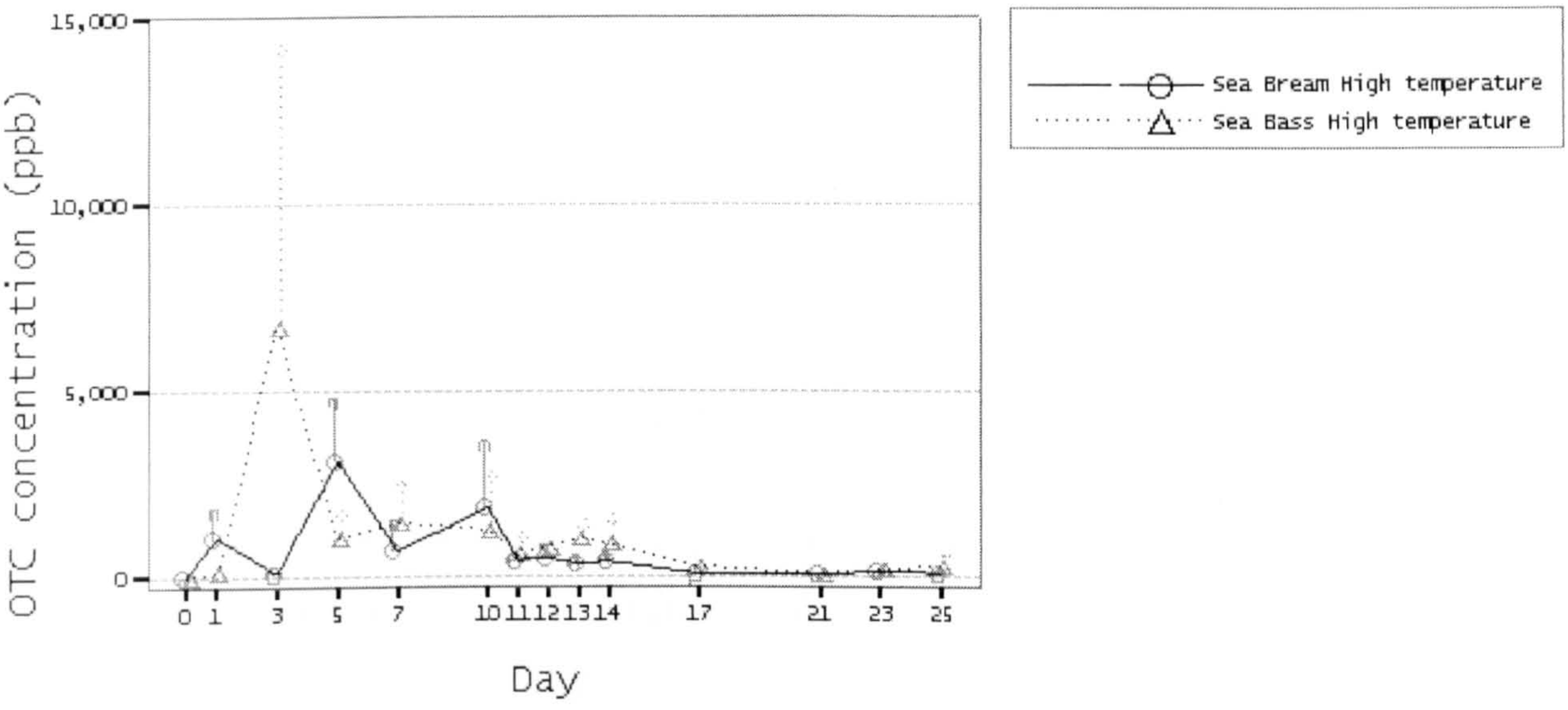


Figure 120: Comparison of OTC mean concentration in the liver of sea bream and sea bass at low temperature

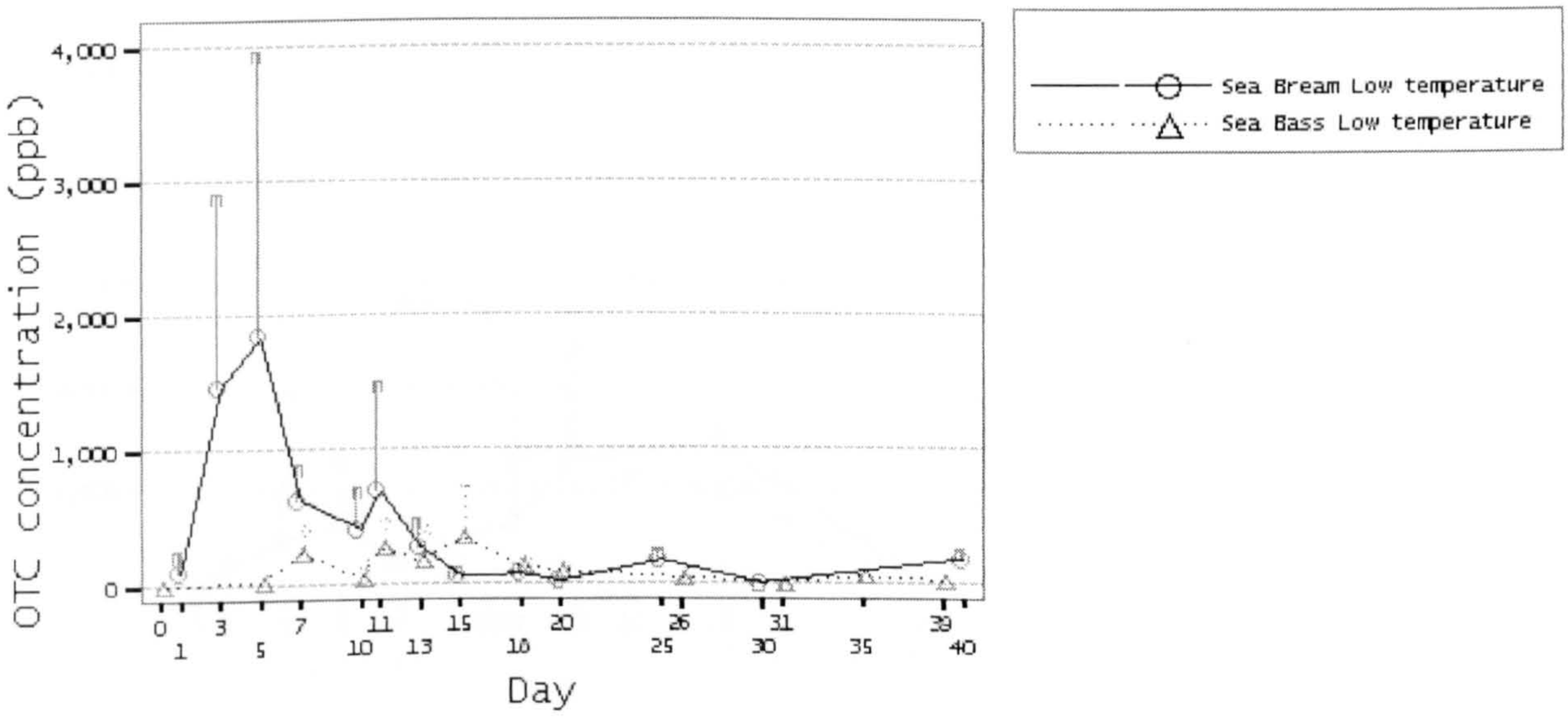


Figure 121: Comparison of OTC mean concentration in the serum of sea bream and sea bass at high temperature

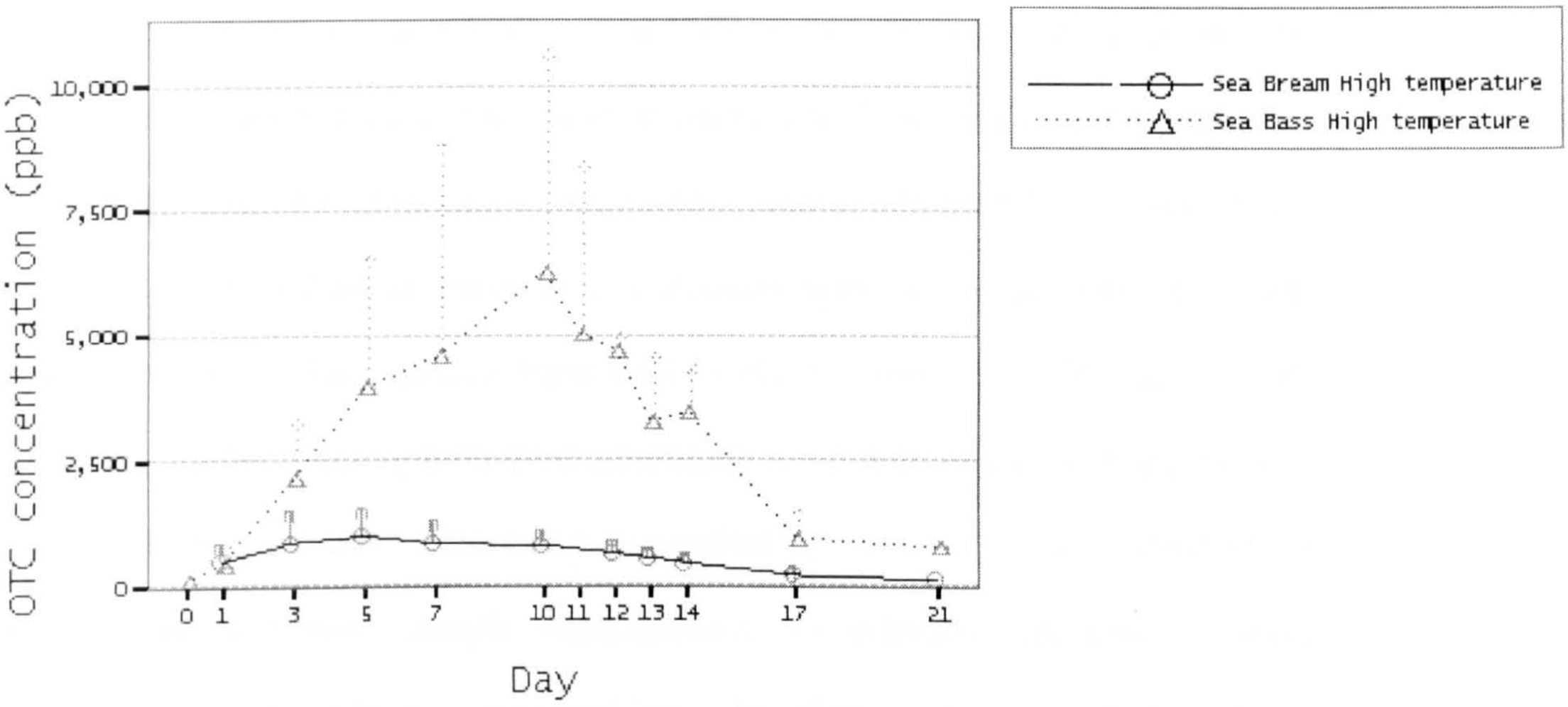
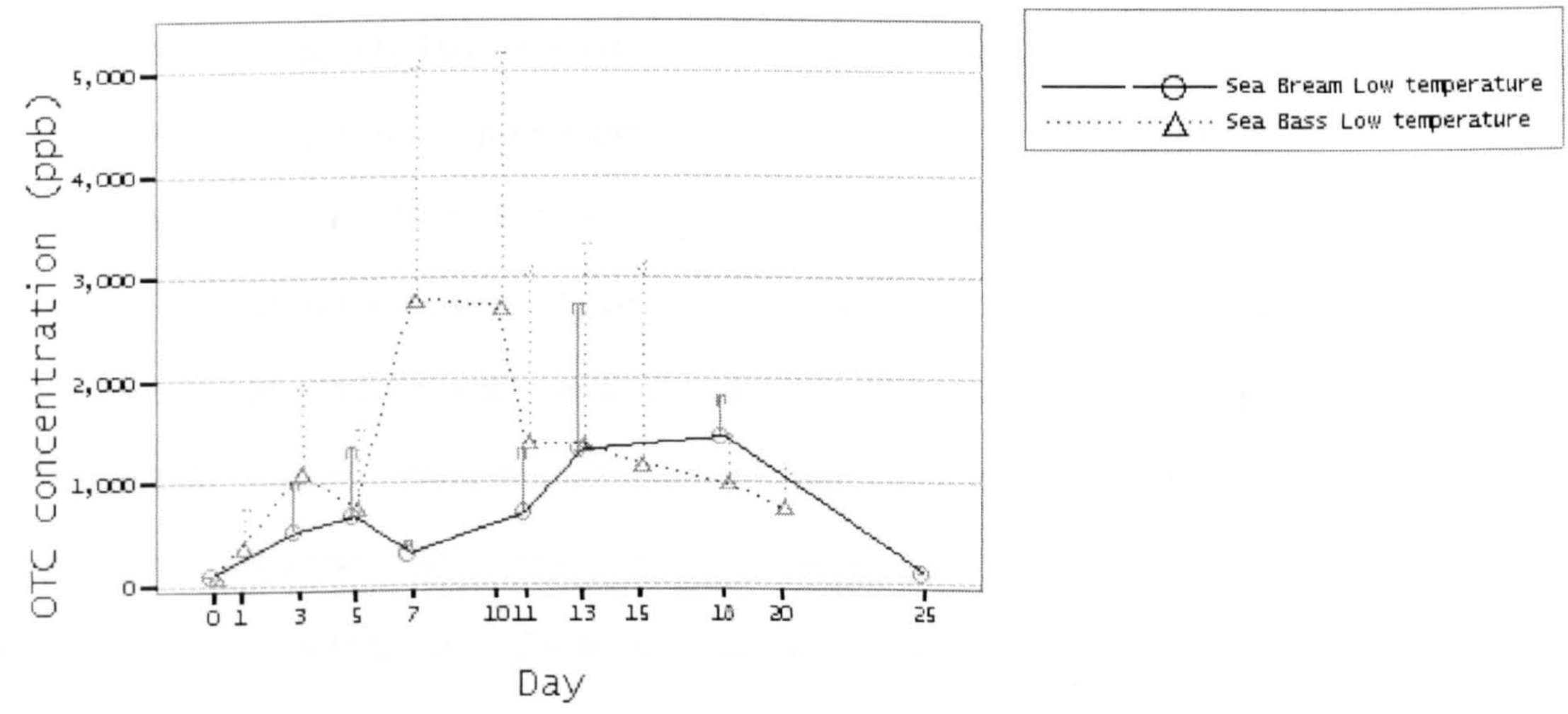


Figure 122: Comparison of OTC mean concentration in the serum of sea bream and sea bass at low temperature



4. Discussion

4.1 Greek Mariculture Industry

Greek Mariculture grew dramatically since 1981 based on ideal climatic conditions and extended sheltered coastline (Papoutsoglou, 2000). Breakthrough in hatchery technology followed by advances in fish nutrition along with private investment and EU funding lead to 20-fold production increased between 1990 and 2000 with dramatic drop in ex-farm prices from €15.57/Kg in 1989 to €4.97/Kg in 1999. (Theodorou, 1999). Cost optimisation strategies in recent years involved gradual replacement of live feeds, production diversification to new species, broodstock selection and improved health management to prevent diseases through vaccination and reduce losses and antibiotic use. Dietary crisis like dioxins, BSE, GMOs, antibiotic residues in farmed animals magnified and often misinterpreted by the media led to consumer awareness and demand of high quality safe and healthy aquaculture products. Mariculture sustainability depends on product quality control and safety supported by codes of conduct and practice (Christofilogiannis, 2000). Despite good prospects presented in a recent EU funded market research (Mc Alister and Partners, 1999) the industry suffers from framework, technology and market perception problems. Supply segmentation, communication gaps between farmers, insufficient sector "image" promotion, rigid legislative framework, competition with coastal zone users, malpractices and lack of presentation of the socio-economic importance in rural communities are only some of the industry's practical problems. Federation of Greek Maricultures (FGM) was founded in 1991 and soon after became the dominant sectoral policy making organisation. FGM took initiatives referring to licensing, legal framework development for aquaculture, technical issues and sector promotion. Funding of this research by FGM in 1994 was unique for the Greek standards. Since 1995, this study was co-supported by the Greek Ministry's of Development YPER programme. Increased consumer

awareness on healthy, safe nutritious final product and need to minimise production cost were the main reasons for this study that aimed to produce "real" life applied research that had clinical, economic and consumer safety relevance. The first goal was to audit the prevalence and the importance of the main bacterial diseases in Greek Mariculture industry and to evaluate by a standard methodology the antibiotic resistance level and the possible reasons for treatment failures. The second goal was to evaluate the effectiveness of antibiotic treatment under "farm" conditions and the dynamics of depletion for the two most commonly used antibiotics namely Oxolinic acid and Oxytetracycline. Since the initiation of this study, considerable effort was devoted in the development of a Quality Assurance scheme for Greek Mariculture. Data from this study have been incorporated in the Greek Mariculture's Code of Practice and to the internal audits protocols in order to ensure compliance and certification of the Greek sea bass and sea bream by AGROCERT, the Ministry' s of Agriculture competent authority for agricultural product certification. This scheme will be launched officially in September 2003. It is important to state that under this close collaboration the industry utilised the technical feedback from this study long before this submission through the FGM Fish Health and FEAP Fish Health committees.

4.1.1 Cultured Species

Sea bass (*Dicentrarchus labrax,.Seranidae*) is also a highly euryhaline and eurythermal species discontinuously distributed around the Atlantic from Moroccan coast to Irish, North, Baltic sea. Ubiquitous also in Mediterranean coasts, enters costal inlets and river mouths. (Barnabe, 1990). Its external colour is silver-grey with silvery sides and stomach, having a cylindrical body. It is not very well known but this species can be adapted to fresh water. Adults leave the lagoons around Mediterranean from October to December in order to reproduce in the open sea.

Sexual maturity develops in males at 2 years of age (23-30cm) and in females at 3 years (31-40cm). Its feeding range includes small fish, prawns, cancers and cuttlefish (Whitehead et al., 1986). Gilthead sea bream (*Sparus aurata,.Sparidae*) is an euryhaline,eurythermal species that reproduces from October to December in the open sea. It is discontinuously distributed in the Mediterranean sea, but rarely in the Black sea, and also in Atlantic from the British islands to Verde Is cape and the Canary islands (Whitehead et al., 1986). Sexual maturity develops in 1 to 2 years of age (20-30cm) in males and in 2 to 3 years of age (33-40cm) in females. Sea bream is protandrous hermaphrodite. Interesting characteristics are its sensitivity at low temperatures and at low dissolved oxygen levels. This species is the first that leaves the lagoons in early autumn, for the open sea. Its feeding range includes molluscs, crustacean, small fish and plants. (Whitehead et al., 1986) New species production of 10 million fry including Sharpsnout sea bream (*Puntazzo puntazzo*), Dentex (*Dentex dentex*), Pagrus (*Pagrus pagrus*), White sea bream (*Diplodus sargus*), Red Pandora (*Pangelus erythrinus*), and Flatfish (*Solea sp.*) is about 5% of the total production (FEAP 2002).

4.2 Bacterial Diseases

4.2.1 Epidemiological Surveys in Mediterranean Mariculture industry

Bacterial Fish diseases remain a limiting factor for the viability of the marine fish culture (Paperna et al., 1977; Colorni et al., 1981; Le Breton, 1999; Christofilogiannis 2002). Bacteria of the genera *Vibrio, Aeromonas, Pseudomonas, Myxobacteria*, are ubiquitous in water, present on undamaged skin of the fish, and form an important part of the intestinal heterotrophic microflora of sea bream and sea bass (Colorni et al., 1981; Le Breton, 1989). However high levels of mortalities occur under unsuitable environmental conditions such as, high loads of organic material, unbalanced nutrition, extreme or rapid salinity or temperature fluctuations, low water oxygen levels and stress due to overcrowding or handling. (Fernardes, 1977; Colorni et al, 1981; Moller and Anders, 1986). Before the current study only a few surveys of bacterial fish diseases in Mediterranean were initiated in Israel (Paperna et al., 1977; Paperna et al., 1980; Colorni et al., 1981; Balebona et al., 1993) France (Breuil et al., 1989) Spain (Aquigrup, 1980; Balebona et al, 1998) Italy (Salati et al., 1997) and Tunisia (Bakhrouf et al., 1995).

4.2.2 Current Bacteriological survey

Due to funding by the Federation of Greek Maricultures this study had to address "real "issues like the spectrum of fish bacterial pathogens in Greece between 1994 and 1997, their distribution and relevance in different areas (Christofilogiannis, 1996), their clinical importance as primary or opportunistic pathogens, their associated pathology -parasitological and histopathological data gathered and analysed during the same period (Christofilogiannis, 1997a), are not presented here - and most important of all in terms of production cost, which antibiotics are effective to combat these diseases in order to avoid treatment failures due to

antibiotic resistance. Therefore the scope of this research had to be broad and practical and we consider this type of approach more relevant for the industry and for the knowledge development between fellow fish pathologist on the field, compared to highly technical focused in a certain issue niche research. This study is the first in Mediterranean to include along with the biochemical screening and identification, two tests like Minimum Inhibitory Concentration (MIC) and Quantitative Antibiogram in order to quantify resistance to antibiotics and initiate a data bank on resistance profiles, the results of which could be compared with new data of future studies as long as the methodology will be kept the same. API 20E has been widely used to identify bacterial fish pathogens (MacDonell et al., 1982; Kent, 1982). Although apart from the reaction profiles of *V.alginolyticus, V.parahaemolyticus* and *V.damsela*, none of the other fish pathogenic vibrios are incorporated in the API 20E Analytical Profile index, 3rd ed. (1989), this test-strip was employed for the identification of the bacterial strains in this study (Christofilogiannis et al., 1997). Large biochemical variability exists between strains of Vibrio *spp* and especially *V.anguillarum* (Egidius and Andersen, 1977). Mac Donell et al., (1982) suggested the application of 2% marine salt solution for the inoculation of the API 20E strip and the characterisation of marine and estuarine bacteria. In this study however the use of a 3% salt solution gave consistently better results. In this survey Sea bass contributed the majority of the strains (86,13%) followed by sea bream (almost 17%) while the new species had very low contribution (less than 8% between them). We have to consider two things to explain this distribution. In the period of the survey (1994-1997) minor species were just in the process of being accepted by the fish farmers and only sharpsnout bream (*Puntazzo puntazzo*) managed to have a fair share (about 5%) while still the rest are being evaluated. On the other hand the vaccine application in order to combat diseases like Vibriosis or Pasteurellosis in sea bass just started to be really implemented after 1997 due to the perception that sea bass was extremely prone to

stress due to handling and farmers were extremely sceptical of practices such as ip injection vaccination or even immersion vaccination. These perceptions changed with the first optimistic results and due to recent application of effective oral vaccines for the major diseases. Saronikos, South Evoikos and North Evoikos were the areas most widely surveyed due to the large number of farms, due to the proximity to the National Centre of Marine Research in Athens and due to the presence of two farms namely Kantali Ltd and Makronisos that were followed more closely. Both farms had no veterinarian responsible and both suffered from antibiotic misuse. Another important finding in principle but not – up until now – in clinical practice was that for the first time Aeromonas salmonicida was isolated from sea bass (1,15%) as well as the isolation of Gram-positive cocci in the new species *Mugil cephalus* and *Dentex dentex*, while Gram negative cocci were isolated in sea bream. It was not possible to identify all bacterial isolates based on API20E analysis. Commercial miniaturized kits for biochemical tests (Pathotec and Minitek) were found to be inadequate for marine fish pathogen identification. (Colorni et al., 1981). Despite the limitations, in real practice the API 20 E test is a valuable tool for biochemical identification. Cluster analysis-Eucleidian distances dendrogram analysis was employed in order to demonstrate different clusters mainly between the Vibrio strains. Different phenotypes of *Vibrio alginolyticus* formed a distinct cluster, while *V.anguillarum phenon* 4b and phenon 2 formed closely related clusters, which agrees with findings reported by Crisez et al. (1991). Statistical interpretation of API 20E data according to the NORDIC manual and employing the ALO test (ADH / LDC / OX) revealed that the Alo test could be used in order to separate *V.anguillarum 1 phenon 4b* and *V.anguillarum 1 phenon 2* along with *Listonella damsela* and *V.alginolyticus* different phenotypes. It was not however possible to be employed for the discrimination between *V.parahaemolyticus, V.vulnificus* and *V.alginolyticus* which formed in this type of statistical analysis the same cluster. The bacterial pathogens isolated during this study as well as those

listed in the literature with potential to cause mortalities in sea bass and sea bream are listed in Figure 123.

4.2.3 Gram negative bacterial pathogens

Most bacterial fish pathogens are Gram-negative rods usually facultative pathogens that initiate diseases only under certain conditions (Austin, 1985a; Moller and Anders, 1986).

4.2.3.1 Vibrio Spp.

Vibrionaceae are small Gram negative, straight or curved rods with a single polar flagellum. This family includes Vibrio spp ubiquitous in the marine environment and *Aeromonas spp.* that predominantly occurs in freshwater conditions. *Vibrionaceae* are predominant in necrotic tissue of the Mediterranean marine fish while *Pseudomonadaceae* in the necrotic tissue of Atlantic marine fish (Gauthier et al., 1984). Vibriosis is one of the more serious problems in marine fish (Christofilogiannis, 1993).

Different species of Vibrio have been described as pathogenic for sea bass and sea bream either in larval stages or in juveniles and adults (Toranzo and Barja, 1990). To date 7 species of Vibrio have been considered as fish pathogens (*V.anguillarum, V.ordalii, V.alginolyticus, V.carchariae, V.cholerae no 01, L.damsela* and *V.vulnificus*) (Vera et al., 1991). Colwell and Grimes (1984) gave detailed information on causative agents of Vibriosis of marine fish. Vibrio species pathogenic for marine fish include *Vibrio alginolyticus, V. anguillarum, V. carchariae, V. cholerae, L. damsela, V. ordali, V. vulnificus,* and *V. parahaemolyticus*. The strains most often isolated belong to the taxon *V.anguillarum* of which 16 different serotypes have been already identified but not all of them pathogenic to fish. Genovese and Micale (1991) confirmed the presence of non-pathogenic strains of *V.anguillarum* in sea bass. *Vibrio spp.* can be separated from *Aeromonas spp.* by exhibiting sensitivity to Novobiocin as well as to Vibriostat compound 0/129 (2,4-diamino 6,7 diisopropylpteridine phosphate) (Shotts et al., 1989). Disease caused by *Vibrio spp.* manifests as an "ulcerative haemorrhagic septicaemia". Handling injury is the primary factor in the aetiology of acute septicaemia by non-specific Vibrio spp. in sea bream in warm water conditions (20-28°C). Grisez et al. (1997) gave a comprehensive review on the intestinal microflora of larval sea bream and sea bass and on the factors influencing Vibriosis outbreaks in larvae. Fluctuations in the composition of the dominant microflora appeared to reflect the bacterial composition of the ingested live feed. Selection towards the genus Vibrio was not observed until the larvae reached the end of the larval life stage. *Vibrio anguillarum* and *Vibrio alginolyticus* exhibited significant adhesion to and chemotactic abilities towards mucus collected from the skin, gills, and intestine of gilt-head sea bream (Bordas et al, 1998). It is generally known that adhesion of bacteria to mucosal surfaces is an initial step for many microbial infections. Balebona al., (1995) demonstrated the role played by the mucous layer of fish, especially that of sea bream, during colonization of fish skin by saprophytic and pathogenic bacteria.

4.2.3.2 *Vibrio anguillarum* serotype I

In this study, the principal bacterial pathogens in sea bass included *V.anguillarum 1 phenon* 4b (33.3%) and *V.anguillarum 1 phenon 2* (6,9%) while *V.anguillarum 1* was isolated also form *Puntazzo puntazzo* (40%). This pathogen is the most important for sea bass farming but has very low significance in sea bream farming. This is important because combined vaccines used in Greece against Pasteurellosis in sea bream include an unnecessary component. It would be important however in the future to include other vibrios that are pathogenic to sea bream like *Listonella damsela* and *V. alginolyticus*. Paperna 1984 reported that this pathogen was the most important and common pathogen that affects only juveniles and adult sea bass but has not been described in sea bream. *Vibrio anguillarum*, one of the most frequent bacterial pathogens of marine fish also occurs in freshwater fish. *V. anguillarum* was often detected from sea water especially when water was below 20°C. In vitro experiments indicated that the organism persisted in seawater but perished within 3 to 5h in fresh water (Muroga et al., 1986). The main biochemical profiles isolated in this study were API 20E: 3047524 belonging to the *V. anguillarum* phenon 4b and API 20E: 1047524 which belongs to the *V.anguillarum* phenon 2.

Clinical demonstration of the disease in the current study agrees completely with the descriptive reports present in the literature (Shotts 1989; Vera et al., 1991;). The acute form of the disease is characterised by absence of apparent clinical symptoms and sudden high mortalities. The acute form of the disease is frequent and mortalities rise up to 40%. The disease is stress related occurring during periods with fluctuating water temperatures such as spring and autumn mainly when water temperature is between 15 and 20 °C. The chronic infections unlike the acute form are often associated with specific clinical symptoms. Infected fish are inactive, lethargic, torpid, dark in colour, with loss of scales. Echymoses, petechiae and

dermal haemorrhages are visible between the scales on the head, the ventral side of the body, the base of the fins and mainly around injuries (Vera et al., 1991). Skin lesions tend quickly to form ulcers that lead to distinct degradation of the skin and exposure of the underlying skeletal muscle, while tail and fins begin to fray. Internally haemorrhagic septicaemia is linked to congestion and haemorrhages of the liver, spleen, kidney, peritoneum, swimbladder and intestinal wall capillaries which may lead to necrotic lesion formation while the spleen is enlarged and haemorrhagic granulomatous lesions are apparent in the muscle. Plasmid-mediated toxins are responsible for the swollen viscera and the haemorrhages (Shotts et al., 1989). The intestine and mainly the rectum, that is often distended or full of clear mucoid liquid was considered a significant port of entry (Olsson et al., 1996). The gall bladder is also often distended and full of bile. A rapid ELISA method for the diagnosis of Vibriosis in sea bass was first described by Romestand et al. (1993).

4.2.3.3 *Vibrio anguillarum* other serotypes

V.anguillarum strain V62 caused mortalities in hatcheries and in on-growing units on the Mediterranean coast of France (Breuil et al., 1990), while sea bass and turbot are very susceptible to Vibriosis caused by *V.anguillarum* strain 408, which is used for vaccine production (Dec et al., 1990). A new serotype of *Vibrio anguillarum* has been implicated in sea bream larvae (150DD and 350DD). Fish become lethargic, inappetant and stay with the head down. Histological examination reveals intracytoplasmic bacteria in the epithelial cells of the intestine. *Vibrio anguillarum serotype 03* has been reported to induce septicaemia in sea bass in France (Vigneulle et al., 1993). None of these serotypes was isolated during this study.

4.2.3.4 *Listonella damsela*

Listonella damsela was isolated in sea bream (about 12%) and sharpsnout bream (20%) and is an important pathogen for these species but not for sea bass. This strain is one of the emerging pathogens in sea bream farming. It has been described in different fish species and was associated with human wounds. Vera et al. (1991) reported it from juvenile sea bream. The main clinical symptoms are emaciation, darkening of the skin, lethargy, distended abdomen with haemorrhages at the base of pectoral fins and in the perianal area. The gut and the rectum are distended, full of clear smelly fluid. The clinical picture mirrored the description in the literature. The bacterium has also been associated with large abscesses in the kidney of broodstock.

4.2.3.5 Other Vibrio strains

V. alginolyticus is a misinterpreted bacterial pathogen. Conflicting views are included in the literature on whether this is an opportunistic or a facultative fish pathogen. In this study it comprised 8% of the bacterial isolates from sea bass, about 12% for sea bream and 20% for *Puntazzo puntazzo*. Clinical practice suggests that this bacterium is more important for *Sparidae* than for sea bass where it is usually present as a secondary pathogen. However in sea bream and sharpsnout bream fry V.alginolyticus can cause significant mortalities. Several reports of mortalities in sea bream and sea bass caused by *Vibrio spp.* exist in the literature. Early reports of Vibrio infections in cultured sea bream in Israel by Paperna et al. (1977) were followed by more detailed reports of *V.alginolyticus, V.parahaemolyticus* and *V.anguillarum* infections in sea bream by Colorni et al., in 1981. In the latest case, *V.alginolyticus* was isolated also from the fishmeal used to make the pelleted feed, which might be significant for the disease's epidemiology. In juveniles and adult fish *Vibrio harveyi* and *Vibrio alginolyticus* have been

isolated from deep ulcerative lesions of sea bass and sea bream. *Vibrio alginolyticus* has been associated with septicaemia inducing severe losses in juvenile sea bass in hatcheries at 26°C. The *in vivo* and *in vitro* pathogenic activities of whole cells and extracellular products of *Vibrio alginolyticus* for cultured gilt-head sea bream were evaluated by Balebona et al. 1998. The strains examined had the ability to adhere to skin, gill, and intestinal mucus of sea bream and to cultured cells of a Chinook salmon embryo cell line. In addition, the *in vitro* ability of *V. alginolyticus* to adhere to mucus and skin cells of sea bream was demonstrated by scanning electron microscopy. The activity of extracellular products of *V. alginolyticus* was hydrolytic and able to degrade sea bream mucous. *V. alginolyticus* was cytotoxic for fish cell lines and lethal for sea bream. Moreover, the extracellular products could degrade sea bream tissues. However, experiments performed with the bath immersion inoculation technique demonstrated that *V. alginolyticus* should be considered a pathogen for sea bream only when the mucus layer is removed and the skin is damaged (Balebona et al, 1998). *Vibrio carchariae*, that is closely related to *Vibrio alginolyticus* (Colwell and Grimes, 1984) has been isolated from dermal lesions in sea bass. Cruz e Silva et al., 1997 reported for the first time in Portugal a co-infection of the monogenetict Trematodes of the genus *Microcotyle* and *Lamellodiscus ignoratus*, the protozoan *Trichodina* and the presence of epitheliocystis, *Vibrio alginolyticus* and *V. vulnificus* in cultured seabream. Deniz et al, 1993 considered *V. harveyi* as an opportunistic pathogen, which causes disease in *Sparus aurata* and rainbow trout when they are affected by stress.

In the current study *V.ordalii* was isolated only in sea bream (6%) but is considered an important pathogen for this species. *V.ordalii*, formely *V.anguillarum biotype II* is a well known pathogen in North America. In Europe this strain was isolated from sea bream with haemorrhagic septicaemia. *Vibrio ordalii* -like bacteria, close to *Vibrio sp.*

INFL described in Japan (blank intestine syndrome) causes similar pathology in sea bass larvae (200DD-400DD). In another study *Vibrio ordalli* was isolated from three species of Pacific salmon (Ransom et al., 1984).

In the current study Vibrios like *V.fluvialis, V.vulnificus* and *V.parahaemolyticus* are often secondary pathogens for sea bass and were isolated at a low percentage with *V.parahaemolyticus* being the most important and common of the three strains and the one with possible but unsubstantiated human health implications. *Vibrio parahaemolyticus* that is pathogenic to humans was not described in the literature as a real fish pathogen. It was suggested that fish simply seem to function as passive carriers of the bacterium. Other vibrios of minor importance for sea bass and sea bream like *V.pelagius, V.harveyi* or *Vibrio sp* associated with *DGS* were not reported in this study. An epizootic of *Vibrio pelagius* in juvenile turbot (*Scophthalmus maximus*) reared in an experimental system located in the *Vigo Estuary* was described by Angulo et al., 1992. *Vibrio harveyi* is one of the environmental Vibrios often linked with mortalities in different production systems. In addition *Vibrio harveii* was linked with mortalities and isolated from marine fish in Kuwait (Saeed, 1995). Distended Gut Syndrome (DGS) was initially reported in the late 80s. Unable to digest rotifers and brine shrimp nauplii, the larvae develop a swollen abdomen, exhibit a disoriented spinning motion and are swept passively with the current. Bacterial or viral infections were suspected. Virus-like particles 80 nm in diameter were observed in necrotic cytoplasm of midgut wall cells (Colorni, 1990). DGS still remained a problem in hatcheries with poor hygiene throughout the 90s. The aetiological agents of the abdominal swelling affecting farmed larvae of sea bream, *Sparus aurata* L., were studied. Four Vibrio strains were isolated from larvae of S. aurata affected by this disease, and all strains reproduced the disease in healthy larvae under controlled infection experiments, producing a significant increase of the mortality rates compared to the control (non-inoculated larvae) (Sedano et al., 1996).

4.2.3.6 *Photobacterium damsela subsp. piscicida*

Photobacterium damsela subsp. piscicida isolates comprised small a percentage in sea bass (2,3%) but much higher for sea bream (almost 12%). The relative importance of this pathogen is high in Greek Mariculture and in Marine farming in general. It is possible that at least for sea bass the percentage stated above is largely undervalued. The technical problem with this pathogen is its slow growing nature that caused many cases to go unrecorded because the bacterium was overgrown by more common and fast growing Vibrios especially when plate cultures were send to NCMR during the summer period when the disease is usually manifested. Pasteurellosis caused by *Photobacterium damsela subsp. piscicida* is one of the most common and serious bacterial diseases affecting yellowtail, (*Seriola quinqueradiata*) aquaculture in Japan (Yoshida et al., 1997) as well as in gilthead sea bream and sea bass, in Mediterranean countries. *Photobacterium damsela subsp. piscicida* (*Pasteurellaceae*) is a small Gram-negative non-motile rod shaped bacterium that requires at least 0.5% NaCl for growth in BHI, TSA agar at 17-31° C. It differs from other *Pasteurellaceae* by its lack of growth at 37°C, its inability to reduce nitrates and also its requirements for NaCl and sensitivity to *Vibriostat* agent.

Fish Pasteurellosis first appeared in the Mediterranean area in 1990 causing heavy losses in fish farms and affecting also wild populations (Quaglio et al., 1991). Pasteurellosis in sea bass develops in an acute and a chronic form. The disease affects fish of O group causing darkening, anorexia, and high mortalities (acute form). Focal necrosis of the gills is the only external sign often observed. Internally the spleen is dark and enlarged. During the chronic form lower mortalities occur and white granuloma visible to the naked eye are found mainly in the spleen. There is evidence that carriers under stressful conditions could suffer from reinfection. Necropsy shows haemorrhagic petechia on many tissues and organs and

congestion in the intestines, which contained mucous faeces. Moreover fish are afflicted by splenomegaly, with small whitish nodules also in the liver and kidney parenchyma (Giorgetti and Ceschia, 1994). The first documented outbreak of fish pasteurellosis in an offshore cage farm for sea bream in Malta, was described by Bakopoulos et al. (1997a). The pathogen entered the farm with juvenile fish and the disease occurred within 2 months of their arrival. The first epizootic pasteurellosis in sea bream cultured in a fish farm located in South Gran Canaria (Spain), was described at the beginning in January 1996. (Real et al., 1997). In this study haemorrhagic septicaemia was found in small fish as well as chronic granulomata in bigger fish. High mortalities ranging between 50 - 100% in affected batches are usually observed as soon as larvae reach 40 days. At this stage resistance to antibiotics can develop within a week. In sea bass Pasteurellosis outbreaks have been reported for juveniles over 30g. Histopathology reveals characteristic granulomata in haemopoietic tissue of the spleen and kidney, which in later stages release bacteria to produce a generalised haemorrhagic septicaemia. In an exceptional study Matsuoka and Kamada, 1995 described a significant part of the epidemiology of the disease, the dynamics of bacterial shedding from experimentally infected Yellowtail (*Seriola quinqueradiata*) weighing 40-92g. Fish were infected with an OA resistant strain of *Photobacterium damsela subsp. piscicida* and it was shown that infected fish began to shed bacterial cells one or two days before death and the shedding was maintained for about 5 days after death.

In a microcosm study *Photobacterium damsela subsp. piscicida* cells survived in water and sediment for only 6 to 12 days, depending on the strain and demonstrated capacity to enter a viable but not cultivable state. These dormant cells were always resuscitated by the addition of fresh medium to the microcosms, were smaller (dwarf cells) had increased surface hydrophobicity but maintained their infectivity and

pathogenicity (Magarinos et al., 1994). It is obvious therefore that bio-available antibiotic residues in the seawater /sediment environment accelerates the selection of the above persistent strains. Roberts, (ed., 1978) reported that misinterpretation often occurred with atypical *Aeromonads* and the need to differentiate from Mycobacteriosis and granulomatous lesions of nutritional origin Diagnostic techniques include Immunohistochemistry (IHC) (Maniatis et al, 2000) and Western blotting. (Pretti et al, 1999). The onset of disease is temperature dependent occurring at above 20°C in ongrowing units and 16-17°C in hatcheries. Magarinos et al., (2001) studied the effect of water temperature on the development of pasteurellosis in 60-day-old gilthead sea bream larvae obtained from asymptomatic carrier broodstock. It was suggested that larvae obtained from asymptomatic carrier broodstock are also carriers and could develop pasteurellosis when the water temperature was increased. This is extremely important for disease eradication in hatcheries to control the broodstock. carrier status. This is one of the first steps that along with adequate water filtration, strict hygiene practices and lately broodstock vaccination could help in the eradication of the disease in the hatcheries. However, it was possible to control the disease by maintaining larvae at low temperatures (Magarinos et al., 2001). This is useful in nurseries that use filtered seawater where correct scheduling of small fry stocking in periods when temperature is low avoids the disease establishment. Quantitative Antibiogram studies are necessary for the effective treatment of the disease and for the monitoring of the emergence of antibiotic resistance during or after treatment. Systematic prophylactic antibiotic bath treatments of sea bream larvae are not recommended. Treatments through live food can be used in infected hatcheries prior to handling and grading. Antibiotic treatment is problematic in Japan due to the presence of R-plasmids conferring transferable resistance to commonly used drugs (Roberts, 1978). Experimental vaccine development has been studied since the late 80s but in the last decade effective commercial vaccines were developed. This is due to the weak antigenic

properties of *Photobacterium damsela subsp. piscicida.* The mechanism of entry of *Photobacterium damsela subsp. piscicida* were studied by Jung et al (2001). Immunohistochemistry (IHC) using monoclonal antibodies revealed colonies of bacteria in the kidney, spleen and liver of sea bass, infected with pasteurellosis. Colonies were observed around the mucosal layers of the intestinal tissue, especially within the lamina propria and were associated with red blood cells and blood vessels of the organs examined. This was evident under the microscope in all Pasteurellosis incidences reported in this study. Turbot (*Scophthalmus maximus*) mucus (most likely a glycoprotein component) inhibited the growth of the *Photobacterium damsela subsp. piscicida* but mucus from sea bream and sea bass did not. This is consistent with clinical experience where turbot was not susceptible to *Photobacterium damsela subsp. piscicida* (Magarinos et al, 1995)

Another study of the peritoneal exudates cells (PECs) of sea bream concludes that the resistance of sea bream to *Photobacterium damsela subsp. piscicida* depends on the size of the fish and may be related to the efficiency of its phagocytes (Noya et al 1995). This explains the failed attempts to immunise 0.5g fry against Pasteurellosis, in nurseries where poor water filtration and quality initiated concurrent disease outbreaks. Current practice allows immersion vaccination in the nurseries when fish are 1.2-1.5 g mean weight in order to immunise fish against Pasteurellosis. Extracellular products, notably phospholipase and haemolysins, siderophores and capsular polysaccharides have been identified as putative virulence factors (Yoshida et al, 1997). The extracellular products (ECP) are good candidates as protective antigens in the vaccine and are still under investigation (Mazzolini et al, 1997). Noya et al. 1993 compared the haematological and histopathological changes caused in fingerling gilthead sea bream, *Sparus aurata* by the intraperitoneal injection of *Pasteurella piscicida* or different concentrations of its extracellular products (ECP) while a similar study was performed in sea bass

(Magni et al., 1998). All ECP products were cytotoxic for fish possessing phospholipase and haemolytic activity (Magarinos et al., 1992).

4.2.3.7 *Aeromonas sp. - Pseudomonas sp.*

Paperna et al. (1977) described deep ulcers filled with yellowish exudate in the interorbital region of mature sea bream associated with *Aeromonas* and *Pseudomonas sp.* *Aeromonas hydrophila* was isolated in juvenile and adult sea bream reared in land-based systems in Italy and from adult sea bass in Greece (Bovo et al., 1995; Doukas et al., 1998). This strain was often associated with *Pseudomonas anguilliseptica* during "Winter Disease Complex" acting more like an opportunistic than a primary pathogen (Domenech et al., 1999). In this study isolation of *Aeromonas salmonicida* from sea bass with haemorrhagic septicaemia might refer to an "old" finding but at the same time might indicate a largely undervalued component that is confused and misdiagnosed in practice due to confluent vibrios in the usual media for bacterial isolation. Winter disease is a sea bream disease syndrome where *Pseudomonas anguilliseptica, Aeromonas hydrophila,* a virus, a *Parvolike* virus, and a *Reovirus* are thought to be the aetiological agents along with a nutritional disorder. (Christofilogiannis et al., 1994; Berthe et al., 1995). Sea bass has also been reported to be naturally susceptible (Berthe et al., 1995). The syndrome develops as haemorrhagic septicaemia with keratitis. High mortalities occur at the end of the winter and early spring when the temperature is between 10°C and 14°C. When the temperature reaches 16°C mortalities (so-called *Picoma-like)* start to decrease. Clinical signs include abdominal distension ("belly-up syndrome"), redness of the pectoral fins and the peri-anal area, haemorrhages in the liver / intestinal tract and congestion in the brain. Isolation of the bacterial strain from the brain and the liver is possible. *In vivo* antibiotic treatments have been unsuccessful. Prevention is based on disinfection,

drying-out periods and appropriate feeding rates. It has been reported from November to April in marine fish farms on the Croatian side as well as the Italian side of the Adriatic Sea. (Sarusic, 1999; Contessi et al., 2000; Sarusic and Bavcevic, 2000). Winter syndrome caused significant losses in fish farms off the Northeast coast of Spain linked to histopathological lesions in intestine, pancreas and white muscle in immunosuppressed sea bream in winter months without however isolation of bacterial pathogens. (Tort, et al., 1998) In another study *Pseudomonas anguilliseptica* was isolated in incidences of average mortality rate of approximately 10-15%, although in some fish farms mortality reached 30% and the link to stressful environmental conditions was discussed (Domenech et al., 1997). Several bacterial isolates obtained from diseased sea bream, sea bass *Dicentrarchus labrax*, and turbot *Scophthalmus maximus* showed phenotypic similarities with the *Pseudomonas anguilliseptica* in French Mediterranean and Atlantic coasts. It was indicated that this represents a potential hazard for many farmed fish species and could cause serious economic losses. (Berthe et al., 1995) Microbiological investigations of winter syndrome cases in Italy detected in different outbreaks and times the presence of different agents: *Aeromonas hydrophila. Picorna-like* virus, *Parvolike* virus, *Reovirus* and an unidentified gram-negative rod. (Bovo et al., 1994). Currently, Winter syndrome has no important significance in sea bream in Greece due to adjustments in the feeding regime during the autumn-winter months. However in Italy due to the drop in temperature especially in vallicoltura the disease has become of increasing importance.

4.2.3.8 *Pseudomonas fluorescens*

In this study *Pseudomonas fluorescens* was isolated in sea bass at a low percentage (1,15%), is not commonly cited in the literature to affect sea bass and sea bream but from clinical experience is associated with mortalities following injection vaccination when the equipment is not kept clean. *Pseudomonas fluorescens* is recognised as one of the causal agents of bacterial haemorrhagic septicemia of fish and was first described as a pathogen of mirror and leather carp. *Pseudomonas fluorescens* was usually associated with bacterial haemorrhagic septicaemia generally clinically indistinguishable from motile aeromonad septicaemias, and as with the aeromonad infections was associated with concomitant environmental stress, especially with high temperatures or overcrowding. Pond fishes are most commonly affected but aquarium tropical fish, marine fish and salmonids may be secondarily infected in chronic viral infections and following traumatic injury. Association with traumatic injury agrees with the clinical finding of a *Pseudomonas fluorescens* link with ip injection vaccination. Chronic infections by non specific *Vibrio spp.* and *Pseudomonas spp.* seemed also to cause a distinct syndrome with white coloration of the head ("White head disease") in Israel. Haemorrhagic inflammation of the cephalic branches of the lateral line and integumental necrosis, were the main clinical signs (Paperna, 1984).

4.2.3.9 Myxobacteria

In this study *Flavobacterium meningosepticum* was the API20E characterisation of some pathogens belonging to (*Flavobacterium / Cytophaga spp.*). This was isolated from the kidney of sea bass with no signs of haemorrhagic septicaemia but exhibiting low mortality levels. Myxobacteria are an important cause of mortalities in sea bass fry but it was difficult to culture them because they require lower nutrient culture media for initial isolation and are easily overgrown by *Vibrio spp.* It is thought however that this is the first report of *Flavobacterium meningosepticum* in sea bass.

It remains to be seen if this type of pathogen could arise as an important pathogen in the future but is more likely to play a role of a secondary pathogen masked by more common bacteria like Vibrios. Myxobacteria (*Myxobacteriales*) are long, thin, flexible bacteria showing oscillating or gliding movement, and are diagnosed only by examination of fresh smears. In fish muscle-sea water broth they demonstrate the typical movement, while they grow poorly on solid and semi-solid media. Myxobacteria have been found to be the etiological agent in focal necroses of the gill filaments, coupled with dermal lesions in high mortalities that occurred in juveniles and adult sea bream, in Israel, usually following netting operations although spontaneous infections occur occasionally. In some cases the "gill rot" due to Myxobacterial gill infection was associated with heavy infections with the Monogenean parasite *Furnestia echeineis*. (Paperna et al., 1977) which develops 2-3 days after handling (Colorni, 1981). Remaining filaments assume a dark brown colour. There is an increased prevalence of Myxobacterial gill infection in sea bass with deformed opercula (Paperna, 1984). Infected fish remain near the surface of the water, moving to the edge of the holding facility, they swim aimlessly but slowly and the opercula may not close normally.

Flexibacter maritimus has developed as a common problem in Mediterranean mariculture. *Cytophaga -like* bacteria (CLB) have been isolated from sea bass. Affected juveniles and adults develop ulcerative skin lesions. In juveniles haemorrhages can be observed on the mouth as well as thick yellowish mucus. Gills exhibit focal necrosis and excess mucus in which long bacterial rods show typical gliding motion. Following severe outbreaks of a disease with dermal necrosis, several bacterial strains were isolated from sea bass (*Dicentrarchus labrax*) reared on French coasts. A phenotypic study revealed that the isolates closely resembled *Flexibacter maritimus* (Bernardet et al., 1994). Antibiotics have been used by bath or oral route. Prevention of stress, regular grading to reduce cannibalism and reduction

of mechanical injuries through gentle handling will reduce the risk of infection. Vaccine testing in Japan has been unsuccessful. An important problem arose in the treatment of Myxobacteriosis in sea bass fry when nitrofurans the most effective medicines for surface infections were banned leaving no other approved effective alternatives. Two reports of *Edwardsiella spp* (Blanch et al., 1990) and *Yersinia ruckerii* (Vignuelle, 1988) isolation in sea bass refer to scientific observations rather than clinical diseases of practical importance.

4.2.4 Gram positive bacterial pathogens

4.2.4.1 Gram positive cocci

In this study six Gram-positive cocci (6,9%) were isolated in sea bass, two in *Dentex dentex* and one in *Mugil cephalus*. Although there was no further identification at a genus level, this finding was not odd. It was consistent with another study from Italy where *Staphyloccocus sp.* was isolated from the kidney of diseased cultured sea bass *(Dicentrarchus labrax)* under poor environmental conditions (Tassi and Salati, 1996).

4.2.4.2 *Mycobacterium spp.– Nocardia spp. – Streptococcus iniae*

A group of bacterial pathogens like *Mycobacterium marinum, Nocardia spp.* and *Streptococcus iniae* were reported in the literature but not isolated yet in Greek Mariculture. However they are mentioned briefly because it is believed that they might soon cause a significant problem in the Mediterranean mariculture industry, due to their zoonotic component, to their limited effective means of therapy and eradication and due to the fact that they make fish unmarketable. *Mycobacterium spp.* are acid fast, slightly curved to straight, Gram positive, non-motile rods. Only three of the 150 species observed in fish are considered to be fish pathogens. (*M.marinum, M.fortultum, M.chelonei*) (Shotts et al., 1989). Mycobacteriosis is a chronic progressive zoonotic disease. (Shotts et al., 1989). There is evidence of oral

transmission of the disease to humans when raw contaminated fish products are used in the diet, while the cutaneous route also plays a significant role. Infected fish become lethargic, separate from the others, have skin ulcerations, small perforations on the fins, pigment alteration or may develop spinal curvature. In addition bi-lateral exophthalmos may occur. The only effective diagnostic technique in asymptomatic fish is the Polymerase Chain Reaction (PCR) test (Colorni et al., 1993). Internal pathology includes grey-whitish nodules or granulomas in most organs but mainly in the kidney, liver and spleen (Shotts et al., 1989). Many lesions are purulent while others are necrotic with dark yellow or brown material in the centre.

Feed medicated with Rifampicin for 6 months and subsequently with Cycloserine for 4 months had no curative effects (Colorni, 1992). Diagnosis can be made by staining of histological sections with Ziehl-Neilsen and by culture on selective medium. A PCR technique made possible screening of new stocks for latent infections, the selective removal of infected individuals from brood stocks as well as tracing the possible source of infection (water, feed, or diseased parents). (Knibb et al, 1993a) *Mycobacterium marinum* has been isolated from sea bass both in the Mediterranean coast of Israel as well as the Red sea (Colorni, 1992; Knibb et al, 1992, 1993b). Sea bream (*Sparus aurata*) and red sea bream (*Pagrus major*) as well as wild fish in the vicinity of the cages have also been found infected. The disease is impossible to eradicate with antibiotic treatment and makes the affected fish unmarketable and may represent a hazard to human health. Sanitation, disinfection and destruction of the carrier fish especially spawners is essential for the control of this disease. Diamant et al., 2000 discussed the implications of spreading of M. marinum infection in wild fish populations in the Gulf of Eilat. Eradication of the pathogen was impossible with any of the treatments (Colorni et al., 1998).

Nocardiosis is one of the most important diseases of cultured yellowtail *Seriola quinqueradiata* in japan but Vigneuelle et al. (1993) have reported the disease for the first time in adult sea bass and broodstock fish. Infected fish exhibit haemorrhagic cutaneous zones on the sides near the lateral line, the operculum, the pectoral and pelvic fins. Yellow white nodules are present on the epidermis. Internally abscesses are detected on the head and the branchial arches. Typical bacteria can be observed on fresh smears from the nodules. Treatment with antibiotics in vivo is not very effective. Long treatment of 21 days with Sulphonamide compounds in Japan had limited success. In order to prevent the disease, handling that may lead into skin injuries has to be avoided. *Streptococcus iniae* was isolated from diseased wild fish collected near a mariculture facility where sea bream and sea bass exhibited a similar infection. Species-specific PCR and ribotyping confirmed that a single *S. iniae* clone infected wild and cultured fish. Wild fish were therefore potential amplifiers of pathogenic *S. iniae* strains (Zlotkin et al., 1998). This bacterium along with *Lactococcus spp.* has been devastating the trout industry in Spain and Italy in the last three years and a potential significance to mariculture industry is expected within the next few years.

4.3 Antibiotic Application

4.3.1 Available Compounds

According to legislation the Greek National Drug Organisation must issue and/or approve the marketing authorisation for any veterinary medicinal product pharmaceutical or biological for fish disease prevention and control (such as antibiotics, vaccines, anaesthetics), for either internal or external use. The medicines which have been approved for use in fish are oxolinic acid 50 and 24%, Oxytetracycline 75.5% and 20% and trimethoprimsulfadiazine 40 and 50%. The process for the use of chemicals is under the consultation prescription and control of veterinarians – fish pathologists.

4.3.1.1 Oxolinic Acid – Other Quinolones: clinical efficacy

Oxolinic acid is registered as a fish therapeutant in Japan and several European countries and is proven efficacious in vitro against select isolates of *Vibrio* spp. *(V. anguillarum, V. ordalii, V. tubiashii, V. carchariae, V. damsela), Aeromonas* spp. *(A. hydrophilia,* A. *sobria,* A. *caviae,* A. *salmonicida), Pasteurella piscicida, Pseudomonas fluorescens, Y. ruckeri,* and *Edwardsiella tarda* with the MICs ranging from less than 0.075 to 0.3 µg/ml. (Austin et al., 1982; Ledo et al., 1987, Bowser, 1990; Bowser and Babish, 1991). It was proven efficacious in furunculosis treatment in rainbow trout (Austin et al., 1983) and was considered successful in lowering bacterial numbers (*Cytophaga sp* and *Pseudomonas fluorescens*) on the surfaces of eggs of rainbow trout, *Oncorhynchus mykiss* (Barker et al., 1990). Quinolone and fluoroquinolone antibacterials examined in-fish have included nalidixic acid, oxolinic acid, piroidic acid, flumequine, norfloxacin, ciprofloxacin, sarafloxacin, and difloxacin. Fluoroquinolones are 6-fluorinated carboxylic acid compounds, second-generation derivatives of first-generation quinolones such as nalidixic acid and

oxolinic acid. These drugs are bactericidal and act by selectively inhibiting prokaryotic topoisomerase II (DNA gyrase), an enzyme necessary for replication, recombination, and transcription of a bacterial (circular) chromosomal DNA (Drlica and Coughlin, 1989; Zimmer et al., 1990). The net result is that the treated bacteria are unable to reproduce. Fluoroquinolones are bioavailable via enteral and parenteral routes, are widely distributed throughout tissues including intracellular sites, and have a broad antibacterial spectrum of activity, especially active against gram-negative bacteria and intracellular bacteria such as mycoplasma, rickettsia, chlamydia, and mycobacteria. *(*Stoffregen *et al, 1996).* Vetoquinol, an oxolinic acid ester was also compared by oral administration against furunculosis in Atlantic salmon in seawater (Samuelsen et al., 1999, 2000) while another comparative study was performed against Vibriosis in Atlantic halibut *Hippoglossus hippoglossus* (Samuelsen, 1997). In two other comparative studies Florfenicol was more effective against *Aeromonas salmonicida* and *Vibrio salmonicida* infections in Atlantic salmon, *Salmo salar* L., *smolts* compared to oxolinic acid and trimethoprim/sulphadiazine (Nordmo et al., 1994, 1998).

4.3.1.2 Oxytetracycline - Tetracyclines: clinical efficacy

Tetracycline antibacterials are bacteriostatic, binding to the 30S subunit of the microbial 70S ribosomes and inhibiting protein synthesis (mRNA translation) by blocking the attachment of aminoacyl-tRN units (Jawetz, 1987). They can be administered enterally or parenterally, have good distribution to tissues including intracellular sites, and have a broad spectrum of antibacterial activity. *(*Stoffregen, et al, 1996*)*. OTC is the most widely used antibiotic in fish farming and several in vitro as well as in vivo studies have been made in order to demonstrate its clinical efficacy against a range of pathogens in different species and production systems. In certain cases it was also used in unconventional applications. OTC has been

applied by injection in pre-spawning female salmon *Oncorhynchus kisutch* in order to control vertical transmission of *Renibacterium salmoninarum*, causative agent of bacterial kidney disease (Brown et al., 1990). OTC has also been used to control secondary bacterial infections even in clinical cases where the aetiology was viral. Fish experimentally treated with oxytetracycline or a combination of OTC and chloramine-T had a significantly lower mortality rate than untreated larval and juvenile halibut *Hippoglossus hippoglossus* (Cusack et al, 2001). In a similar situation in Greece in cases of a new invasive strain of Lymphocystis virus affecting sea bream OTC is often employed to combat the bacterial component of the syndrome due to the subsequent development of Pasteurellosis. OTC application for the control of bacterial microflora associated with rotifers, used as live food, in a marine fish hatchery (Minkoff et al., 1992) is never employed in practice in order to reduce the bacterial flora in mass cultures. In addition treatment failures following oral OTC application have been recorded in Atlantic salmon fingerlings experimentally infected by *Vibrio salmonicida* by intraperitoneal injection (Cox and Rainnie, 1991).

4.3.2 Potential Oxolinic acid and Oxytetracycline Side Effects

Several side effects were associated with Oxytetracycline and Oxolinic acid application in fish although several laboratory studies were not able to demonstrate them. These include immunotoxic immunosuppressive effects and even skeletal deformities for Oxytetracycline. (Table 60) The importance of these effects on actual clinical practice and their effect on the success of an antibiotic treatment is generally unknown. However registration of both drugs was successful a long time ago and their importance in clinical veterinary practice vastly dominates the possible minimal side effects. No side effects were recorded during the commercial application of OA and OTC during this study.

Table 60: Oxolinic acid and Oxytetracycline Side effects reports in literature

Antibiotic	Side effect	Species	Literature
Oxytetracycline	Humoral response	Rainbow trout	Kreutzman, 1977; Rijkers et al., 1980; 1981; Grondel et al., 1985)
Oxytetracycline	Immunosuppresion	Carp	Rijkers et al., 1980;
Oxytetracycline	Leukocyte mitogenic and allogenic responses	Carp	Grondel and Boesten 1982 Grondel et al. 1985
Oxytetracycline	Deformities of the spinal column	Rainbow trout	Madsen et al., 2001
Oxytetracycline	Photosensitivity	African catfish	Stacell and Huffman, 1994
Oxytetracycline	Hepatic necrosis	Atlantic salmon	Bruno 1989 Stoffregen et al., 1996
Oxytetracycline	Modulation of the fish resistance	European eel	Van der Heijden et al., 1996
Oxytetracycline Oxolinic acid	Suppression of antibody production	Rainbow trout	Lunden et al., 1998
Oxytetracycline Oxolinic acid	Mitogen-induced lymphoid cell proliferation	Rainbow trout	Lunden and Bylund, 2000
Oxolinic acid	Cellular histopathological changes	Rainbow trout	Moutou et al., 1997
Oxytetracycline Oxolinic acid	Immunotoxic effects	Carp	Studnicka et al., 2000
Oxolinic acid	hepatic microsomal cytochrome P450 monooxygenases	Rainbow trout	Moutou, 1998
Oxolinic acid	haematological disturbances adverse effects of liver	Marine fish	Miyazaki et al.,. 1984

OTC was indicated initially to be of low toxicity to fish (Herman, 1969a,b). However more recent studies indicate that humoral response is susceptible to low OTC levels while cellular response is unaffected (Kreutzman, 1977; Rijkers et al., 1980; 1981; Grondel et al., 1985). OTC is bacteriostatic and not bactericidal therefore careful consideration regarding prolonged administration is essential, especially when the fish immune system may already be compromised by environmental conditions, husbandry techniques, and infectious diseases (Grondel and Boesten, 1982). OTC injection caused delay in the rejection of allogenic scale transplants in Common carp (*Cyprinus carpio)* demonstrating an immunosuppressive effect (Rijkers et al., 1980). In similar studies OTC caused a reduction of leukocyte mitogenic and allogenic responses in a dose-dependent manner (Grondel et al., 1985) while in another study kinetics of the PFC response were not affected but the PFC numbers were decreased and the anti-SRBC antibody production of the pronephros was delayed. (Grondel et al., 1987) Although OTC caused spinal deformities in Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*) with spinal fractures observed only in the first species (Toften and Jobling, 1996) it did not have the same effect in rainbow trout (*Oncorhynchus mykiss*) (Madsen et al., 2001). Exposure to ultraviolet light following OTC injection caused photosensitivity in channel catfish (*Ictalurus punctatus*) with necrotic ocular and dorsal epidermal lesions (Stacell and Huffman, 1994). A series of studies at histopathological cellular level indicated extensive hepatic necrosis in broodstock Atlantic salmon furunculosis survivors following OTC injection (Bruno, 1989). This was initiated by oxytetracycline via enterohepatic recycling and compromised excretion, resulting in this novel hepatopathology (Stoffregen et al., 1996). Limited affectivity of OTC versus Flumequine treatment in European eel (*Anguilla anguilla*) infection with the parasitic swimbladder nematode *Anguillicola crassus*, points to a modulation of the fish of the cellular rather than the humoral response as a result of the drug treatment. (Van der Heijden et al., 1996). Both oxytetracycline and oxolinic acid significantly suppress antibody production as

well as the level of circulating white cells, especially lymphocytes, in rainbow trout when administered in association with immunisation. The phagocytic activity of whole blood leukocytes was stimulated by oxolinic acid and slightly suppressed by oxytetracycline (Lunden et al., 1998). Both OA and OTC suppressed the mitogenic response of the head kidney cells in rainbow trout (*Oncorhynchus mykiss*). T-cells were more vulnerable than B-cells (Lunden and Bylund, 2000). OA application caused compensatory, rather than degenerative haematological, (Miyazaki et al., 1984), enzymatic, (Moutou, 1998) and cellular changes in the liver of rainbow trout (*Oncorhynchus mykiss*) (Miyazaki et al., 1984; Moutou et al, 1997; Moutzouris et al., 2000). Although non drug-specific these effects of oxolinic acid were delayed and longer lasting compared with flumequine (Moutou, 1998).

4.3.3 Current Practice Auditing

The framework in Norway where the Norwegian Medicinal Depot a national drug monopoly system is used along with authorised feed mills dispensing medicated feed is an ideal system to collect valid and reliable sales data on drugs in fish farming (Grave et al., 1990). In Greece like most of the other EU countries such a monopoly does not exist and sales of antibiotics by wholesalers and pharmaceutical companies only recently started to be collected by the National Drug Organisation. Although a common EU legislative framework exists, implementation and control is usually problematic due to state inefficiencies, lack of resources and personnel. This is the reason why no official data of drug use in Greek mariculture are presented in this study. Personal communication with fish vets in the FGM Fish Health Committee indicated that total cost of antibiotics used in Greek Mariculture per Kg of fish is around €0.05/Kg, while in this empirical figure if biologicals (fish vaccines) are included, the cost is more than €0.1/Kg of fish produced. A shift from treatment to prophylaxis led to a rapid increase of fish vaccine application against vibriosis and

pasteurellosis in sea bass and, to a lesser extent in sea bream. The current practice includes the following series of events in relation to antibiotic treatment in a fish farm. The fish vet (full-time employee) or a regional fish vet visits the farms on the notice of increased mortalities, or samples are sent to the laboratory for analysis. Usually this is basic bacteriology along with non-quantitative basic antibiogram. Due to the limited number of fish veterinarians, laboratories in institutes perform this basic diagnostic service. While it is legally compulsory, there is a misuse of veterinary prescription and only big organised companies accompany orders of antibiotic to pharmacists by vet prescription. The drug quantity ordered arrives in the farm to be mixed in a cement mixer to be oil top coated onto the fish feed (this was the sole practice in early 90's when this study was initiated). This on farm mixing poses many questions about homogeneity, concentration of the medicated feed mixed, the possible lack of therapeutic effect and the hazards of exposure for the workers (Grave et al., 1990). Recently, a few feed mills are licensed to manufacture medicated feeds and more fish farms, usually the big companies, are using this method to treat fish diseases or to incorporate successfully oral vaccines. However, the logistics of such operations, the remote rural nature of mariculture and the added cost of production line clean up / delay of production of these feed mills still make this option less attractive.

Reports of antibiotic utilisation in different countries come mainly from salmon and trout farming in different countries like Norway where Growe et al., 1990 described in a comprehensive review for the period 1980-1988 the gradual shift to feed mills and the decrease in antibiotic utilisation despite the concurrent increase in production from below 40,000 to more than 120,000 tons. OTC was the most frequently prescribed antibiotic used, mainly against *V. anguilarum*. This situation was improved gradually in Norway with the development of effective vaccines for Furunculosis and Vibriosis as well as for some of the viral diseases. Sales estimates

of antimicrobial products used as veterinary medicines in UK in 2000 indicate that tetracyclines accounted for 49% of the total sales for use in food animals while fluoroquinolones for about 1 tonne as in previous years. Approximately 56% of antimicrobial products in food animals were administered via feedstuffs mainly in pigs and poultry. It was also described that while 10 tonnes of active ingredient was used in 1993 (59,000 tonnes salmon & trout production) only 2 tonnes of active ingredient were used in 2000 despite the massive increase in production (130,000 tonnes). Selected salmonid fish farms in England and Wales were sent a questionnaire designed to collect data on enteric redmouth disease (ERM) vaccination and the usage of antimicrobial agents. In the farms, which employed vaccination, the commonest chemotherapeutant used against ERM was oxolinic acid. However, oxytetracycline was the most frequently used antibiotic on farms not employing a vaccine (Rodgers, 1991).

4.3.3.1 Clinical Efficacy

The terminology of drug metabolism includes terms like palatability of the drug (pleasant in taste), leaching, digestibility (ability to be assimilated), absorption (ability to be absorbed in the gut epithelium), bioavailability (potential of achieving high concentration of "active" drug in the target tissues), excretion (expulsion of waste material as result of the metabolism) and depletion (reduction of drug residues in the fish tissues). Oil coating of Oxolinic acid and Oxytetracycline was initially tried for the kinetics studies but poor palatability, leaching and the creation of an oily film on the surface of the tanks led to the preparation of fish feeds where the antibiotic was incorporated in the pellet. This strategy improved the acceptability of the feed pellets (even of OTC pellets to sea bass that are known to refuse OTC treatments due to palatability) in both antibiotics and species. Following these observations the

research group in NCMR decided to tackle the issue of leaching and palatability initiated by study by Rigos et al., 1999.

4.3.3.1.1 Palatability

The most convenient method for treating fish with antibiotics is the use of medicated feed. However because of the reduced feeding of fish suffering from bacterial diseases and the unpalatability of feed containing drugs (Hustvedt et al., 1991), only 20-30% of the antibiotics administered are absorbed through the intestinal tract. The remaining 70-80% passes into the environment (Samuelsen, 1989a,b, 1991). Sea bass was proven in the pilot stages of this study to be reluctant to take OTC oil-coated medicated diets, therefore incorporation of the antibiotics in the feed was selected as the method of choice. This significant reduction of OTC oil-coated feed consumption in sea bass at two temperatures was confirmed in a study initiated in NCMR after these findings while no reduction was evident with OA oil-coated medicated feed (Rigos et al., 1999). This study simply confirmed findings of a previous study in rainbow trout (*Oncorhynchus mykiss)* using radioactively labelled diets. (Hustvedt et al., 1991a). However this negative effect is not present in all species. OTC for example did not seem to be a feeding deterrent for Arctic charr (*Salvelinus alpinus*), possibly indicating that the char is a rather unselective feeder (Toften et al., 1997).

4.3.3.1.2 Leaching

In several studies leaching was proven to be reduced when drugs were incorporated in the feed compared to top coating (Rigos et al., 1999) or drug sprayed on the feed (Xu and Rogers, 1994). OTC and OA alginate-coating on fish pellets has also been used to reduce leaching (Duis et al., 1995). Usually OTC is leaching is more significant compared to OA leaching as it is readily dissolved in water while OA requires a pH shift to alkaline in order to be dissolved. Increased water temperature induced a significant effect on the leaching of both drugs when mixed with the feed,

but did not affect the loss of oil-coated drugs (Fribourgh et al., 1969; Xu and Rogers 1994; Rigos et al., 1999). Often if oil-coating cannot be avoided the concentration of OTC is increased or a higher feeding rate is used to compensate for the loss of OTC due to leaching. The amount of OTC that must be added to feed depends on type of feed, water temperature, and time of consumption by fish (Xu and Rogers, 1994).

4.3.3.1.3 Digestibility

In several studies digestibility was proven to be higher for OA than for OTC (Cravedi et al., 1987) and was increased proportionally for both antibiotics with increasing water temperature (Rigos et al., 1999). A significantly higher digestibility of OTC (59-85%) and OA (64-94%). in sea bass was reported compared to other species.(Rigos et al., 1999) with at least OTC digestibility being similar to that reported in humans (60-80%) (Weinstein, 1970). However in many other studies OTC digestibility was 10 fold lower in other fish species (Cravedi et al., 1987; Rogstad et al., 1991). Low digestibility at lower temperature raises the significance of the interactions between fish therapy and marine environment. Digestibility is increased with decreasing particle size. Effects of ultra-fine size oxolinic acid on absorption by yellowtail were studied by a high performance liquid chromatographic method (Endo et al., 1987).

4.3.3.1.4 Bioavailability

Hustvedt et al., (1991a), discussed limitations on the OA bioavailability studies. OA serum levels in Atlantic salmon (*Salmo salar*) after injection and after oral administration indicated that mean relative OA bioavailability by the oral route compared to the intraperitoneal route was about 50% (Hustvedt et al., 1991b). This can be explained to be due to either malabsorption or first pass metabolism (i.e metabolism in the gut epithelium or in the liver) or due to the use of carboxymethylcellulose (CMC) as thickening agent in the medicated diet that reduces bioavailability by 10-20%. Low bioavailability after intraperitoneal injection could probably be explained by absorption into the intestinal lumen and possibly by

leakage from the injection site. Bioavailability of Oxolinic acid in Atlantic salmon was reported to be 30.1% (Martinsen and Horsberg, 1995), while Hustvedt et al., (1991b) reported bioavailability 19.9% at 7.5°C and 21.4% at 9°C. However, confusion exists on the dependency of OA bioavailability on administered dose level. Dose independent OA bioavailability in Atlantic salmon (*Salmo salar*) was low (20-22%) after the low and high oral dose at two low temperatures (Hustvedt et al., 1991). Contrary to that a more recent study by Rogstad et al., 1993 suggested an inverse correlation between OA dose level and bioavailability. Bioavailability of the oxolinic acid was found to decrease with increasing dose rates in Atlantic salmon (*Salmo salar*) at 5°C being 25% at a dose rate of 50mg/kg b.w/day and 40% at 25mg/kg b.w/day (Rogstad et al., 1993). OA oral bioavailability was 13.6% in freshwater cultured rainbow trout (*Oncorhynchus mykiss*) (Bjorklund and Bylund, 1991). Comparative OA per os bioavailability studies in channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*) at two temperatures indicated early plasma peaks but increased bioavailability and sustained concentrations for a longer period at lower temperature (Kleinow et al., 1994). While in one study OA bioavailability in turbot *(Scophthalmus maximus)* following single oral dose was 27,9% (Poher and. Blanc, 1998) in another more recent study poor OA metabolism was described in turbot (*Scophthalmus maximus*) after a single oral dose indicating that it was dissolved in the liquids present in the gut and absorbed in a non-dissociated form depending on the gradient of concentration between gut lumen and the environment. In this study 81% of the dose was rejected along with the faeces in the environment - of which at least 28% was in a solid form - which can accumulate in the sediments (Guichard, 2000). OA Bioavailability was enhanced by particle size reduction (Endo et al., 1987).

OTC bioavailability was studied less but it was found to be very limited after oral application although it was positively correlated with water temperature (Hustvedt et

al., 1991, 1991a). In rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) studies oral administration was linked to low bioavailability (Grondel et al., 1987a; Rogstad et al., 1991), while intramuscular application gave high bioavailability (Grondel et al., 1989). Therapeutic efficacy of OTC via the oral route in common carp (*Cyprinus carpio*) was therefore questioned due to extremely low bioavailability possibly due to lack of a stomach and absence of pH variations in this species (Grondel et al., 1987). High, but not significant different bioavailability was observed in rainbow trout *(Salmo gairdneri)* and African catfish *(Clarias gariepinus)* following i.m. OTC administration (Grondel et al., 1989). Oral bioavailability of Oxytetracycline HCl investigated in Atlantic salmon (*Salmo salar* L.) in seawater at 7-8°C was found to be only 2% (Elema et al., 1996). A significant difference in OTC bioavailability was revealed between healthy and vibriosis-infected ayu (*Plecoglossus altivelis*), whereas elimination was similar in both fish (Uno 1996).

4.3.3.1.5 Tissue distribution

Oxolinic acid taken into fish was partly biotransformed to 6,7-diOHOA by microsomal monooxygenase,this was further metabolised to 7-OHOA or 6-OHOA, and these metabolites as well as the unchanged form were conjugated with glucuronic acid. The difference in the retention among the species may be due to the difference in activity of microsomal monooxygenase. Oxolinic acid was retained at a lower concentration and for a shorter period in fish acclimated to seawater than in fish raised in fresh-water due to the faster excretory rate in seawater fish. Metabolic rate was affected by the activity of microsomal monooxygenase and the physiological function of fish such as osmotic regulation (Ishida, 1992a). Bjorklund et al., (1991) reported that maximum OA concentration in the muscle and serum was achieved at the same time at three temperatures. Maximum concentration of O.A in

rainbow trout occurred in the order bile>liver>kidney>muscle>serum. In a similar study by Ueno et al., (1988) organ distribution of OA level was kidney>liver>muscle>blood. Distribution and elimination of OA after oral administration in Nile tilapia (*Oreochromis niloticus*) were delayed at lower temperature, but significant difference of the peak concentrations in the tissues of the group at 15°C and 25°C was not revealed. However, the changes of maximum concentration, absorption and elimination rates in the tissues of fish following bath treatment depending on different temperatures were more significantly different from the results of the studies with oral administration. OA pharmacokinetics in tissues of diseased fish, the main target of drug treatment, also appeared to be distinguishable from those of healthy fish (Kim et al., 1998). The distribution profile of flumequine in the various compartments of fish appeared to be different from that of oxolinic acid following a single oral dose with higher concentration in plasma and liver (Rogstad et al., 1993). Highest organ OTC concentrations were found in liver, gills, kidney, and gonad (Black et al., 1991) and gastrointestinal tract, liver, vertebral body, skin, and mucus (Rogstad et al., 1991). OTC affinity and chelation to calcium namely bone and scales made OTC tagging application widespread for salmonids (Koenings and Lipton, 1986). OTC tissue distribution and elimination in salmonids in seawater generally decreases in the order of liver>kidney>skin>muscle at the end of the treatment period. These results show that the pharmacokinetics of OTC in Atlantic and Chinook salmon are very similar after per os administration (Namdari et al., 1998). In a previous study OTC tissue concentrations in chinook salmon (*Oncorhynchus tshawytscha)* was about twice as high as those of the coho salmon. (*Oncorhynchus kisutch*) (Namdari et al., 1996). The tissue disposition and bioavailability of JH-tetracycline were examined in the channel catfish *(Ictalurus punctatus)* after intravascular and *per os* application. The drug was highly concentrated in both hepatobiliary and urinary compartments. The concentrations in the edible flesh were the lowest of any compartment examined (Plakas et al., 1988).

OTC kinetics and tissue distribution was studied in rainbow trout (*Oncorhynchus mykiss)* following bolus i.v administration (Black et al., 1991; Uno et al., 1997). The highest drug levels were found in the liver and the lowest were in the brain (Black et al., 1991).

4.3.3.1.6 Excretion

Four points clearly emerge from the literature. Temperature is an important factor in elimination of drugs from fish tissues. However, generalisations concerning this relationship should be made cautiously as the type of drug and the species of fish are also important factors in determining elimination rates. The current conversion of referring to withdrawal periods in fish in "degree days" was often questioned (Ellis, 1991). Drugs are cleared at different rates from different tissues. Elimination from muscle may be relatively rapid but certain drugs may accumulate and persist in bone, scales and kidney. Factors such as species maturity and health status affect elimination rates. Environmental parameters play a significant role (eg. Sea water versus Freshwater). This study revealed marked differences between Oxolinic acid and Oxytetracycline excretion rates. Factors like tissue and water temperature were proven more important than species differences. The concept of the relevance of Degree-days was questioned as it happened in some studies in the literature. Factors like fish health status (only healthy fish were used in the experiments) and environment were not evaluated in the current study.

Temperature is an important factor affecting clearance rates. Some data available indicate that the residue time in muscle is approximately proportional to temperature and concentrations in the muscle. However large individual variation in elimination rate occurs in rainbow trout in seawater and to allow for the slowest elimination rate it has been recommended that at temperatures above 10^0C there should be a withdrawal period of 60 days and from 7-10^0C a period of 100 days (Salte and

Liestol, 1983). These recommendations are in line with other recommendations (Jacobsen, 1989) for rainbow trout in fresh water for achieving levels of less than 0.01µg/g in whole gutted carcasses. The important point here is that OTC was retained in bone and scales for long-periods (Jacobsen, 1989). OTC has also been reported to persist in high concentration in the pronephros of carp (Grondel et al., 1987a). For these reasons, it has been recommended that whole gutted carcasses should be analysed rather than just muscle on the assumption that skin may be eaten (Jacobsen, 1989). To examine the hypothesis that kinetic processes are higher at warmer environmental temperatures in fish several studies that were conducted, reported that in warmer water absorption peaked faster in the serum and muscle. Terminal elimination rates were consistently more rapid at higher water temperatures in all tissues, including muscle (Bjorklund and Bylund, 1990; Rogstad et al., 1991) OTC elimination rate in rainbow trout and ayu was not affected by temperature, with a tendency for faster elimination at lower temperatures. Recommended withdrawal times are 20 days (Jacobsen, 1989). In contrast, other workers (Ueno et al., 1988a,b) reported that elimination rates in rainbow trout were temperature dependent (muscle residues cleared in 18 days at 10° C; 12 days at 15° C). Species differences could be marked. *Amago salmon* at 15° C took at least 30 days to eliminate residues from muscle (Ueno et al., 1988b).

Ellis (1991) argued that the effect of temperature is not always linear and doubt must be cast upon a general "degree days" convention for setting withdrawal times. Data for many drugs does not include measurements of elimination rates at different temperatures and research reports (eg Salte and Liestol, 1983; Jacobsen, 1989) did not use the "degree days" convention but instead recommended withdrawal times for specific temperature ranges. A further limitation in the "degree days" convention plasma of rainbow trout held at 12^0 C in 29 days (=348 ^{0} days) and from the plasma of catfish held at 25^0 C in 31 days (= 775 ^{0} days) (Grondel et al., 1987a). At 400

degree-days the catfish plasma still contained 2 µg OTC/ml. Thus if a "degree days" convention is to be used it would have to be set specifically for each fish species. Ellis (1991) suggested withdrawal times for freshwater rainbow trout treated with these drugs were not linked to degree days and for OTC were 90 days below 6°C, 70 days between 6 and 12°C and 60 days above 12°C; and for OA, 20 days at approximately 18°C (Jacobsen, 1989). Based on serum concentrations of OA post-treatment in Atlantic salmon (*Salmo salar*), withdrawal periods of 38 days at 4-6°C and 31 days at 13-15°C were necessary to ensure that the drug levels would remain below 1 ng/ml serum (Hustvedt et al., 1992).

It is apparent that elimination times of certain drugs differ for different tissues. Most available data apply to muscle residues, as it is the muscle which is directly consumed by humans. However the CVMP monograph for the O.A provisional MRL indicates that the sample for residue analysis should contain muscle and skin in natural proportions. If skin and bone are retained in edible portions of flesh it is possible that leaching into the flesh may occur under certain circumstances. Further, there is growing interest in silaging diseased carcasses for processing into fishmeal. The fate of antibiotics during such processing is not known (Ellis, 1991). Both liver and kidney participate in the excretion of oxolinic acid, although OA elimination through the gills must also be considered. Dauble and Curtis (1989) showed that in rainbow trout branchial elimination was the primary route of excretion of quinoline, a nitrogen-heterocyclic compound with structure similar to Oxolinic acid. Quinaldine (2-methylquinoline) on the other hand is also eliminated in rainbow trout by diffusion through the gills. Neither Bjorklund et al. (1991) or Ueno (1988) studies have shown any identified peaks, which could be interpreted as OA metabolites in serum, bile or tissues of rainbow trout or amago salmon (*Oncorhynchus rhodrus*). However, in a study on yellowtail (*Seriola quinqueradiata*) two metabolites, a glucuronid conjugate and an unidentified compound were found in liver and bile after a single oral

administration of OA (Ueno et al., 1988). Ishida (1992b) reported three metabolites of OA in the bile of rainbow trout namely O.A glucuronide (O.A–G) and two other glucuronides and decomposition products (7-OHO.A-G and 6-OHO.A-G). Six to nine different metabolites, including conjugates of OA have been found in tissues and urine of rabbit, dog and man. Thus compared to mammals, fish seem to have limited capacity of metabolising oxolinic acid (Bjorklund et al., 1991). Approximately 50% of the OA at therapeutic levels is unbound and can freely interact with the secretory system in the nephrons.

In fish and mammals, tetracyclines are removed from the blood by the liver, concentrated and excreted by the biliary route and then reabsorbed in the stomach and the upper small intestine resulting in an enterohepatic circulation (Fanelli and Nigrelli, 1963; Cravedi et al., 1987). Therefore, this class of drug may persist for some time after the cessation of therapy (Huber, 1986). The persistence of OTC may be related to liver function or to the physiological condition of the fish. In case of severe infection, due to damage of the bileducts in man the excretion rate of oxytetracycline may be significantly reduced resulting in the accumulation of toxic concentration (Dowling and Lepper, 1964; Weinstein, 1970). Other factors, such as water temperature also influence antibiotic excretion rates (Herman et al., 1969). OTC i.m. Injection monitoring revealed that the drug was accumulating in pronephros, bone tissue and scales (Grondel et al., 1987). Jacobsen (1989) and Kasuga et al. (1984) indicated that OTC is quickly eliminated from muscle and gutted carcasses of rainbow trout and ayu.

Species difference and salinity as high as 24‰ do not play an important role in the absorption and elimination of OTC by the salmonids. Moreover, freshwater trout may be used as a model salmonid to study OTC pharmacokinetics in seawater salmon and vice versa (Abedini et al., 1998; Namdari et al., 1999). Other factors affecting elimination rates include

maturity and health status. For example, the elimination of OTC in fasting Atlantic salmon broodstock was far slower than in growers and furthermore, elimination was also prolonged in fish suffering hepatic lesions associated with a past furunculosis outbreak (Bruno, 1989).

OA was retained at higher concentrations and for longer periods in freshwater than seawater Coho salmon (Ishida et al., 1990) and sea trout (Ishida, 1992b) and OA excretion in seawater Coho salmon was similar to that in other seawater fish like Japanese mackerel, red sea bream, yellowtail and flounder (Ishida et al., 1990). This difference in regulation was considered to be the most probable function responsible for the difference in the excretory rate despite the similarity in the metabolic pathway of OA between freshwater and seawater fish (Ishida, 1992a). Digestibility as well as excretion could be influenced not only by the feeding rate but also by the drinking of fish in different environments. Drinking in Atlantic salmon commenced after abrupt transfer from fresh water to seawater. In unfed fish the rate steadily increased 4 days post-transfer, apparently in response to increasing plasma ion concentrations. Drinking rate was independent of gut food content but was significantly higher in feeding fish than in unfed fish (Usher et al., 1988). On the other hand urine flow in salmonids is dramatically reduced in seawater compared to freshwater (Hunn, 1992).

4.4 Antibiotic Resistance

Resistance is always a relative term. A strain can be classified as resistant to a specific antibacterial agent if it is able to function, survive and persist in a concentration of antibacterial higher than the members of its parental population. A species on the other hand can more loosely be classified as resistant when its members can function, survive and persist in higher concentrations of an antibacterial agent than the members of other species (Smith, 1995). Bryan (1989) suggested that there are two forms of antimicrobial resistance: positive function resistance and bacterial persistence. Positive function resistance is the form where a microbe gains the capability to resist one or more antimicrobial agents by the acquisition of a gene function. Usually this results from the transfer of plasmid DNA into the bacterial strain. The gene functions are for the most part concerned with inactivation or modification of the antimicrobial agent, the addition of an efflux system or the specification of a new target or the enzymatic modification of the target. In each of these circumstances there is relatively little disadvantage to the cell in possessing these additional gene functions. Thus there is a tendency for these mechanisms to be relatively stable in a strain and for bacterial populations derived from these to contain predominantly resistant organisms. Humans for a substantial period of time and from individual, institutional and community reservoirs may carry the resistant strain. Control can be achieved in two major ways, either by infection control or by the development of new agents or inhibitors, which are insensitive to or overcome the resistance mechanism. It is also possible that control could be achieved in the long term by restriction of a particular agent, as the strains are not likely to be perfectly stable. This is the main mechanism by which bacterial pathogens acquire resistance to Oxytetracycline. In relation to courses of antimicrobial therapy positive function resistance is unlikely to appear during therapy unless acquired by cross-infection during a course of treatment or more rarely by

selection of a minority population of the pathogenic strain. It is likely that this form of resistance will be readily detected by susceptibility testing because of the relatively high levels of resistance normally specified and the high frequency of resistance in the particular bacterial population. Therefore, positive function resistance would usually be found at the onset of therapy. Tetracycline inhibits bacterial growth by interfering with protein synthesis at the level of ribosome. Plasmid-mediated resistance to antimicrobials has been identified in a number of bacterial fish pathogens, including *Aeromonas salmonicida, A. hydrophila, Vibrio anguillarum, Pseudomonas fluorescens, Pasteurella piscicida* and *Edwardsiella tarda* (Starliper et al., 1993; Klein et al., 1995; Aoki, 1988) and *Yersinia ruckerl* (DeGrandis and Stevenson, 1985). Transferable Rplasmids have been found in *A. salmonicida* in Japan and in Ireland (Aoki, 1997). Plasmid-mediated resistance to 4-quinolones has not been reported in fish pathogens, presumably because they appear to effectively inhibit the process of conjugative plasmid transfer (Nakamura et al., 1976), so that the spread of quinolone resistance by gene transfer is unlikely. In the case of *A. salmonicida,* laboratory studies have shown that resistance to oxolinic acid can readily be selected in the presence of the antibacterial (Tsoumas et al., 1989). The study was carried out with *Y. ruckeri,* selecting for resistance against oxolinic acid, oxytetracycline and potentiated sulphonamide. Stamm (1989) reported a similar study with the new fluorinated quinolone, sarafloxacin, which was being developed specifically for aquaculture applications. There are several studies that reported R-plasmid presence in freshwater fish pathogens (Aoki and kitao, 1981; Toranzo et al., 1984; Hedges et al., 1985; Brazil et al., 1986; Aoki et al., 1988; De Paola et al., 1988; Inglis et al., 1991, 1993a). The majority of the literature refers to antibiotic resistance profiles of *Aeromonas salmonicida* and other important salmonid bacterial pathogens. Widespread resistance in *A. salmonicida* to approved antimicrobials and the capacity of this bacterium to become resistant in the fish culture environment was described by Starliper et al. (1998). Adams et al., 1998

using broth conjugation, transferred OTC resistance encoded by R-plasmids from Aeromonas salmonicida to Escherichia coli and to both environmental and clinical isolates of Aeromonas spp. Transfer of R-plasmids between different genera of fish bacteria was demonstrated with *Aeromonas salmonicida* and *Aeromonas sobria* showed donor capicity to *Yersinia ruckeri.* (Klein and Boehm, 1994).

The second form of resistance can be termed 'persistence' and the mechanisms responsible, mechanisms of persistence. In these circumstances persistence is the result of resistance from mutation or from temporary resistance due to gene regulatory events or phenotypic changes that would only become evident during exposure of a bacterial population to an antimicrobial agent. Thus persistence is only seen during courses of therapy. These changes allow a microbe to persist during therapy by giving some levels of antimicrobial resistance. However, they are frequently deleterious to the organism in some manner causing growth impairment to a variable degree and in some cases a temporary reduction in virulence. Mechanistically, these changes operate by reducing permeability or target affinity for a drug or by turning on chromosomally specified enzymes capable of degrading the drug. In contrast to positive function resistance these changes result in loss or reduction of gene function or a functional change that provides a metabolic load to the organism. Such forms of resistance are most likely to be effective where there is a local or general deficiency of host defences, where drug entry is poor or where the local environment is antagonistic to the action of the drug. Persistence may impair the organism but after removal of the antibiotic there are strong selective forces for the parental form to return or for loss of the regulatory or phenotypic changes because of better growth and virulence characteristics. The effect of persistence is to slow therapeutic response, establish colonization, or produce outright therapeutic failure. This is the mechanism by which bacterial pathogens acquire resistance to Oxolinic acid. *Flavobacterium psychrophilum* isolates showed resistance to oxolinic

acid in rainbow trout freshwater farms in Denmark (Dalsgaard and Madsen, 2000). Resistance to five fluorinated 4-quinolones against the fish pathogen *Aeromonas salmonicida* appeared to be fairly stable, with only a small decrease in activity after 10 successive passages of the test strains on drug-free tryptone soya agar (Barnes et al., 1990,1990a). Persistence mechanisms were illustrated after sequential subcultures of strains of *Yersinia ruckeri*. The MIC increased by 20-, 16- or 16-fold for oxolinic acid, oxytetracycline and the potentiated sulphonamide, respectively. Subsequent attempts to decrease this induced resistance to oxolinic acid were unsuccessful. However, it was possible to decrease the induced MICs for Oxytetracycline (6-fold) and the potentiated sulphonamide (4-fold), also in a stepwise fashion (Rodgers, 2001).

4.4.1 Current Antibiotic resistance survey

This study exhibited some unique characteristics. It is the first survey of antibiotic resistance among fish bacterial isolates in Greece and it included a large number (24) of farm /sources from all over Greece (9 distinct marine areas). It is among the very few in the literature to compare "Quantitative" antibiogram data with MIC values for the two most widely used antibiotics (Oxytetracycline and oxolinic acid) and to address these data statistically according to the clinical relevance of the bacterial isolates. Most MIC studies are focused in certain fish pathogens (see 4.4.3.4) but in this study all bacterial isolates from 5 fish species (sea bass, sea bream, *Mugil cephalus*, *dentex* and *Puntazzo puntazzo*) were screened for their antibiotic resistance profile and level.

The quantitative antibiogram evaluation of the bacterial isolates during this survey (1994-1997) was based on the condition that a zone of inhibition between 6-15mm refers to resistant isolates, 15-25mm zone to intermediate resistance and finally zone greater than25mm in diameter indicates susceptible strain. The relevance of

this condition was evaluated by the success of the antibiotic treatment and was proven to address the issue of clinical relevance. The quantitative antibiogram was performed in Mueller Hinton agar with 3% salt without the addition of Mg^{++} and Ca^{++}. The Standard practice of cation addition reduces the availability of antibiotic and the zones of inhibition. However, it was found in subsequent studies on sea bass and sea bream pathogens - not described in this report – that the effect of these cations is variable depending on the bacterial strain, therefore the induced variation reduces the extrapolation of clinically relevant results. The analysis of the resistance profiles (quantitative antibiogram) revealed the resistance of *V.anguillarum 1 phenon 2* to Oxolinic acid, Potentiated sulphonamides and Furazolidone while all cocci both Gram negative and positive were resistant only to Oxolinic acid. Oxolinic acid resistance was expected because at the period of the survey treatments have been switched from Oxytetracycline to the newer antibiotic available (Oxolinic acid) due to repeated resistance problems. It was encouraging however that the most common bacterium isolated *V.Anguillarum 1* (API 20E-3047525) was susceptible to most antibiotics. Statistical analysis revealed malpractices in certain farms (21) where only Oxytetracycline and Potentiated sulphonamide intermediate and resistant strains were isolated, while the only multiresistant strain in the study with resistance to 3 antibiotics was also isolated from this farm. In addition in four other farms (2,10,16 and 17) only Oxolinic acid resistant bacterial strains were isolated. Cross tabulation analysis revealed that 12 % of the strains were resistant to two antibiotics while 69% exhibited intermediate resistance or resistance to at least one antibiotic. A link of the resistance pattern between Oxolinic acid and Furazolidone was evident in 74% of the strains. It can be argued that in these type of studies even when the conditions in the media employed are identical several factors might interfere with and affect the validity of the data produced. The medium used for the quantitative antibiogram was Mueller Hinton a widely used and accepted medium. However as pointed out in the relevant workshop for the production of common standards on

antibiotic resistance monitoring (Alderman and Smith ,2001) and following more recent studies on the importance of Mg^{2+} and Ca^{2+} addition in the medium is now currently employed as the method of choice in all media used generating higher MIC values. Another potential argument is that the same conditions were applied for all fish pathogens and the same inoculum (adjusted by O.D) was employed in all quantitative antibiograms for both slow and fast growing bacteria giving a time benefit to the fast growing bacteria (e.g Vibrios) that could be able to maintain smaller zones against any antibiotic due to confluent growth. All these technical issues however are faced daily by most researchers and high variability of techniques used results in data which are not readily comparable. However these results stand as a clinically relevant report of the antibiotic resistance level in Greek Mariculture between 1994-1997.

The workshop on the new standards for antimicrobial resistance interpretation clearly recognised that the formulation and publication of new standard methods could not be taken as a criticism, implied or otherwise, of the many methods that have been developed by laboratories working in this area. It was accepted that many well-specified and useful methods are currently being employed in many laboratories. It is further accepted that in some of these laboratories, the use of these methods has, over time, allowed the specification of breakpoints relevant to the clinical interpretation of the data generated by the methods. Such interpretative schema was, however, method dependent and could not have general applicability. (Alderman and. Smith, 2001) An initial part of the resistance profiles from this study were presented for the first time in 1997 (Christofilogiannis et al., 1997).

4.4.2 MIC Studies

Antimicrobial sensitivity and resistance are relative terms and provide an interpretation of the clinical significance of concentrations of an antimicrobial, which inhibit the growth of an organism or kill it in laboratory systems (*in vitro*). The commonly used measure of such concentrations is the Minimal Inhibitory Concentration (MIC). This has been defined by the British Society of Antimicrobial Chemotherapy (Working Party, 1991) as the lowest concentration of antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.

MICs are determined according to guidelines for antimicrobial sensitivity testing and the basic information upon which this study is based. MIC determinations are carried out using agar methods or broth dilution methods, and under strictly controlled circumstances, using antimicrobial discs. In the disc method, discs impregnated with a known concentration of antimicrobial are placed in a lawn of bacteria grown on an agar plate, incubated overnight and examined for evidence of growth inhibition. This method is widely used in clinical laboratories, especially veterinary ones. When the thickness of the agar, its composition, the concentration of the inoculum, and the conditions of growth especially the time are all carefully controlled and specified, the results are reproducible. The size of the zone of inhibition of growth may be measured to provide the MIC of the organism under test by interpretation and previously prepared calibration curves obtained using known concentrations of antimicrobial and reference strains of organism. This type of MIC determination is frequently reported in the literature as the Kirby Bauer Method (Bauer et al., 1966) and is accurate when the conditions have been defined. In most literature cited here, veterinary diagnostic and other bacteriology laboratories have not used calibration curves and careful controls, but have applied a clinical isolate to the agar surface, made a visual assessment of the size of any zone of inhibition and

recorded the result as 'sensitive' or 'resistant'. In agar or broth dilution methods, antimicrobial of known potency is diluted in the medium according in a manner, which must be defined. It is common to use two-fold dilutions (doubling dilutions), but ten-fold dilutions or non-standard series of dilutions may be used. The antimicrobial containing media are then inoculated with a suspension of the bacteria under test, which should be of known concentration and are then incubated for a standard period of time. On solid media growth is detected by examination for colonies and in broth media by examination for visible growth. The presence or absence of growth may be quantified by the use of optical density readings. The potential sources of error in these methods have been discussed by the British Society of Antimicrobial Chemotherapy Working party (Working party, 1991). In many cases where these methods are used in clinical practice, proprietary kits in plate form are used and the presence or absence of growth in an individual well is recorded. In research studies, particularly of difficult organisms or new or uncommon antimicrobials, the basic technique used is clearly defined. It is clear from the above that, an MIC recorded in the literature may have been obtained in a number of different ways and that the accuracy of the MIC is important in assessing the quality *of* the data provided.

The British Society for Antimicrobial Chemotherapy recommendations for the point at which a Minimal Inhibitory concentration is sufficiently high to indicate resistance ("break-point") is critical to the objective evaluation of sensitivity and resistance of bacteria to antimicrobials. The use of different methods to establish MIC has been outlined. In many studies sensitivity or resistance are used in the text to describe results obtained. Where these are based on MICs and the formula specified above (or another comparable formula) they can be justified, but in many cases, particularly in clinical veterinary medicine, the MIC was never determined and only a visual assessment of size of the zone of inhibition around an antimicrobial disc has

been made. More recently the use of breakpoint concentration methods become more common in medicine and in vet medicine. Here the growth of a suspension of the organism under test is assessed on or in medium containing the breakpoint concentration of the antimicrobial under test. The media may be prepared in the laboratory concerned, but increasingly are supplied in kit form as multi well plates containing the appropriate concentration of antimicrobial.

4.4.2.1 MIC – Temperature effect

The most pronounced effect on the MIC studies for OTC and OA is the fact that the level of MIC increases with Incubation temperature increase for OTC while it decreases for OA. This means that at low water temperature OTC is more effective while OA becomes less efficacious. Studies confirmed the effect of incubation temperature on the in vitro activities of 4-quinolones and Oxytetracycline against *Aeromonas salmonicida subsp. salmonicida* (Barnes et al., 1990b), atypical A. *salmonicida, Vibrio salmonicida, Vibrio anguillarum*, and *Yersinia ruckeri* using a drug microdilution method. When tested against *A. salmonicida subsp. salmonicida*, all of the quinolones examined had MICs higher at 4°C than at 15°C. Similarly higher MICs were recorded for all of the quinolones except sarafloxacin at 4°C than at 15°C when the drugs were tested against *V. salmonicida*. In contrast to those of the quinolones, the MICs of oxytetracycline were lower at 4 °C than at 15 °C against all of the bacterial species tested (Martinsen et al., 1992). In contrast MICs for oxytetracycline against *V. anguillarum* and *V. ordalii* showed a two-fold increase as incubation temperature increased but this pattern was not confirmed seen for *A. salmonicida* (Giles et al., 1991). Higher MICs for OA at lower temperature could be explained by decreased drug diffusion into bacterial cells diminishing drug availability at the site of action and slower bacterial growth rate and lower susceptibility to DNA gyrase-inhibitory activity of quinolones at lower temperatures.

A hypothetical explanation refers to reduced quinolone affinity for the DNA gyrase complex at lower temperatures, inducing inefficient inhibition of DNA synthesis (Martinsen et al.,. 1992). Lower MICs of OTC were obtained at low temperature against all of the bacterial species tested which could indicate a response to decreased incubation temperatures but it could hardly be explained by membrane permeability variations. However, alterations in the energy-dependent active transport mechanisms for tetracyclines in bacterial membranes, or a temperature dependent mode of action at the bacterial ribosome, could be possible explanations (Martinsen et al., 1992). In fish, bioavailability and plasma drug levels of quinolones and oxytetracycline are reduced at low temperatures and their specific effects may, in turn, also be reduced. Regarding the quinolones, one might assume that the elevated MIC at low temperatures could further impair their clinical efficacy. On the other hand, the lower MICs of oxytetracycline could partly compensate for the reduced bioavailability at low temperatures, so that low water temperatures might less influence the outcome of oxytetracycline treatment. Whether these theoretical considerations are of clinical relevance remains to be clarified. Potential mechanisms of resistance have been investigated and the development of cross-resistance between oxytetracycline and oxolinic acid has been studied. In terms of MIC, the fluoroquinolones were highly active against oxolinic acid-sensitive strains of *A. salmonicida* and considerably more active than oxolinic acid against oxolinic acid-resistant strains. Oxolinic acid showed poor bactericidal activity against *A. salmonicida* but in contrast, the fluoroquinolones were rapidly bactericidal for the bacterium. The frequency of occurrence of mutations leading to resistance against the fluoroquinolones was lower than with oxolinic acid. Some oxytetracycline-resistant mutants of *A. salmonicida* showed significant levels of resistance to oxolinic acid (Barnes et al., 1990b).

4.4.2.2 Drug Comparative studies

Comparisons between Oxolinic acid and the new fluoroquinolones along with cross-resistance development between them and between OA and OTC are widely discussed in the literature. Oxolinic acid showed poor bactericidal activity against *A. salmonicida* while Flumequine and fluoroquinolones were more potent were rapidly bactericidal and also had much lower frequency of mutations (Barnes et al., 1990b; Barnes et al., 1991a,b 1992a; Martinsen, et al., 1992). On the contrary in other studies OA performance was better than those of the newest quinolones. OA was the agent that caused highest inhibitory effect against *Vibrio anguillarum O4*, and *Photobacterium damsela subsp. piscicida* while Tetracyclines displayed the strongest bacteriostatic and bactericidal effect against *Vibrio damsela* strains (Lopez et al., 1993). The problem of complete cross-resistance between quinolones has previously been demonstrated for various bacterial species. This was confirmed in a study for the four 4-quinolones examined, as tested against strains of *A. salmonicida* subsp. *salmonicida.* However, the cross-resisitance between the old 4-quinolones, such as oxolinic acid, and the new and more potent fluoroquinolones, such as enrofloxacin and sarafloxacin, might not be of clinical significance. Clinical resistance depends on the MIC for the bacterial strain together with the pharmacokinetic profile of the drug in the fish species treated. Consequently, strains showing decreased in vitro quinolone susceptibility might nevertheless be clinically susceptible to potent fluoroquinolones (Martinsen, et al., 1992). Some oxytetracycline-resistant mutants of *A. salmonicida* showed significant levels of resistance to oxolinic acid (Barnes, et al 1990b).

4.4.2.3 Methodology and interpretation

Methodology and interpretation difficulties constitute the third element strongly presented in the MIC related literature. Different laboratories have employed and are employing a wide variety of methods and according to evidence presented they are generating widely different results. As a direct result of the use of different methods to determine laboratory sensitivity, there were severe problems in comparing data generated by different laboratories. Possibly of greater significance is the difficulty that method variation presented for the development of appropriate breakpoints to facilitate the clinical interpretation of laboratory data on sensitivity (Alderman and Smith, 2001). Some of the difficulties in methodology and interpretation of antimicrobial sensitivity testing of fish pathogens have been discussed by Smith et al. (1994). It should be noted that as yet there is no agreed standard method for determining the MIC of fish pathogens to be used in different laboratories, so that interpreting the practical meaning of reported differences is difficult. Proposals to move towards selecting an agreed method have been made by the European Association of Fish Pathologists and by the Working Group on Diseases and Pathogens of Marine Organisms of the International Council for the Exploration of the Sea (ICES WGDPMO) (Alderman and Hastings, 1998). A workshop funded by the European Union as a Concerted Action by DG XIV under the FAIR programme-FAIR CT 97-3760 attempted to resolve this chaotic situation. The intention of the workshop was to bring together representatives of the major European Community laboratories (plus a limited number from outside Europe) working on aspects of the use of chemotherapeutants, particularly upon antibiotics and the problem of drug resistance in aquaculture. The primary theme of the Workshop was to enable these experts to re-examine these problems and, in particular, to discuss and gain agreement on recommendations for uniform methods for determining minimum inhibitory concentrations for the most important

antimicrobials against the most important fish pathogens. (Alderman and Smith, 2001). The Workshop recognised that the determination of sensitivity was composed of two separate but interrelated issues. The first was associated with the laboratory methods employed and the second was related to the meaning, in terms of clinical relevance, of any data generated by such methods (Alderman and Smith, 2001). The purpose of these methods was to function as 'reference methods'. They aimed to provide means of communication between laboratories, means of comparing the data generated by local methods and means with which, microbiological, pharmacokinetic and clinical data, relevant to the setting of breakpoints, could be exchanged between laboratories. Further technical issues refer to the means for selection of resistant isolates, the media and the method most adequate for the estimation of the level of bacterial resistance without interference, the media preparation and validation protocols, the inoculum size etc.

The reports in the relevant studies refer to three different schools of thought in terms of antibiotic level for resistant bacterial isolate selection: Initially the concentration can be set according the distribution in the levels of resistance in undisturbed microflora. Other scientists refer to a standard breakpoint concentration (OTC 2-4 µg/ml) at which a high percentage of the natural microflora in an undisturbed environment could grow. The third approach is to set the concentration at such level that it would select all cells containing R-plasmids. Datta et al., (1971) for example set OTC concentration for the selection of R-plasmids to a level of 10µg/ml.

The culture media for marine pathogens used in these studies usually incorporate seawater. In one study Samuelsen et al. (1992a) introduced TSCA (trypton soya citrate agar) - citrate counteracts the inhibitory effect of the 70% seawater also used in the medium. The reported level of reduction of OTC activity was about 80%. Tetracyclines have a number of potential metal binding sites. The ability of tetracyclines to form complexes with di- and trivalent cations has long been known.

This ability is strongly pronounced for hydrophilic tetracyclines (mainly tetracycline and oxytetracycline). Lypophilic tetracycline derivatives, such as doxycycline, complexes the presence of di- or trivalent cations has also been known to mediate the binding of tetracyclines to macromolecules (Lunestad and Goksoyr, 1990). Several studies exist on research for appropriate media for OTC MIC testing. Despite improvements with new media versus Mueller Hinton large strain-to-strain variation gave inconclusive results (Pursell et al., 1996).

Arguments are presented that the media currently being employed in studies of the frequency of resistance in the vicinity of marine fish farms are inappropriate. In order to maximize the number of colonies that can be enumerated from the marine environment any media must contain Mg^{++} and Ca^{++} ions in addition to NaCl (Baumann and Baumann, 1981). Seawater with a salinity of 35‰ contains 54 Mm Mg^{++} and 10 mM Ca^{++}. The concentration of Ca^{++}, Mg^{++} and Fe^{++} in seawater have been shown to significantly limit OTC biological activity in marine environment (Lunestad and Goksoyr, 1990). In studies of the frequency of resistance to oxytetracycline in sediments under marine fish farms Samuelsen et al. (1992a), employed a Tryptone Soya Agar (TSA) made up with 70% sea water and attempted to counteract the inhibitory effect of the divalent cations by incorporating citrate ions at 10g/l (TSC). Kerry et al. (1994; 1995) employed a medium (2216V), which incorporated only 6% seawater and made no compensation for the concentrations of divalent cations introduced. It was suggested that media specifically formulated for work with antimicrobial agents, such as Iso-Sensitest Agar (ISA) and Mueller Hinton Agar (MHA) would be more suitable. Seawater is routinely added to media used to enumerate bacteria from the marine environment. The ions present in seawater, particularly Mg++ and Ca++, inhibit the antimicrobial activity of oxolinic acid. A considerable underestimation of the level of OTC resistance may occur when media prepared without seawater are used. In sea water 95% of the OTC is bound by the

magnesium and calcium ions present in the water. (Lunestad and Goksoyr, 1990) Use of seawater in media for susceptibility testing of amoxycillin or potentiated sulphonamides is less important (Barnes et al., 1995). The Mg^{2+} content of seawater of salinity 35‰ can be in excess of 50 mM (Potts and Parry, 1964). Indeed, the results of recent research have shown that such concentrations of magnesium ions can have a marked effect on both the antibacterial activity and uptake by fish of various antimicrobials (Lunestad and Goksoyr, 1990). The problem of drug interaction with seawater prior to entry into the fish may be avoided to some degree by encapsulations of the antibiotic in feed pellets such as Aqualets (Apathekernes Laboratorium AS). However for the drug to be taken up from the fish's gut, it must be in the liquid phase, thus interaction with seawater ions at this stage would seem to be unavoidable. Beside the medium composition debate refers to media preparation & validation protocols. Valid breakpoint concentrations for the determination of the frequency of oxolinic acid resistant bacteria in marine environments should consider clinical, pharmacokinetic and microbiological data. It is indicated that the incorporation of 0.5µg/ml oxolinic acid in Tryptone Soya Agar (TSA) would provide a suitable breakpoint for selecting resistant strains of A. salmonicida. High MIC values should be interpreted carefully because for example Oxolinic acid is not active against anaerobes, non-fermenting gram-negative rods and gram-positive strains. Thus physicochemical factors oxygen tension and environmental quality support the growth of these microorganisms increase in the frequency of resistance. Species innately resistant include mesophilic Aeromonads, *Yersinia, V.parahaemolyticus*, gram-negative species and, yeasts. Yeasts represent 25% of the sediment microflora in freshwater and are usually resistant to OTC.

4.4.2.4 Specific Pathogen MIC studies

4.4.2.4.1 Aeromonas salmonicida MIC studies

A limited number of studies concerning the sensitivities of truly marine fish pathogens and, therefore, quantitative estimates of appropriate breakpoints have been made by reference to data on the sensitivity of *Aeromonas salmonicida*. In a study by Martinsen et al. (1992) it was suggested that therapeutic drug concentrations in plasma and tissue around 4 to 10 times the MIC are often insufficient if selection of resistant mutants is to be minimized. In another study resistant mutants of *Aeromonas salmonicida* developed at lower frequency to the fluoroquinolones than to oxolinic acid or oxytetracycline at 5, 10 and 20 times the MIC. However, mutants selected with one quinolone were cross-resistant to all the other quinolones tested (Barnes et al., 1991a,b). Four bacteria commonly associated with diseases of fish (*Aeromonas hydrophila complex*, *Aeromonas salmonicida*, *Flexibacter columnaris-like* organisms and *Pseudomonas fluorescens*) were evaluated in a serial dilution system for susceptibility to four antibiotics commonly used in the treatment of bacterial fish diseases (chloramphenicol, erythromycin, furpyrinol and oxytetracycline). Findings were expressed as minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC). MIC and MBC's for oxytetracycline were complicated by the presence of R-plasmids, but were less than 4 µg/ml for those without plasmids. Pseudomonas fluorescens was susceptible only to oxytetracycline (Nausbaum and Shotts, 1981).

4.4.2.4.2 Vibrio spp MIC studies

Ledo et al (1987) reported that M.I.C values for Oxolinic acid for different bacterial pathogens like *Vibrio anguillarum, V.ordalii, V.tubiashii, V.damsela, V.carchariae, Pasteurella piscicida, A.caviae, A.salmonicida, Pseudomonas fluorescens, Yersinia ruckerii* and *Edwardsiella tarda* ranged between 0.075 and 0.3 µg/ml while for OTC

it was around 1.56 µg/ml. Twelve times OTC concentration greater than the average MIC of 0,26 µg/ml cited in the literature for sensitive strains of *Vibrio spp.* was used in treatment of post larvae of shrimp *Penaeus monodon* with OTC encapsulated Artemia (Rogue et al., 1998).

4.4.2.4.3 Photobacterium – Pseudomonas spp. MIC studies

In a series of papers for the evaluation of drug sensetivity (MIC) of *Photobacterium damsela subsp piscicida* strains using the method of the Japan society of Chemotherapy isolated from cultured yellowtail between 1984-1985 and 1986-1988. MIC OA was shifted from 12.5 to 6.15 µg/ml, while MIC OTC was estimated at a level of 100 µg/ml during the first period (Kusuda et al., 1988; Kusuda et al., 1990). The MIC_{90} values of OA, oxytetracycline, against *P. Piscicida* were 2.66, 35.70, µg/ml, respectively (Takahashi and Endo,1987).

4.5 MIC Analysis

Drug resistant bacterial fish pathogens reported to cause problems in aquaculture (Watanabe et al., 1971; Toranzo et al., 1984; Aoki and Kitao, 1985; Takashima et al., 1985; Hjeitnes et al., 1987; Sorum et al., 1988; Torkildsen et al., 2000). Philips (1986) suggested that for bacterial pathogens that exhibit a widely separated bimodal distribution of MICs and when MICs of the sensitive strains are very low, predictions on the clinical outcome can be made with a high degree of reliability. Several studies referred to high number of strains of intermediate sensitivity in potentiated sulphadiazine (Tzoumas et al., 1989) and oxytetracycline (Schlotfeldt et al., 1985). Breakpoint recommendations were presented from several committees and societies like the National Committee for Clinical Laboratory Standards in a study based on human strain studies (Waltman and Shotts, 1986) as well as the Japanese Society of Chemotherapy (Aoki et al., 1981; 1985; Aoki and Kitao 1985;

Aoki, 1992). In addition studies like those by Tzoumas et al. (1989) where MIC pharmacokinetic and clinical data were considered and Inglis et al. (1993,1993b) where breakpoints were based on zone sizes are included in a spectrum of studies, which however are extremely difficult to compare. It is important to state that the medium, the method (96-well plate) and the inoculation procedure followed a MAFF protocol, initially tested during the current author's MSc studies (Christofilogiannis, 1992). It was proven that the general guidelines of the method were valid, so many of its characteristics were included in the proposed "reference method" (Alderman and Smith, 2001)

In this study an "epidemiological" way of analysis of MIC values to Oxolinic acid and Oxytetracycline was employed in order to correlate these data with the quantitative antibiogram data obtained from the same bacterial isolates. The statistical analysis of all bacterial isolates and the correlation between Quantitative Antibiogram data and M.I.C values for Oxolinic acid and Oxytetracycline was used in order to establish M.I.C breakpoints in relation to the categories obtained from the Quantitative Antibiogram. The condition in order to establish M.I.C breakpoints in this study was the following: For each of the categories described in the Quantitative Antibiogram, M.I.C breakpoint was considered the M.I.C concentration under which at least 75% of the bacterial isolates of this category belonged in. Employment of this condition made it possible to consider the following M.I.C breakpoints for Oxolinic acid and Oxytetracycline.(Table 61)

Table 61: MIC values for O.A and OTC vs quantitative antibiogram classification

Bacterial strain classification (Zone of inhibition)	O.A M.I.C breakpoints (µg/ml)	OTC M.I.C breakpoints (µg/ml)
Susceptible (6-15mm)	< 0.3	< 0.625
Intermediate (15-25mm)	0.15 - 1.25	0.15 - 2.5
Resistant (>25mm)	> 1.25	> 2.5

Strains of intermediate resistance are considered to belong to a "grey" area and regarding the extrapolation of % incidence the presence of strains of intermediate resistance with M.I.C that ranged from 0 to 1.25 (Oxolinic acid) was taken into consideration. In a further step similar analysis was performed for each species with sea bass and sea bream being the most important due to their economic importance and due to the number of bacterial isolates from these species. The aim was to deduct correlations between Antibiogram data and M.I.C values for each antibiotic and possibly for each fish species. The interpretation of the actual values seemed to be a more effective tool in order to estimate M.I.C breakpoints of clinical relevance. The Minimum inhibitory concentration which inhibited the growth of at least 80% of the bacterial isolates of this group (Tables 62,63) was defined as the M.I.C breakpoint for each antibiotic and each bacterial strain group (susceptible / of intermediate resistance / resistant).

Table 62: MIC values for O.A and OTC vs quantitative antibiogram

Classification in sea bass

Bacterial strain classification (Zone of inhibition)	O.A M.I.C breakpoints (µg/ml)	OTC M.I.C breakpoints (µg/ml)
Susceptible (6-15mm)	< 0.3	< 0.625
Intermediate (15-25mm)	0.3 - 0.625	0.625 - 5
Resistant (>25mm)	> 0.625	> 5

Table 63: MIC values for O.A and OTC vs quantitative antibiogram

Classification in sea bream

Bacterial strain classification (Zone of inhibition)	O.A M.I.C breakpoints (µg/ml)	OTC M.I.C breakpoints (µg/ml)
Susceptible (6-15mm)	< 0.625	< 0.625
Intermediate (15-25mm)	-	-
Resistant (>25mm)	> 0.625	> 0.625

The precision of these M.I.C breakpoint values for different species depends on the number of the bacterial isolates under consideration. The limited number of bacterial isolates from the minor species does not give scope for further elaboration on the results recorded. The clear definition of susceptible and resistant strains regarding sea bream bacterial isolates could be the result of either limited number of bacterial isolates and / or limited use of antimicrobials in sea bream farming. A hypothesis could be that increased use of antimicrobials leads to increase of the % incidence of bacterial isolates of intermediate resistance to the antimicrobials used. This type of analysis was not performed in other studies because in we have always to refer MIC values of a certain bacterial strain to a specific antibiotic. This was not mistaken but it was found that a "general" classification of this type for all isolates in a survey or even for the main species studied has important clinical relevance and could provide

a generic tool for the veterinarians in regional labs that need to interpret, by the results of an "in vitro" method the clinical importance of these findings.

The British Society for Actimicrobial Chemotherapy (1991) reported that the only clear property of breakpoint antibiotic concentrations ("breakpoint': A discriminating concentration which may be used in the interpretation of the results of many types of sensitivity testing method) was that they are largely arbitrary, based as they are on consensus decisions related to pragmatic considerations. They seek to achieve two objectives, which may be irreconcilable: therapeutic relevance and laboratory reproducibility. They are nonetheless perceived as necessary by most clinical microbiologists, regulatory authorities, and manufacturers of media and discs and antibiotic producers. In almost all contexts, the clinician expects and the laboratory provides information that designates organisms as sensitive, intermediate (moderately sensitive) or resistant. Sensitive implies that the infection is likely to respond and resistant that it is unlikely to respond, while intermediate implies that an intermediate or indeterminate response is likely, except perhaps in special, defined circumstances, such as when high doses can be used, or the antibiotic is concentrated at the site of infection.

The Working Group on Antimicrobial Chemotherapy (BSAC, 1991) suggested a certain formula for the calculation of MIC breakpoint values. This formula and the associated assumptions are presented in Figure 124.

BSAC Working Party 1991: MIC Breakpoint = (C_{max} * f * S) / t * e

C_{max} = "Peak" blood concentration of microbiologically active drug

f = Protein Binding factor

Protein Binding level	f value
< 70% binding	1
70-90% binding	0.5
>90% binding	0.2

t = Half Life Factor

Half - Life (T)	t value
< 1h	2
1-3h	1
>3h	0.5

e = Times that Serum concentration should be higher than MIC in Vitro

s = Shift factor for reproducibility

s=1	No shift
s<1	Downward shift
s>1	Upward shift

The results are rounded to the nearest MIC value in the doubling dilution series

Figure 124: BSAC MIC breakpoint calculation

A practical example of an application of the above formula in this study is attempted taking a worst-case scenario. The achieved OTC in serum of sea bass at low temperature (far lower than the achieved serum OTC at high temperature), protein binding of 70-90% due to the OTC chelating nature, half life of more than 3h taking into account several single injection studies and the assumption that serum concentration should be 4 times higher than MIC values (Martinsen et al., 1992). The rounded value of MIC breakpoint for Oxytetracycline was 1μg/ml (Figure 125).

MIC OTC Breakpoint - Example of BSAC formula application

Mean OTC serum concentration in sea bass (max) - 7nth Day - 2,78 μg/ml

BSAC MIC OTC Breakpoint (sea bass) = (2,28 * 0,5 * 1) / 0,5 * 4 = 0,695

that rounds up to 1μg/ml

Assumptions

f=0,5 (protein binding =70-90%)

t=0.5 (Half life >3h)

e=4 ((Martinsen, et al. 1992)

s=1 (No shift)

Figure 125: MIC OTC Breakpoint – BSAC formula application

Similar studies with references to MIC breakpoints for OTC and OA include the study by Tzoumas et al 1989 for *Aeromonas salmonicida* (Table 64).

Tzoumas et al 1989			
MIC Breakpoints Aeromonas salmonicida			
Antibiotic	Susceptible	Intermediate	Resistant
Oxolinic acid / Flumequine	0.0625	0.125-0.5	>1
Oxytetracycline	<1	2 to 4	>4
Potentiated Sulfonamides	<2	2 to 16	>16

Table 64: Aeromonas salmonicida MIC breakpoints

A similar analysis was performed in this study for *Vibrio anguillarum* that was proven as the most important bacterial isolate due to the high isolation frequency as well as due to the high clinical and economic importance. This approach had two steps. Initally the M.I.C values of 32 Vibrio anguillarum strains were plotted for OA and OTC (Table 64) and then an analysis of those MIC values was made with reference to their zones of inhibition in the quantitative antibiogram (Table 65).

Table 65: V.anguillarum MIC values for Oxytetracycline and Oxolinic acid

OXYTETRACYCLINE MIC - Vibrio anguillarum (32 strains)			
MIC VALUE	FREQUENCY	PERCENT	CUMMULATIVE PERCENT
0.075	4	12,5%	12,5%
0.15	3	9,4%	21,9%
0.3	11	34,4%	56,3%
0.6	9	28,1%	84,4%
1.25	4	12,5%	96,9%
160	1	3,1%	100,0%
	32	100,0%	

OXOLINIC ACID MIC - Vibrio anguillarum (32 strains)			
MIC VALUE	FREQUENCY	PERCENT	CUMMULATIVE PERCENT
0.075	18	56,3%	56,3%
0.15	6	18,8%	75,0%
0.3	3	9,4%	84,4%
0.6	1	3,1%	87,5%
1.25	3	9,4%	96,9%
80	1	3,1%	100,0%
	32	100,0%	

Table 65: V.anguillarum MIC values versus zones of inhibition in

Quantitative antibiogram studies

MIC record		Vibrio anguillarum (32 strains)	
Classification	Antibiogram zones of inhibition (mm)	MIC OA	MIC OTC
RESISTANT	6 to 15	160	80
INTERMEDIATE	15 to 25	0,625 - 1,25	0,625 - 1,25
SUSCEPTIBLE	>25	0,075 - 1,25	0,075 - 1,25

The oucome of the above analysis is that an MIC concentration of more than 1,25 µg/ml for both OA and OTC seems to inhibit growth of all but the resistant strains of *Vibrio anguillarum* while it is effective for more than 95% of the 32 bacterial strains (Table 66).

Table 66: *Vibrio anguillarum* MIC breakpoints for OA and OTC

MIC BREAKPOINT	OTC	OA
Vibrio anguillarum	>1,25	> 1,25
Effective for more than 95% of Vibrio anguillarum strains		

This is in accordance with the BSAC formula calculations for OTC mentioned above and regarding Oxolinic acid it compares to MIC breakpoint for *Aeromonas salmonicida* (Tzoumas et al., 1989). The difference is that in the above study the MIC OTC breakpoint for OTC for *Aeromonas salmonicida* (> 4μg/ml) was far higher than the MIC breakpoint calculated in this study for *Vibrio anguillarum.* The above data suggest a moderate level of antibiotic resistance to OA and OTC especially for the most important bacterial pathogen, *Vibrio anguillarum* for sea bass. This type of analysis partly explains why despite problems in tissue distribution, large fish variation and although low levels of OA and OTC are achieved in sea bass and sea bream culture, treatments with the above antimicrobial agents are clinically relevant and succesfull. As it is suggested in the conclusions achieved antibiotic levels mean nothing without the clinical interpretation. It is common however in the literature for scientists to draw conclusions of clinical relevance only from bibliographic data.

4.6 Consumer Safety

4.6.1 Antibiotic Residues

Since it is a well-established principle that residues of medicines given to livestock animals should not be found in food for human consumption, considerable efforts are made to prevent drug transfer in this way. In the case of OA, the drug is also rather thermostable and no reduction in antibacterial activity could be observed when an aqueous solution of the drug was boiled for 15 min to simulate conditions resembling normal treatment of fish (Samuelsen et al., 1992b).

This study was designed not to give ideal kinetics records of sea bream and sea bass that will probably never be realized under commercial conditions. The aim was to treat fish in as natural as possible enclosures (experiments in lab and in the fish farm) with antibiotic incorporated in the feed rather that oil top coated. The feeding was performed early in the morning and was topped up if needed with regular feed in the afternoon. The idea was to see the "real" effect in the treated population rather than the "ideal" effect in a single fish. Several studies include stomach catheters or even anal tube for enteric accumulation studies while other studies focused on iv, interartial application include branchial or dorsal aorta canullation for the applications of antibiotics. Usually from these studies ideal graphs arise with minimal standard deviation and clear mathematical description. The limitation of these studies is that they rarely end up presenting the same type of "compartment" model between them for the elimination and tissue distribution of each drug. Usually, each study suggests a different compartment model that statistically better describes their results. Although these studies carry a lot of information on metabolism of different drugs, methods of analysis and interpretation should ideally be harmonized and critical review should be made in order to understand the "real" effect of these studies.

The aim of this study was different. It was the first time in both sea bass and sea bream for Oxolinic acid and first time in sea bass and for the second time in sea bream for Oxytetracycline that the kinetics of these two antibiotics were studied in these species after oral 10 day treatment under "commercial" conditions. Three other studies exist in the literature for the above species. The first (Malvisi et al., 1996) referred to study of OTC oral treatment of sea bream (sea bass in this study gave no results due to sudden mortality) while the other two (Poher et al., 1997 and Rigos et al., 2002) refer to iv single application of OTC and OA respectively in sea bass. Only in the latest study Bioassay assessment was employed for OA residue analysis. No study had employed OTC Bioassay for residue studies in the two mentioned species. Another limitation was that both Malvisi et al., 1996 and Poher et al., 1997 referred to experiments at one prevailing temperature and only Rigos et al., 2002 employed two temperatures for experimentation.

The structure of this study included eight experiments for Oxolinic acid and Oxytetracycline kinetics in two species sea bass and sea bream at two water temperatures. It was performed in a period of almost three years between the NCMR aquarium and Kalloni fish farm. The tissues usually employed for analysis were muscle and liver for Oxolinic acid (initial experiments) and serum (bioassay) and skin were added in OTC experiments. Although according to the legislation skin should be in natural proportions with muscle for residue analysis, at the time of the experiments this was not very clear in the legislation and on the other hand separate analysis aimed to describe tissue reservoirs for OTC (another not tested tissue candidate: the vertebrae). Skin was also analysed (along with vertebrae) in the study by Malvisi et al., 1996. High standard deviation in the OTC concentration achieved under "commercial" conditions was confirmed in the study by Malvisi et al., 1996. This was a prevailing characteristic in all experiments in the present study for both OA and OTC. This deviation could be explained due to low palatability for OTC

but for OA protocol application inefficiencies or hierarchy formation within these populations could only explain this phenomenon. The confirmation however by another study and the repetition in all the current eight experiments (at higher or lower degree) probably indicates a phenomenon that exists in the farm environment with domination of a fraction of the population that develops high residue levels and other fish in the cage which concentrate minimal or very low antibiotic tissue levels. One has to consider that this phenomenon should be worst in the case of an outbreak where sick fish are usually inappetant. Treating a cage of fish has to be considered as a population treatment and sometimes from the environmentalist point of view is a water, sediment and wild fauna treatment. Most likely healthy or healthier fish are treated in a "pro-phylactic or meta-phylactic mode" rather than treating the sick fish that are expected to die during the outbreak. The dynamics of this analysis should be elaborated more in future studies so the vet who prescribes will understand better the implications of any antibiotic application.

4.6.1.1 Detection Methods and Limitations

The methods used for the determination of antibiotics and their metabolites in farmed fish are reviewed and the data presented in tabular form. They can be divided into two general types namely those that use microbiological assessment and those that use physiochemical techniques (McGill and Hardy, 1992). Microbiological methods have certain advantages in that they are relatively inexpensive, simple and can provide an assessment of total antimicrobial activity. Conversely, they lack specificity, are relatively insensitive, do not differentiate between natural antimicrobial substances and synthetic antibiotics that are present, are time consuming using present methods, often lack reproducibility and are only semi-quantitative. Physiochemical methods using chromatographic techniques

coupled with various detectors do not suffer from these disadvantages but unfortunately the equipment is complex and expensive, requires skilled operators and, except where it is known that only a limited range of antibiotics have been used, do not provide an assessment of total antimicrobial activity. Currently, HPLC is the most established technique for testing 4-quinolone and tetracycline residues in fish tissues and is capable of considerable sensitivity. A comprehensive review of HPLC methods and Limits of Detection (LDs) is given by McGill and Hardy (1992) where reported LDs range from 300ppb to as low as 0.5ppb. In contrast LDs using bioassays are generally far higher. Recommended withdrawal times have been based on the detection limit of the assay used, ie zero residues. Obviously this depends upon the sensitivity of the assay (Ellis 1991). In the beginning of the 90s the detection limit of the microbiological test in use in Norway is 0.10 µg/g the detection limits of the different HPLC methods available are 0.005 to 0.10 µg/g (Rogstad et al., 1989; Andresen and Rasmussen, 1990; Steffenak et al.,. 1991). A wide array of methods is used in the analysis of antibiotic residues in fish. Most of the techniques are, or in the case of the microbiological ones could be made, sufficiently sensitive and accurate for safety and legislative purposes. There is need to define standard methods and for this purpose more inter-laboratory calibration exercises are required to compare methods and select the best. A number of analytical problems remain. Perhaps the most important is the assessment of total residues and metabolites. This can only be done in an experimental situation be feeding radioactive drugs to the fish (McGill and Hardy, 1992).

McGill and Hardy, 1992 presented a summary of papers in which reports on the use of microbiological assays have been described. The assays rely to a large extent on the use of agar plates containing an appropriate growth medium and microoorganism. The most commonly used microorganism is *B. cereus* although not surprisingly some microorganisms are more sensitive to specific antibiotics, eg *B.*

subtilis appeared to be most suitable for the determination of sulphonamide drugs (Salte and Liestok 1983; Kanzaki et al., 1984). The tissue sample or extract is applied to wells cut or formed in the agar and is used to impregnate a small disk of filter paper which is then placed on the agar surface. After incubation of the agar plated for the appropriate time and temperature, usually 24h at 30°C, the diameter of the zone in which no growth occurs is measured. The amount of antibiotic present is then estimated from appropriate calibration curves (McGill and Hardy, 1992). The technique is relatively simple, it can integrate total antimicrobial activity, use the whole tissue sample and thus overcome problems associated with sample extraction and is inexpensive to perform, but it does suffer from a number of disadvantages. First of all, many of the methods used lack sensitivity. The detection limits quoted are >0.1 µg/g which may be greater than the maximum residue limit (MLR). Related problems are, the poor differentiation between these and naturally occurring inhibitors of microorganisms, such as the lysozymes, and the accuracy and precision of the methods (McGill and Hardy, 1992). The method is also time consuming in that the total analysis time is in excess of 24h. Many of these drawbacks could be overcome by the use of modern rapid methods now developed for the estimation of bacterial numbers and growth and indeed have been used to demonstrate the presence of bacterial inhibitors in milk (Easter and Gibson, 1989). Such methods however do not appear to be used for general assays of antibiotic residues (McGill and Hardy, 1992). The rapid and sensitive detection of drug residues in farmed fish is essential to meet legislated tolerance levels and to ensure consumer confidence. Several antibiotic diffusion tests were made available for other food markets. For example, a rapid and sensitive Brilliant Black reduction test for the detection of drug residues in farmed animals, flesh and products using *Bacillus steatotheromophilus* as the indicator organism was introduced (Lloyd and Van der Merwe, 1987). The test satisfied the need for speed, versatility and simple handling.

A study using *B. cereus* as the indicator strain in a conventional bioassay format and acid/methanol extraction of tissues (Salte, 1982) suggested a high degree of sensitivity to oxytetracycline (OTC). Kusser et al., 1990 described a modified bioassay for OTC from fish tissue samples, which combined the speed and versatility of the dye reduction assay (Lloyd and Van der Merwe, 1987) with the sensitivity of a *Bacillus cereus* MT 987 strain. To increase the economy and sensitivity of the test, a 96-well microtitre format was designed for the assay and Brilliant Black BN was used as colour indicator. Using this test, a detection limit of 0.005 µg OTC determined. Bioassay with brain-heart infusion (BHI) agar plates inoculated with the indicator organisms *A. hydrophila, Bacillus cereus Vibrio anguillarum,* and *Vibrio ordalii* in another study (Xu and Rogers, 1993). There are few references in the scientific literature to bioassays developed for the detection of 4-quinolone residues in fish tissues. Endo et al., (1973b) described a thin layer cup assay for OA residue detection in carp tissues using E.coli, *Vibrio anguillarum* was used by O'Grady et al., (1986) for OA residues in salmon serum and *Bacillus subtilis* (ATCC 6333) by Stoffregen et al. (1993) for enrofloxacin monitoring in salmon tissues. In another bioassay study, when OA was detected in the intestinal contents. Fish containing OA in faeces usually had high concentrations of the drug in liver, muscle and plasma. Considering the positive correlation with the HPLC method, the microbiological assay was considered valuable in the investigation of drug residues (Samuelsen et al., 1992b).

The Bioassay methods used in this study derived from MAFF (Barker 1994; Barker personal communication). The bioassay for OA detection employed *Yersinia ruckerii* (FDL 39/81) as indicator organism. This method did not employ colour growth indicator. The limit of detection was 250 ppb for muscle and 125 ppb for serum samples. The method was modified from Barker (1994) for application in sea bass and sea bream. On the other hand the bioassay method employed for OTC analysis

included *Bacilus cereus* as indicator organism and had detection limit of 250ppb in serum. Other Bacillus strains like *B.subtilis* and *B.stearothermophilus* were employed in the literature.

Regarding the High Performance Liquid Chromatography methods, a variety of extraction, concentration and analytical conditions have been used even for the same antibiotic and consequently the reported recoveries have varied quite markedly. Furthermore, in many of the reports recoveries for several species or tissue samples have been given.(McGill and Hardy, 1992) In nearly all instances the extractions of the residues have been carried out on the homogenised sample using polar solvents. In so doing most workers have chosen to ignore the possibility of bound residues being present whose extraction can not be mimicked by simply extracting added antibiotics to the tissue sample (McGill and Hardy, 1992). After extraction the samples were usually passed through a concentration and clean up procedure and for this purpose a number of different proprietary solid phase cartridges that have markedly different retention characteristics (particularly which fish extracts) have been used (McGill and Hardy, 1992). A variety of chromatographic methods have been used in final analysis of the extracts most commonly in association with UV or fluorescence detectors. Gas chromatographic analyses are not common in the determination of fish antibiotics even though various forms of this technique have been used in the analysis of sulphonamides (Siegret 1985, Simpson et al., 1985; Holtmannpotter and Their, 1982) and chloraphenicol (Holtmannspotter et al., 1982; Bories et al., 1983, Ginkel et al., 1990) in meat. There are a number of reports on the use of thin layer chromatography (TLC) see Table 60, but notwithstanding the simplicity of the technique it does not seem to be a favoured method even though very low detection levels have been quoted (McGill and Hardy, 1992). The HPLC conditions vary from one group of workers to the next and the quoted accuracy and sensitivities differ accordingly. In

contrast to the microbiological methods, the physicochemical methods are relatively complex and tend to require several analyses if complex mixtures are suspected as being present (McGill and Hardy, 1992). Describing the existing literature in time we could see a gradual shift from laborious and less sensitive methods to rapid more advanced methods. In this study fluorometric detection and ethyl acetate extraction were selected. Oxolinic acid detection method included liquid extraction (Sved et al., 1991), centrifugation, evaporation to dryness, reconstitution in aqueous phase and injection to fluorescent detector. It comprised a simple, robust, fast method of good relative sensitivity and OA recovery of 77%.

Andresen and Rasmussen, (1990) described an automated method by on-line dialysis and column switching in an HPLC system for residue analysis of oxolinic acid and flumequine in liver of Atlantic salmon *Salmo salar* The limit of detection was 4µg/kg for oxolinic acid and 7µg/kg for flumequine with fluorescence detection.

Steffenak et al. (1991) described a simple and rapid method for the simultaneous extraction and determination of residues of oxolinic acid (OA) and flumequine (FQ) in fish tissue, muscle and liver using acetonitrile and ammonia for the extraction. Recovery of oxolinic acid was 101-104% while the recovery of flumequine was 88-94%. The detection limits were 5 ng/g for oxolinic acid and 10 ng/g for flumequine.

Degroodt et al., 1994 described another method for residue analysis of oxolinic acid and flumequine in fish tissues by HPLC and fluorometric detection and ethyl acetate extraction. The quantification limit of the method was 2µg oxolinic acid and 5µg flumequine/kg fish tissue. Since 1994 when a research group (Hormazabal et al, 1994a, 1994b) described a similar HPLC detection method considerable time elapsed since the recent presentation of a simple and much more sensitive method for the simultaneous determination of flumequine and oxolinic acid in fish muscle with attached skin using NH_3 and acetone extraction., The limit of quantification was

30μg/kg for flumequine and 20μg/kg for oxolinic acid. The recoveries were 75-76% and 79-82% for flumequine and oxolinic acid, respectively (Hormazabal et al., 2001). The Oxytetracycline method (Tyrpenou, 1995) was more complicated than oxolinic acid. The difficulty starts from the hydrophilic OTC nature. Following liquid–tissue homogenation, liquid extraction with evaporation to dryness is not possible because OTC on the contrary of OA is concentrated in the aqueous phase. This makes the solid phase extraction also described in the literature (Malvisi et al., 1996;) time consuming, delicate and a necessary expensive step. OTC was eluted by methanol and after evaporation to dryness was re-constituted and injected in the UV detector. Rigos et al., 2002 at a more recent study employed the same method. In general serum residue analysis is far easier than tissue analysis due to the lack of a series of steps for the extraction procedure. The use of ethylenediaminetetraacetic acid (EDTA) as chelating agent was proven critical for the extraction of OTC in several studies.

HPLC method and modified extraction procedure was used to analyze residues of oxytetracycline in channel catfish *(Ictalurus punctatus*). Mean recovery rates in fish muscle were 92.5% for OTC over concentrations of 0.05-1.0 ppm (Du and Marshall et al., 1995). Burns et al., (1996) described a Liquid chromatography (LC) method for the detection of OTC residues in fish and crustacean tissues and fishery products using a reversed phase c-18 column with diode array detection and UV spectral confirmation. At the same time the Central Laboratory of the Directorate of Fisheries in Bergen Norway announced a method to detect the following antibacterial agents in samples of farmed fish: oxytetracycline, nitrofurazolidone, florfenicol, oxolinic acid, flumequine and sulfadiazine/trimethoprim. Segawa, 1995 developed a simple, rapid and sensitive method for determination of oxytetracycline (OTC) in the serum of three cultured marine fishes by use of HPLC. OTC was extracted with sodium oxalate-acetonitrile (4:1,v/v) and disodium

ethylenediaminetetraacetate. After precipitated serum protein, the supernatant was injected into ODS column. OTC and TC were separated with 10mM oxalic acid - acetonitrile (72:18). The average recoveries of OTC were more than 92%. Ueno and Aoki, 1995 described the development of a rapid HPLC assay for oxytetracycline in the serum and muscle of cultured eel with simple pre-treatment. The extraction of oxytetracycline with several solvents from fish muscle was examined. It is known that it is hard to extract antibiotics from animal tissues, and so the authors also examined the effect of ethylenediaminetetraacetic acid (EDTA) on recovery of the drug from tissue. As a result, 90% methanol containing 0.5% EDTA was used as an extracting solution in these experiments. The detection limit of the method was 0.05 µg/ml for serum and 0.1 µg /ml for the muscle sample. This HPLC method did not require time consuming and complex extraction and moreover, did not cause column clogging, peak broadening or variation of retention times throughout the analysis.

Table 67: Characteristics of HPLC methods for the detection of Oxolinic acid and Oxytetracycline

Antimicrobial agent	Tissue	Species	Extraction	Clean up procedure	Mobile phase	HPLC Detector	Limit of Detection	Recovery %	Refference
Oxolinic acid	tissue	fish	metaphosphoric acid-methanol Sep-Pak		phosphate buffer-acetonitrile	fluorescence detection ex 325 em 365	10ppb	83.7-88,7%	Horie M et al 1987
Oxolinic acid	serum muscle liver	rainbow trout	solid-phase extraction					83,6 - 99,7%	Bjorklund HV (1990)
Oxolinic acid	tissue	fish	acetone extraction alumina column clean up		tetrahydrofuran-acetonitrile -phosphoric acid-water		2ppb	80%	Nose N et al 1987
Oxolinic acid			n-hexane-ethyl acetate	amino-type prepaked cartridge	Acetronitrile-methanol- 0.01 M aqueous oxalic acid		50ppb	77,1-95,5%	Ikai Y et al 1989
Oxolinic acid Flumequine	liver	Atlantic salmon	Phosphate buffer	on - line dialysis column switching		fluorescence detection	4 ppb 7 ppb		Andersen AT & Rasmussen KE (1990)
Oxolinic acid Flumequine	plasma	Atlantic salmon	on - line clean up precolumn	column switching		fluorescence detection			Rasmusen et al 1989
Oxolinic acid Flumequine	plasma	Atlantic salmon	solid-phase extraction						Rasmusen et al 1989
Oxytetracycline		meat - fish	aqueous HCL amberlite XAD-2 column washed water & methanol	concentraton under vaccum		UV detection		82%	Onji Y et al 1984
Oxytetracycline	Artemia		acetonitrile-tetrahydrofuran -oxalic acid solution			UV detection 365	10ppb	97%	Touraki M et al 1995
Oxytetracycline	muscle	Bovine, porcine	Homogenise EDTA McIllaine buffer solid-phase extraction (Sep-Pak)	centrifugation precipitation trichloroacetic acid	phosphate citrate-acetonitrile	UV detection Diode array	10ppb		Walsh JR et al 1992
Oxytetracycline	tissue	fish	solid-phase extraction (Sep-Pak)	elution water-acetonitrile			5 ppb (muscle) 10ppb (liver)	89,30%	Rogstad et al 1988
Oxytetracycline	plasma	fish	precolumn			UV detection 350nm	2,5 ppb		Eivindvik K & Rasmussen KE 1989
Oxytetracycline	meat		metaphosphoric acid-methanol Sep-Pak elution methanol	redisolve distilled water-acetonitrile		UV photometer 268nm		75,1-92,1%	Hoshino Y et al 1984
Oxytetracycline	muscle	catfish	C18 column	Acetonitrile-methanol		UV detection Diode array 365nm		80,90%	Long AR et al 1990
Oxytetracycline	tissue	yellowtail rainbow trout red sea bream	metaphosphoric acid-methanol Sep-Pak	methanol elution evaporate to dryness re-dissolve in acetonitrile-water	Acetronitrile-monobasic sodium phosphate		50ppb	68,9 - 91,7%	Horie M et al 1985

4.6.1.2 Oxolinic acid kinetics

In order to draw the necessary conclusions for oxolinic acid metabolism and kinetics in sea bream, mean concentration achieved in the muscle and liver was compared in the two prevailing water temperatures. During the treatment period liver OA concentration at both temperatures was 4 to 5 times the concentration achieved in the muscle. Liver concentration exhibited "odd" peaks at both temperatures after the treatment period. Almost the same levels (slightly higher at low temperature) of OA concentration were achieved in the muscle at both temperatures. An earlier peak (on the 3rd day) was achieved as expected at high temperature, while it was delayed at low temperature (11th day). Consumer safe levels were achieved two days earlier at low temperature. Earlier but lower OA concentration was achieved in the liver on the 3rd day at high temperature, while higher concentration was achieved at the 10th day at low temperature. It is characteristic that at both temperatures liver concentration was reduced to consumer safe levels at the same time.

In sea bass, tissue comparisons of the OA achieved concentration at high (26°C) and low (18°C) temperature indicated that muscle concentration at high temperature was 2 to 4 times the concentration on the liver during the treatment period. Muscle OA concentration was increased gradually while liver concentration was rapid and decreased sharply after the cessation of the treatment. OA concentration in both tissues fell to consumer safe levels on the 13th day. OA concentration in sea bass at low temperature followed the same pattern for both tissues. Liver concentration was 2-3 times the muscle OA concentration until the 20th day of the experiment except on the 10th day when muscle and liver concentration were at the same level. Consumer safe levels were reached much earlier in muscle (afternoon of the 11th day) than in the liver (20th day).

Comparison of muscle OA concentration at both temperatures (26°C and 18°C) indicated that OA concentration was increased rapidly at low temperature and kept 2 to 3 times higher than the concentration achieved at high temperature. In the afternoon of the 11th day of the experiment muscle concentration was the same at both temperatures indicating that OA excretion rate was not related to water temperature. Liver concentration seemed to follow different patterns at high and low temperature. At high temperature slow to moderate increase lead to late peak of 700 ppb on the 10th day, while rapid increase lead to early peaks on the 1st and 7th day. In the afternoon of the 11th day liver concentration was at the same level for both temperatures but for the following days OA concentration at low temperature was kept much higher than at high water temperature.

Comparisons of OA kinetics in sea bass and sea bream muscle at high temperature indicated an earlier and higher peak for sea bream (3rd vs. 11th day) almost 3 times the concentration achieved in the sea bass. It is important however to note that in both species, despite water temperature differences, OA concentration in the muscle achieved fell simultaneously below consumer safe levels (on the 14th day). Comparison of the OA liver concentration achieved in both species at high temperatures indicated similar pattern of OA kinetics in the muscle with OA depletion, in both species, below consumer safe levels on the 12th day, earlier than in the muscle. Comparative study of OA kinetics at low temperature indicated an earlier (7th day vs10th day) but lower peak (450ppb vs. 750ppb) in the muscle of sea bass, but concentration followed almost the same dynamics after the 12th day. The study of OA liver concentration indicated the same picture with the muscle with earlier (7th vs. 10th day) and lower peak (1100ppb vs. 3000ppb) in sea bream and except for the reversal in OA concentration between 12th and 25th day (where sea bream OA concentration

was higher than sea bass liver concentration) OA depletion followed the same pattern after the 25th day.

Three points were clear after this type of analysis: a) the similarity of OA depletion dynamics in both species. b) The apparent inability to predict depletion dynamics using the degree-day concept. c) The ability of sea bream to assimilate and metabolise Oxolinic acid more efficiently than sea bass, obtaining much higher concentration in both tissues and at any water temperature. Higher concentration in the liver compared to muscle was confirmed in a study of Intorre et al (2000). The MIC value of 0,15μg/ml (Table 29a,b,c) was described for the susceptible strains *V.anguillarum* (80% of susceptible strains) in this study. The levels of OA in the liver and muscle of both species and at both temperatures from the 3rd day of the treatment up until the 1st day post treatment (Day 11) were above this level. The only difference was in the liver of sea bass at low temperature where OA concentration remained high up until the Day 20 (10th day post treatment). A limited number of studies have been performed on the kinetics of antibiotics in sea bream and in fact there is no literature on Oxolinic acid kinetics in bream. One study described the pharmacokinetics of amoxicillin (AMX) in sea bream of 90-160 g kept in tanks at 22°C, after in-feed (80 mg/kg b.w.) or intravenous (i.v.) (40mg/kg b.w.) administration. Serum levels were collected at close intervals and AMX detection was carried out using both a microbiological (*Bacillus stearothermophilus*) and radioisotopic (Charm II test for beta-lactams) method. AMX serum concentration following i.v. administration decreased slowly between the first 8-10 h, and the antibiotic was still detectable at 72 h. After in-feed medication very low concentrations of AMX were found in serum falling below detection limit 25 h after treatment (Della Rocca et al., 1998). The kinetics of oxolinic acid in sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) were presented in three previous reports (Christofilogiannis, et al.,

1995;1997b; 1997c). At the same time Poher et al., 1997 published the only other available OA kinetics study in sea bass. The fish in that study were kept in seawater at 15.2°C with a 12h / 12 h photoperiod. Oxolinic acid was injected once in the caudal vein of anaesthetized sea bass. Plasma concentrations of oxolinic acid were determined using two analytical methods, a plate diffusion bioassay using *Escherichia coli* and a high performance liquid chromatography (HPLC) using solid phase extraction and U.V. detection. This report emphasized that the distribution process of oxolinic acid is faster in trout and that the elimination phase is shorter in seawater than in freshwater fish (Poher et al., 1997). Taking into account data previously published (Tsoumas et al., 1989; Lewin & Hastings, 1990; Martinsen et al., 1992), concerning the minimum inhibitory concentration (MIC) of oxolinic acid *in vitro* for many susceptible strains (*Aeromonas salmonicida, Vibrio anguilarum., Vibrio salmonicida* and *Yersinia ruckeri*), ranged from 0.005-0.5 μg/mL (for most strains, < 0.1 μg/mL) Poher et al., 1997 indicated that OA bolus injection of 10 mg/kg in sea-bass kept in seawater at 15°C should maintain the concentration above this MIC value for 2 days or more than 4 days. Moreover, the high partition of oxolinic acid in the octanol phase (Bjorklund and Bylund, 1991) explains the large distribution in skin and muscle, which are only slightly vascularised in fish (Ellis et al., 1989), and where abscesses caused by bacteria may occur. Nevertheless, oxolinic acid is usually administered orally. Therefore it was indicated that for OA large-scale therapeutic application, further studies with oral application were needed in order to determine the species-dependent bioavailability of this drug (Poher et al., 1997). This study attempted to elaborate on these issues. Oral medication of sea bass larvae through the use of *Artemia* nauplii as a carrier vehicle appeared to be a promising approach to be used as an alternative method of treatment (Touraki et al., 1999).

Oxytetracycline Kinetics

The OTC residue depletion from fish tissues of OTC following a multi-day oral medication have been studied for a number of farmed species such as in Atlantic salmon (*Salmo salar*) (Namdari et al., 1998; Pye-MacSwain, et al., 1994), in chinook salmon (*Oncorhynchus tshawytscha*) (Namdari et al., 1996; 1998), in coho salmon (*O. kisutch*) (Namdari et al., 1996; Meinertz et al., 2001), in rainbow trout (*O. mykiss*) (Herman et al., 1969; Jacobsen, 1989; Mirzargar et al, 2000), in striped bass (Xu and Rogers, 1994), and in channel catfish (*Ictalurus punctatus*) in Arctic charr (Salvelinus alpinus L.) (Haug and Hals, 2000), Hybrid striped bass (Xu and Rodgers, 1993), Tench (*Tinca tinca* L) (Reja et al., 1996), eel (Meitong et al., 1997) and in red tilapia (Choo, 1997). For Mediterranean mariculture, only a single study exists on the depletion of OTC in sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) following oral administration (Malvisi et al., 1996). However that study managed to produce data only for sea bream due to sudden mortality of sea bass. In the study by Malvisi et al, 1996 sea bream in cages were fed a commercial medicated diet at a rate of 75 mg./kg b.w/day for 14 days. Muscle, liver, vertebrae, and skin with scales were sampled at different intervals during (2^{nd}, 4^{th}, 6^{th}, 10^{th}, and 14^{th} day) and after treatment (10^{th}, 20^{th}, 30^{th}, 40^{th}, 50^{th} and 60^{th} day). OTC analyses were carried out by HPLC, after SPE extraction. As in this study and most of the oral treatment studies great variation in drug intake was evident from the inter-subject differences in OTC tissue considerations. Individual differences in feed uptake and metabolism of OTC among catfish were considered to contribute to variability in residue (Rawles et al, 1997). The highest recorded considerations were in skin and pooled liver (7.70±6.71 µg/g and 14.65 µg/g at the 6^{th} day, respectively). Vertebrae showed concentrations even six fold higher than those in muscle, and reached steady state concentrations at the 40^{th} day after the end of treatment (1.73±0.92µg/g), lasting until the end of the study. OTC concentrations in muscle were lower than in all the other tissues throughout the entire experiment and declined under 0.1 µg/g 20 days after treatment

ceased (Malvisi et al., 1996). Therefore, the aim of the present work was to provide additional data in order to optimise future therapies as well as to investigate the effect of water temperature on the kinetics of the drug.

In order to draw the necessary conclusions for oxytetracycline metabolism and kinetics in sea bream, mean concentration achieved in the muscle and liver was compared in this study at two prevailing water temperatures. The tissue distribution and residue depletion of OTC in sea bream (*Sparus aurata*) following a 10 day oral medication (75 mg / kg fish / day) at 24 and 18^{0}C water temperature was investigated. Liver and muscle samples were taken at different time points and analysed for OTC levels under HPLC while serum OTC levels were determined with a bioassay. Skin OTC concentrations were measured only at the low temperature. The depletion of OTC was found to be temperature-dependent. Liver, skin and muscle OTC levels displayed biphasic elimination while serum OTC levels revealed a single peak. The OTC concentration in skin at 18^{0}C was calculated to be higher than the other tissues while liver OTC levels were higher than corresponding muscle levels at all time points. At the end of the treatment period tissue OTC concentration decreased in the order of skin > liver > serum > muscle. At 24^{0}C, the highest OTC levels were measured at day 5 for liver (3.089 ppm) and serum (0.98 ppm) and at day 11 for muscle (0.121 ppm), while at 18^{0}C, maximum OTC levels in liver, skin, muscle and serum were found at days 5 (1.849 ppm), 11 (2 ppm), 3 (0.439 ppm) and 18 (1.438 ppm) respectively.

The results of the present work show that the extraction method detecting OTC levels reached 70%, which is within the range reported in previous studies (60-91%) (Bjorkund, 1988; Jacobsen, 1989; Luzzana et al., 1994; Malvisi et al., 1996; Namdari et al., 1996; 1998). The present study also demonstrates that the depletion of orally incorporated OTC from tissues of sea bream is temperature dependent. Several factors that the kinetics of a drug depends upon such as gastric emptying time,

absorption, biotransformation and excretion are temperature-dependent in ectotherm animals (Bjorkund and Bylund, 1990).

The elimination of the drug in sea bream appeared to be faster in muscle where OTC levels reached consumer safe levels on the 12th day (below 100 ppb). Although the drug concentration in muscle at the corresponding time point was also below MRL at the low temperature, detectable OTC levels were evident even at day 40. A markedly temperature-dependent elimination of OTC was reported by Bjorklund and Bylund (1990) in rainbow trout following a single oral dose. This finding is also in accordance with the studies of Namdari et al. 1996, 1998 and Jacobsen 1989 where multiple day treatments were carried out in chinook salmon and rainbow trout respectively.

The effect of increased water temperature on the drug depletion was not apparent in liver tissues where OTC levels remained above MRL (300 ppm) until day 14 at the high temperature, depleting to consumer safe levels on the 17th day. On the contrary, at the low temperature the liver OTC concentrations reached consumer safe levels following the first depletion day. It is likely that an increased appetite and thus a more efficient feeding along with a better distribution and absorption during higher water temperatures may have contributed to this discrepancy. The evaluation of measurements during and post-treatments indicate a biphasic elimination of the drug in all tested tissues and at both water temperatures, with the exception of serum OTC levels. This does not agree with a study where OTC depletion in fish tissues revealed a single peak (Namdari et al., 1996; 1998;). However, Malvisi et al. (1996,) and Meinertz et al., (2001) reported a biphasic decrease of OTC in tissues of sea bream when treatment ceased. Although feeding was careful, the creation of hierarchies was not possible to be excluded. It is assumed that feeding response in sea bream was problematic with a few fish being higher in hierarchy and dominating in each tank consuming most of the feed. This was also stressed in previous investigations where

variation in drug intake was evident due to the inter-subject differences in drug levels (Malvisi et al., 1996; Meinertz et al., 2001).

Distribution and absorption of OTC in sea bream serum and liver seemed to be faster and higher at the high temperature since OTC concentration was higher than the corresponding levels at almost all time points during treatment. In comparison, muscle OTC levels at the high temperature show the effect of increased excretory rates since the drug concentration at all comparable samplings was higher at the low temperature. Skin OTC concentration was measured only at 18^0C revealing the highest concentration among all tissues and its depletion profile showed biphasic elimination. At this temperature, at the end of the treatment period tissue OTC concentration decreased in the order of skin > liver > serum > muscle. The same profile was evident at 24^0C, where no skin samples were measured. Previously in the same species, after completion of treatment, OTC levels followed the order of vertebrae > skin > muscle (Malvisi et al., 1996). In salmonids, OTC concentrations decreased in the order of liver > bone > kidney = skin > muscle (Namdari et al., 1996) or liver > kidney > skin > muscle (Namdari et al., 1998) at the end of the treatment.

The lowest drug levels were measured in the muscle of sea bream. Previous studies have also demonstrate that muscle retain the lowest OTC residues among other fish tissues (Jacobsen, 1989; Malvisi et al., 1996; Namdari et al., 1996; 1998). Liver levels were measured to be many folds higher than muscle, which is in agreement with the aforementioned studies. However when skin samples were included in the analysis, measurements revealed even higher concentration than liver, which is not in accordance with published literature. Due to the fact that a low number of skin samples were analysed, results could be considered provisional. No comparable studies exist for OTC serum levels. In conclusion, the residue depletion of OTC from tissues of sea bream showed a different profile than other farmed fish. A biphasic

elimination dominated the depletion of the drug that in some instances was temperature-dependent. At both water temperatures, the OTC concentration in important tissues fell quickly to consumer safe levels almost when medication ceased. This indicates that common treatments under recommended OTC levels require short withdraw times in sea bream and the market product become safe very fast.

In sea bass OTC experiments were performed at (26°C) and low (18°C) temperature. Muscle concentration at high temperature was 2 to 4 times the concentration on the liver during the treatment period. Muscle OTC concentration was increased gradually while liver concentration was rapid and decreased sharply after the cessation of the treatment. OTC concentration in both tissues fell to consumer safe levels on the 13th day. OTC concentration in sea bass at low temperature followed the same pattern for both tissues. Except the 10th day where muscle and liver concentration were at the same levels up until the 20th day of the experiment liver concentration was 2-3 times the muscle OTC concentration. Consumer safe levels were reached much earlier in muscle (afternoon of the 11th day) than in the liver (20th day). Comparison of muscle OTC concentration at both temperatures (26°C and 18°C) indicated the following: OTC concentration was increased rapidly at low temperature and kept 2 to 3 times higher than the concentration achieved at high temperature. In the afternoon of the 11th day of the experiment muscle concentration was the same at both temperatures indicating that OTC excretion rate was not related to water temperature. Liver concentration seemed to follow different patterns at high and low temperature. At high temperature slow to moderate increase lead to late peak of 700 ppb on the 10th day, while rapid increase lead to early peaks on the 1st and 7th day. In the afternoon of the 11th day liver concentration was at the same level for both temperatures but for the following days OTC concentration at low temperature was kept much higher than at high water temperature. The only other study of OTC kinetics in sea bass following an

intravascular administration (40 mg/kg) at 13.5 and 22 °C water temperature was carried by Rigos et al., 2002. Serum, muscle and liver samples were taken at 1. 2. 4. 8. 16. 32. 64 and 128 h post-injection. Liver levels indicated higher OTC values than respective muscle levels at all time points and for both temperatures. The elimination of OTC from tissues tested was faster at the high temperature. Whereas the drug was eliminated faster from liver compared to muscle when comparisons are made at the same temperature (Rigos et al., 2002). Comparisons of OTC kinetics in sea bass and sea bream muscle at high temperature indicated an earlier and higher peak for sea bream (3^{rd} vs. 11^{th} day) almost 3 times the concentration achieved in the sea bass. It is important however to note that in both species, despite water temperature differences, OTC concentration in the muscle achieved fell simultaneously below consumer safe levels (on the 14^{th} day). Comparison of the OTC liver concentration achieved in both species at high temperatures indicated similar pattern of OTC kinetics in the muscle with OTC depletion, in both species, below consumer safe levels on the 12^{th} day, earlier than in the muscle. Comparative study of OTC kinetics at low temperature indicated an earlier (7^{th} day vs. 10^{th} day) but lower peak (450 ppb vs 750 ppb) in the muscle of sea bass, but concentration followed almost the same dynamics after the 12^{th} day. The study of OTC liver concentration indicated the same picture with the muscle with earlier (7^{th} vs. 10^{th} day) and lower peak (1100ppb vs. 3000ppb) in sea bream and except the reversal in OTC concentration between 12^{th} and 25^{th} day (where sea bream OA concentration was higher than sea bass liver concentration) OTC depletion followed the same pattern after the 25^{th} day.

Two points were clear after this type of analysis: the similarity of OTC depletion dynamics in both species and the apparent inability to predict depletion dynamics using the degree-day concept (Christofilogiannis, 1999).

Considering the OTC MIC breakpoint described in this study for susceptible *V.anguillarum* strains at 0.625 μg/ml (Table 29a,b,c) then analysis of the OTC levels achieved during oral treatment of sea bream and sea bass at high and low temperature reveals that OTC muscle concentration in both species and temperatures remained below this MIC value and this was also true of sea bass liver at low temperature. In general OTC liver concentration remained above the MIC from the first to the 10th day of treatment (except day 3) in sea bream at high temperature and between 3rd and 7th day of treatment of sea bream at low temperature. In sea bass at high temperature OTC liver concentration was higher than MIC between the 3rd and 14nth day of the experiment. An important element is that serum OTC level remained higher than MIC in all species and temperatures between the 3rd and 20th day of the experiment (exception sea bream at low temperature: 3rd –10th). Skin OTC level was analysed only in sea bream at low temperature indicating two distinct peaks at the 3rd and 11th day. A hypothesis can be drawn from these data about the OTC pool in skin and vertebrae that is mobilised post treatment keeping OTC levels in serum at levels above MIC levels of certain pathogens. This is contrary to what is happening in OA kinetics where due to lack of such a pool OA levels decline swiftly after cessation of the treatment.

4.6.2 Kinetics Conclusions

There are considerable differences in the anatomy and physiology between fish species that indicate that the processing of xenobiotics including drugs is not similar in these animals. Previous studies have provided evidence for species-dependent differences in drug kinetics or concentration (Grondel et al., 1989; Kleinow et al., 1994; Namdari et al, 1996) Therefore, it is of vital importance to investigate the depletion of the drug residues from edible tissues of the species of interest in order to re-establish withdrawal times and secure consumer health. Antibiotics are routinely used in aquaculture husbandry practices to control disease. In order to assure the consumer that farmed fish contain only reasonable quantities of residual antibiotics, regulatory agencies, producers and food associations specify or adopt limits for such xenobiotics. While many methods have been reported for the analysis of antibiotic residues in fish, most procedures lacked specificity and sensitivity and very few protocols have been reported for sea bass and sea bream residue analysis. Despite obvious limitations and experimental restrictions involving eight experiments performed for the study of the kinetics of two antimicrobial agents supplied at different dose rates, in two fish species kept at two areas of prevailing water temperatures, some basic comparisons were made in order to give a general idea.

Comparison and analysis of the kinetics of Oxolinic acid and Oxytetracycline in sea bass and sea bream at low temperature indicated some similarities in both species. In both species OTC concentration in the muscle and liver was lower than OA concentration and the elimination rate was much faster even at much lower temperature (sea bass). Both antimicrobial agents achieved much higher concentration in the liver. O.A and OTC demonstrated a two-wave elimination from the muscle of sea bass at low temperature. In both sea bass and sea bream OA

elimination rate was the same in muscle and liver. OTC elimination rate on the other hand was faster in the muscle than the liver in both fish species.

In sea bass kept at high water temperature OA achieved lower concentration and demonstrated faster elimination rate than OTC, probably due to higher temperature (26°C versus 24°C). In all cases liver concentration was higher than muscle concentration but for OA liver exhibited faster elimination rate than muscle. In sea bream probably due to moderate water temperature during OA kinetics experiment (24°C) some controversies were evident. OA muscle concentration was slightly higher than OTC concentration but the elimination rates from the muscle were similar. OTC elimination rate from the liver was much slower than the rate achieved by OA. Liver concentration was always higher than muscle concentration. It is however important to underline that the interpretation of any kinetics data achieved should be compared to MIC levels applicable to certain fish pathogens in the area where kinetics experiment were performed. It is almost irrelevant to compare achieved antibiotic levels in different tissues with MIC values from the literature due to differences in the characteristics of bacterial strains as well as on the methodology employed by different labs. It is important to expand the kinetics studies with modules where clinical relevance of these data should be tested. Otherwise data will be constantly generated, accepted on the context of statistical analysis or even mathematical modelling but they will probably have little population and efficacy relevance.

4.6.3 Fish – Human Bacterial Pathogen interactions / resistance transfer

There are 3 possible threats to human health from use of drugs in aquaculture: a) residues of drugs in fish destined for human consumption, b) development of drug resistance in human pathogenic bacteria and c) direct toxic effects on human from handling the drugs (Bernoth, 1991) Four ecological compartments may be considered as important for the transfer of resistance to antimicrobials; humans, animals, plants and soil-water. The common factors between the four ecological compartments are the antimicrobials, the bacteria, and the genes that code for resistance. Some resistance genes have been shown to move between bacteria in each compartment and it is possible for bacteria to move between the compartments. Bacterial gene transfer is now thought to occur not only in the human and animal intestine but throughout the biosphere, especially in nutrient-rich sites such as aquatic systems, sediments, soils, in the vicinity of plant roots, and in the sludge of the biological sewage treatment systems. Antimicrobial resistant bacteria have been isolated from all of these sites. Resistance may also be spread from plants and vegetables treated with antimicrobials or fertilised with wastes containing animal or human faecal residues. Thus resistance should be considered as a phenomenon of global genetic ecology. The crucial questions are whether resistance genes are transferable between environmental microorganisms and mammalian pathogens, and whether there are cascades of exchanges between related species or genera. The chain of resistance transfer is probably much more complicated and longer from plant pathogens to mammals than from animals to man. At present, no definitive antimicrobial resistance rates and predictive models are available. The Advisory Committee on the Microbiological Safety of Food in UK (2001) suggested that there are three potential routes of spread of antibiotic resistance from fish to human pathogens. The drinking

water pathways despite the barrier between temperate environment fish pathogens and human pathogens, dilution factors and complex formation / reduced antibiotic bioavailability/availability, refers only to antibiotics where transferable resistance occurs. In kitchen contamination the route involves all viable bacteria that could contaminate uncooked food and wound infection is the last route involving handling hazards for fish farm and fish processing unit personnel. There is considerable evidence to support the view that antimicrobial use in animals, both in the therapy of infections and as feed additives, is associated with an increasing prevalence of bacteria exhibiting resistance to the agents used. Many drugs used in animals can select for bacteria, which are resistant to antimicrobials used in man. An important question is to what extent the increasing prevalence of antimicrobial resistance in animals contributes to the increasing prevalence of resistance among human pathogens. Transmission to man of zoonotic agents such as *Salmonella* spp. and *Campylobacter* spp. is of particular importance in assessing this relationship. Humans will acquire both pathogenic and non-pathogenic antibiotic resistant organisms from animals. This can be partly controlled but not entirely prevented by good food hygiene. Zoonotic bacteria like salmonella therefore have to be controlled primarily during food production, according to the concept of pre-harvest pathogen control (WHO, 1983). Austin (1985b) in experiments with 4 fish farming sites found that during treatment with oxolinic acid, oxytetracycline, or a potential sulfonamide, the total count of bacteria in the effluent was not markedly altered, but the spectrum slightly sifted to Gram-positive species and resistances increased, returning to usual levels with 9 days after cessation of treatment (Bernoth, 1991). During a second OTC treatment in a microcosm study a significant increase in the number of sediment OTC resistant bacteria was observed highlighting the need for prudent use of antibiotics in aquaculture (Vaughan et al., 1996). Resistance in microflora has been reported in several studies (VanHouweling, 1971; Jacobsen and Berlind, 1988; Bjorklund et al.,

1990; Samuelsen et al., 1992a; Coyne et al.,., 1994; Kerry et al., 1994; 1995; 1996a; 1996b; 1997). The quantification of resistance to antimicrobial agents in the fish farm environment is based on the study of frequency of resistance in simulated environments (McPhearson et al., 1991; Nygaard et al., 1992; Samuelsen et al., 1992a,b; Hansen et al., 1992a; Spaanggaard et al., 1993; Ervik et al., 1994a; Kerry et al., 1994). While some researchers generally suggest that antibiotic use in fish farming may add to the environmental resistance pool (Spanggaard et al., 1993) one must remember that bacterial isolation using a limited range of culture methods and media, gives only a small percentage of the actual flora present and of any changes brought about by the antibiotic use or ecological changes induced in the fish farm environment (Smith et al., 1994). Problems arise in simulated environments because culture methods are fundamentally inefficient (Buck, 1979) supporting growth on any medium, in a fraction below 1% of the total viable count (Hoppe, 1976) or for other researchers around 0.3% (Buck, 1974). McPhearson et al., (1991) identified aquaculture ponds in south eastern US as potential reservoirs of antibiotic resistant bacteria. Regardless of whether resistance was mediated by exposure to antibiotics or by other mechanisms that pool of antibiotic resistance bacteria may have public health significance. Health risks and implications associated with plasmids that were detected in a strain of Salmonella, other members of the *Enterobacteriaceae* and several Pseudomonas species were discussed after the occurrence of these antibiotic-resistant human pathogenic bacteria in integrated fish farms in India (Twiddy and Reilly, 1995).

During OTC treatment periods, bacterial populations in sediment markedly declined but recovered once treatment once treatment had ceased. No increase in resistance to antibiotics was recorded among isolates screened during the first treatment period with OTC. However, during the second treatment period a significant increase in numbers of sediment bacteria resistant to OTC was observed, highlighting the need

for prudent use of antibiotics/chemotherapeutants in aquaculture (Barker and Alvarez, 1993). In a study of horizontal distribution of OTC residues and OTC resistant bacteria in the sediments under fish cages resistant bacteria were distributed over a wider area than the antibiotic. In addition mussels, microflora growing between the cages and the sediments have shown much greater frequencies of resistant bacteria than the sediments This implies that they are selected elsewhere and most probably in the fish intestine and reach the sediment by a different route than the antibiotic (Kerry et al., 1993). The study of the intestinal flora of marine fish has been limited (Sera and Isida, 1972; Silva et al., 1992) but in a very comprehensive research Guichard (2000) described the effect of medicated feed in the intestinal flora of turbot (*Psetta maxima*). Kerry et al. (1994) in a study of spatial distribution of elevated frequencies of oxytetracycline resistance at a farm noted that their distribution showed an independence of the dominant current flow. As argued by Smith et al. (1994) and Smith (1995) increases in resistance frequency may arise as a result of the increase in the frequency of innately resistant bacteria, bacteria with multiple non-specific resistances or bacteria with specific, possible plasmid encoded, resistances. Of these, only the latter will have any public health significance or any potential to transfer their resistance's to fish pathogens. In a previous study (Christofilogiannis, 1992) was possible to transfer R-plasmids in the fish farm environment of a trout farm between a multi-resistant environmental isolate to a susceptible isolate of *Aeromonas hydrophila* without the selection pressure of a concurrent antibiotic treatment. Another mechanism of resistance besides plasmid mediated and Chromosomally induced involves alterations in bacterial outer membrane proteins as an avoidance mechanism (Barnes et al., 1990b, 1992b). Austin and Austin (1987) proved that the intestine of wild fish acts as reservoir of *Yersinia ruckerii* and *Aeromonas salmonicida*. Simultaneous presence of OA and the fish pathogenic bacterium *A. salmonicida* in the gut of both cultured and wild fish was reported illustrating the potential of development

and dispersion of resistance (Samuelsen, 1992a). Although transfer of antibiotic resistance from a fish pathogen can take place in the environment of fish intestinal content it was not proved in vivo so the likelihood of this being a high-risk route for acquisition of resistance by bacteria that can affect humans is low (Del Rio-Rodriguez, et al, 1997). While some studies indicated that the gastrointestinal tract of the fish serves as a niche for the selection of resistant bacterial strains Spanggaard et al. (1993) others suggest that the intestinal microflora of goldfish (*Carassius auratus*) is not affected markedly by the oral administration of OA (Sugita et al., 1989). Supporting the findings of the last study Bjorklund et al. (1991) indicated that bacteria isolated from the fish farm sediment were OTC resistant while bacteria isolated from fish did not show the same degree of resistance. Controversial studies have linked human diseases with antibiotic resistant bacteria of animal origin. Potential human implication was examined because *Aeromonas hydrophila* and *Plesiomonas shigelloides* isolated from intestine of catfish as well as water and sediment and aquaculture ponds were potential human pathogens. Polyculture of fish with birds (ducks) and mammals (pigs) is rather common in Asia. Acquisition of plasmid-transferred resistance by bacterial pathogens of homeo therms is then of great concern (Michel, 1986), especially when, in these geographical regions, *Vibrio cholerae* may be widespread. This organism is a ready recipient of R-plasmids from aquatic bacteria (Arai et al., 1980) Levy et al., (1988) described in an extensive study, high prevalence of resistant bacteria in the gut flora of ambulatory and hospitalised individuals whether or not they were taking antibiotics. Under experimental conditions transfer of R-plasmids in the human intestine from animal strains to resident strains has been demonstrated (Smith, 1969). Epidemiological evidence of Shoemaker et al. (1992) also suggests that R-plasmids originating in animal strains can transfer to human strains and that such transfer may have occurred in human intestine. Levy (1986) demonstrated the transfer of a plasmid from chickens to two workers in a

chicken farm. In each case the plasmid was isolated on only one occasion and did not persist in the human host. In a more detailed study O'Brien et al. (1993) examined the restriction fragments of plasmids originating from strains causing urinary tract infections in women working in a poultry processing plant. These were compared with the fragments from plasmids isolated from the poultry microflora. They detected no evidence for the transfer of plasmids from the microflora of the chickens to the pathogens infecting the women. Walton (1992) suggested that with respect to intestinal microflora: "It is highly likely that the physiological barriers present in man, animal and birds prevent the successful colonisation by bacteria from an unrelated species whether the bacteria are antibiotic-resistant or not". The diversity of R-plasmids subsequently present in both animal and human isolates (O'Brien et al., 1993) present significant logistical problems in detecting such transfers in the natural environment. The use of antibiotics in veterinary medicine probably had an extremely low influence on the treatment of human disease either by the direct transfer of resistant pathogens from animals to humans or indirectly by the transfer of R plasmids from animal to human pathogens (Weidemann, 1993). Although the frequency of R-plasmids is elevated in the fish farm environment there are reasons to believe that such plasmids make only a very small and transient, contribution to the numbers of R-plasmids in human pathogens. The ecological differences between the aquatic environment and the human intestine decrease the possibility of resistant transfer between aquaculture isolates and human pathogens. Although there is evidence that antibiotic resistance can be selected for in normal therapeutic use in aquaculture, the risks of transfer of such resistance to human consumers by any of the possible routes appears to be low (Alderman and Hastings, 1998). Smith et al. (1994) in a comprehensive review suggested that there was no evidence of a continuing increase in the frequency of resistance in most human pathogens in the developed world. The level of resistance in these pathogens is primarily a function of the use of antimicrobial

agents by the medical profession. With the exception of the case of the Salmonella there is little compelling evidence that the use of antimicrobial agents in veterinary medicine has had an adverse effect on the therapy of human pathogens. Epidemiological and ecological considerations of pathogenic bacteria associated with fish suggest that resistant variants are unlikely to cause any impact on human pathology. In developed countries, the possibility that antibiotic resistant bacteria deriving from fish farm application of antimicrobial agents might reach the human consumer through the drinking water chain appears to be remote. Factors that need to be taken is to consideration include very high dilution factors and the fact that most fish pathogens present in temperate climates are not capable of affecting humans. In addition, antimicrobials such as oxolinic acid and oxytetracycline (although more available in fresh water than in salt water) are complexed by calcium and magnesium ions readily available in the aquatic environment and therefore are poorly bio-available. There is also a multiplicity of other anthropogenic sources of antibiotics in ordinary river water, including those derived from land run-off and, more especially, the presence of effluents from sewage plants that may be derived from human antibiotic usage. Against all of these factors and sources the comparative input from such farming antibiotic use must be recognized to be small. The treatment processes ending in chlorination routinely practised in treatment of drinking water should ensure that there are no viable microorganisms in that water when it leaves the water treatment plant. Thus, the likelihood of transfer of any transferable antibiotic resistance to the drinking water consumer from fish farming use should not be regarded as a significant possibility (Alderman and Hastings ,1998).

4.7 Environmental Impact

The environmental problems caused by fish farming are different in the different countries due to geographical, topographical, and physical conditions (Braaten, 1991). Environmental Impact Assessment is preclusive in order to lease the site and obtain permission for the operation of a fish farm, together with production management details and a description of the specific area in terms of physical, chemical and biological parameters of the water body (Papoutsoglou 1996; Papoutsoglou et al., 1996; Karakassis et al., 1998). Unfortunately all data needed are usually obtained from published sources, rather than from specific monitoring programmes at individual sites. Annual predicted production, stocking density/m^3 or production per water surface unit, require site-specific environmental data (Papoutsoglou, 1991). The criteria for land-based farms are covered by existing legislation, which requires an approved special water discharge study, which includes recommendations for the treatment of effluents before their discharge.

Superficial sediment layer beneath sea cages fluctuates between 2cm (February) to 5-6cm (July), suggesting a recovery procedure during the winter months when feeding rate is reduced the potential for inorganic fermation is increased, the wind generated currents that shake up the sediment surface. Loose top segment of the sediment consists of 95% water, it has increased chlorophyll due to phytobenthic organisms or due to the elevated phytoplankton production beneath the cages due to ammonia and phosphorus increase. However, most of the phosphorus is organically bound and not bioavailable, which is why the risk of phytoplankton blooms is less in the Mediterranean where phosphorus is the limiting factor. In comparison to studies of sediment recovery following different activities the sediment recovery in aquaculture activities is faster because the scale impact in space is confined, the precipitation material (fish feed, fish waste) is more bioavailable than all other urban or industrial

waste. A series of factors affect these bio-geo-chemical procedures (Karakassis et al., 1998). Karakassis et al., 1998 suggested the following measures for the reduction: a) avoidance of sites with muddy substance, b) avoidance of low degree of water exchange or high level of fluctuation of environmental parameters and c) selection of at least 500m minimum distances between farms in order to avoid transfer of diseases, parasites etc. (Figure 121). HPLC and bioassay methods have been utilised for the detection of the impact of the antimicrobials in the aquatic environment (Hansen et al., 1992a; Ervik et al., 1994a,b). Large fraction of antibacterial agents, typically in the form of in feed treatment is not absorbed and retained, but is released to the environment through any of three routes. First, some fraction of the medicated feed is not ingested and instead falls directly to the substratum. Estimates of this feed loss in salmonid net-cage culture range from 15% to 40% (Rosenthal et al., 1988), and are likely to be highest during a disease outbreak when the cultured fish tend to feed less. This proportion of uneaten medicated feed is 30% in another study (ICES, 1987,1987a,b). It is reasonable to assume that 20% of the feed administered to fish is not eaten and passes through the cage and will be deposited on the sediment in the vicinity of the cages. Sandaa et al. (1993) have suggested that during a disease outbreak this percentage may rise. A proportion of the antibacterial agent leach from the pellets, and a further proportion is excreted by the fish. OTC and OA are practically un-degradable in sediments, and 60-98% of these chemicals are not absorbed in the gut of the fish (Braaten, 1991). Fate of uneaten medicated feed depends on fish farm site characteristics. The nature of the environment may significantly alter the biological activity, and therefore the extent of the environmental impact, of a particular concentration of an antibiotic. The significance of a chemical in the environment is not a function of its presence but its impact on processes in that environment (Smith et al., 1993). In marine cage sites with fast currents and large tidal flows there will be greater dispersal of solid and particulate material than in sites with

poor flows. In addition the nature of the agent affects its fate in the marine environment. The area of sediments containing measurable oxytetracycline residues was very localised, under the cages and to a distance of 30 m, but absent 100 m from the site (Capone et al., 1996). At the end of OTC treatment elevated frequencies of resistance were detected up to 75 m from the edge of the cage block and ceased one-month later (Kerry et al., 1994).

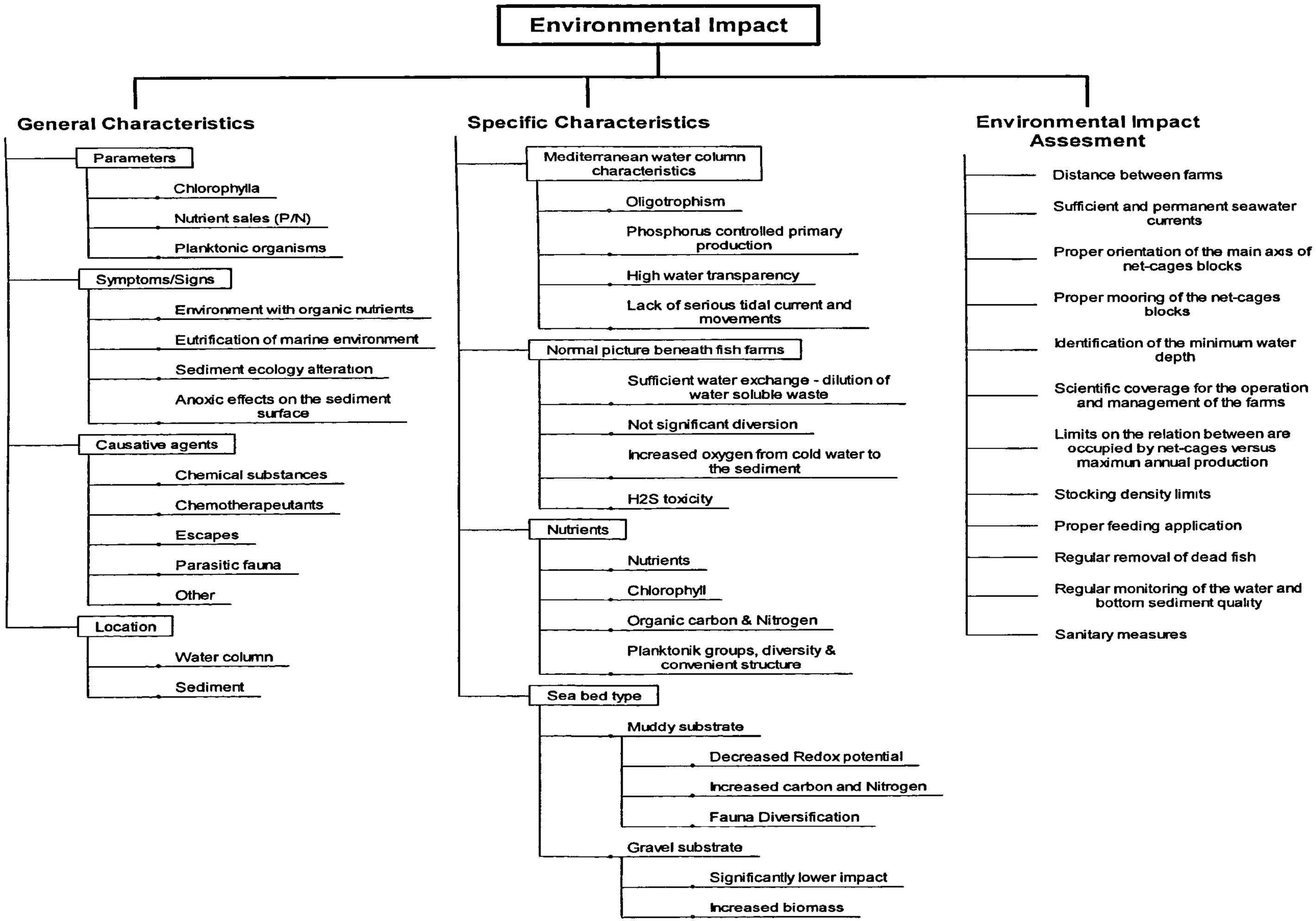
OTC is poorly absorbed especially in marine fish where the intestinal uptake is substantially reduced due to complex formation in seawater as compared to fish in fresh water (Smith, 1996). A major portion of the OTC administered to farmed salmonids inevitably ends up in the environment, especially in the sediments under aquaculture facilities. No mechanism is known for biodegradation of OTC and thus it can remain in the sediments long enough to affect the indigenous bacterial flora and induce resistance (Lunestad and Goksoyr, 1990; Smith and Samuelsen, 1996). Large differences exist in the literature on the level of sediment antibiotic residues following treatment in a cage farm. While in Norway, Samuelsen et al. (1992b) detected high OTC concentrations other workers in Ireland have reported considerably lower concentrations (Coyne et al., 1994). In addition OTC half-life differences exist in several studies ranging from 2 weeks (Coyne et al., 1994). to more than 1,5 years (Bjorklund et al.,1990) depending on the nature of the sediment. The nature of the sediments of farm ponds and beneath cage bottoms therefore seems to exert a major role in the persistence of antimicrobials in that environment (Hektoen et al., 1995; Alderman and Hastings,1998).

Aquaculture operations are often linked with increased densities of wild fish and invertebrates in the vicinity due to the shelter provided by the culture structure or enhanced food resources (Carss, 1990). Fish and invertebrates in the vicinity of the farm may also consume uneaten medicated feed pellets (Nausbaum and Shotts 1981;

Samuelsen et al., 1992a). Several studies on the impact of medicated diets on wild fauna around fish cages include studies in blue mussels *Mytilus edulis* (Moster, 1986; Ervik et al., 1994b; Coyne et al., 1997), sea trout Bjorklumd et al. (1990) coalfish, *Pollachius virens*, cod *Gadus morhua*, mackerel *Scomber scombus*, ballan wrasse *Labrus bergylta* and haddock *Melanogrammus aeglefinus* (Carss, 1990) oysters (*Crassostrea gigas)* (Capone et al., 1996), Dungeness crab (*Cancer magister)*and red rock crab *(Cancer productus)* and scallop (Capone et al 1996; Skjoelstrup et al., 2000). OA toxicity to the freshwater crustacean *Daphnia magna* indicates the potential to cause adverse effects on the aquatic environment (Wollenberger et al, 2000). A fraction of the antibacterial is not absorbed during gut passage and is released to the environment via the faeces. The fraction of antibacterial released through this route may be higher that 90% for oxytetracycline (Cravedi et al., 1987). Finally, some antibacterials such as oxytetracycline are excreted via the urine and bile fluid in an unmetabolized, microbially active form (Bjorklund and Bylund, 1990). Compared to OTC, OA reached significantly higher tissue levels in the farmed fish in spite of the fact that the OA doses administered were eight to eleven-fold lower than the OTC doses. This indicates that, compared to OTC, OA is much better absorbed and distributed in the fish when administered mixed in the feed (Bjorklund, 1991). Oxolinic acid and oxtetracycline, in particular, have been reported at many farms (e.g. Jacobsen and Berglind, 1988; Samuelsen, 1989; Bjorklund et al., 1990,1991; Hansen et al., 1992b; Samuelsen et al., 1992a; Coyne et al., 1994). These residues may be quite persistent under some conditions, with half-lives exceeding 100 days (Bjorklund et al., 1990; Samuelsen et al., 1992a). These residues could be of environmental significance if they affect the sedimentary microbial community (Samuelsen et al., 1988; Hansen et al., 1992a; Herwing et al., 1997; Herwing and Gray, 1997) and affect the viability of continued culture if they alter the rate of organic matter degradation or promote proliferation of antibacterial-resistant strains of bacterial pathogens. (Capone

et al, 1996) The presence of *Aeromonas salmonicida* and resistant strains in sediments beneath Norwegian fish farms illustrate the potential danger from persistent antibiotic concentration as selection factor in the sediment (Husevag and Lunestad, 1995). The area of sediments containing measurable oxytetracycline residues was very localized, however, detectable residues existed only under the cages and to a distance of 30 m, but were absent from a 100 m site (Smith et al., 1994).

Figure 126: Environmental Impact Considerations



The persistence of OTC in fish farm sediments may depend significantly on the type of sediment, chemical characteristics (O_2, H_2S, pH, etc), temperature and bacterial activity in the sediment. Compared to OTC, OA showed a faster loss of antibacterial effect in the fish farm sediments. Sediment porosity due to bioturbation by *Capitella 'capitata'* may accelerate antibiotic elimination while in other cases a white carpet of the blue/green algae *Beggiatoa* may decrease the antibiotic diffusion (Coyne et al., 1994; Smith et al., 1994), but in cases of extreme enrichment even this species may be eliminated. Quinolones may not be broken down because there are no enzyme systems to target these synthetic molecules. In that case any breakdown will depend on physical factors such as photolysis. Oxytetracycline is very soluble in seawater and solution and diffusion process is most likely a major mechanism of OTC escape from the sediment. OTC degradation in water is much faster than in sediment. Tetracyclines in water solutions are degraded by photodecomposition and half-lives depend on temperature, pH, air saturation and light intensity (Samuelsen, 1989a). Coyne et al., (1994). Oxytetracycline, Oxolinic acid and Flumequine photodegrade in the upper levels of the water column (Lunestad et al., 1995) while they exhibit no degradation in the sediments (Samuelsen et al, 1992a,b). Only OTC and not OA showed reduced antimicrobial activity when exposed to underwater light intensities. Photo-degradation is the process in which a component is directly altered by the action of light or indirectly altered by the action of the product of another component, which has absorbed light. A prerequisite for direct photo-degradation is that the chemical absorbs light in the UV – visible region (190-800 nm). Substances that do not absorb in this region cannot be directly photo-degraded. Therefore, photo-degradation will only have a limited effect on the drugs when administered as medicated feed. However, applying bath-treatment (O'Grady et al, 1988) where drugs are dissolved in water and kept in tanks, photo-degradation can be an important tool to decompose the drug and thereby

decrease the environmental impact (Lunestad, 1995). OTC, whose binding to mineral and organic compounds of the bivalve tissues is stronger than OA, was eliminated more slowly from the bivalve tissues than OA. The biotransformations of the antibacterials agents by the bivalves did not seem to play a prominent part in the contamination and decontamination of the bivalve tissues (Pouliquen et al., 1996). New methods of monitoring overfeeding and collecting surplus feed and dead fish have been developed using Lift up feed collector systems or hydroacoustic feed detectors. This equipment can reduce the environmental impact of antibacterial agents used in fish farming (Coyne et al, 1993; Ervik et al, 1994a).

4.8 Immunostimulants and Fish Vaccines

Immunostimulants in sea bass increase total leukocyte count, blood leukocytes, oxidate leukocyte radical production, phagocytosis, lysozyme activity and total protein levels. The effect of glucans was greater than levamisole. The glucans ability to play an important role in the prevention of diseases in sea bass was proven although in high dose rate they exert an immunosuppressive effect (Jeney et al, 1994). The in vitro effect of several beta -glucans on the respiratory burst of turbot and sea bream phagocytes was examined in another study. Three particulated beta -glucans from yeast, *Saccharomyces cerevisiae* and a particulate glucan from the fungus *Schizophyllum commune* were used. High concentrations of glucan directly induced respiratory burst and led to exhaustion. Low concentrations of glucan primed the phagocytes to be capable of enhanced production of reactive oxygen species on subsequent activation of the respiratory burst. The former may increase disease susceptibility, the latter increase resistance (Castro et al, 1999). In a challenge study sea bream juveniles were fed a diet containing beta -1,3 Glucans (0.1%) and then were exposed by bath to

Photobacterium damsela subsp.. piscicida. The effects of such diet were: low mortality, low pathogen recovery and low number of (apparently intact) colonies of bacteria in the spleens. These results seem to indicated that beta-1,3 Glucans could play an important role in the prevention of diseases in fish culture (Albuquerque et al., 1999).

Fish Vaccines marketed in Greece to protect against serious bacterial systemic diseases of farmed sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus auratus*) comprise of formalin-killed bacterins monovalent and divalent either water based or oil-adjuvanted products. The available vaccines may offer protection against several pathogenic serotypes of *Vibrio anguillarum* and/or the *Photobacterium damsela subspecies piscicida* causing pasteurellosis. The application methods and the vaccination schemes that are suggested by the vaccine distributors differ, but all employ either the immersion in the hatchery and/or in the cages and intraperitoneal injection as booster vaccination. Oral vaccination started to be employed three years ago producing consistent results (Locatelli et al., 1999). The practical needs in terms of personnel, cost, and time, differ according to fish size, the type of unit (tank, raceway or cage) and the application method employed. Injection vaccination on the cages is considerably more time-consuming and labour-intensive, as the fish have to be anaesthetised and treated individually. On the other hand, immersion vaccination proceeds fast as groups of fish are added simultaneously to a vaccine dilution. Oral vaccination is the most attractive option for booster vaccination on the cages. Vaccination schemes depend not only on the epizootiology of the site and the duration of the production cycle compared to the expected period of immunity, but also on the structure and availability of time and labour on each particular unit. New trends in fish health management of marine species in Mediterranean include full vaccination programmes covering the fish during their whole production cycle

coupled with other prophylactic methods, including good hygiene practices, husbandry and nutrition management (Le Breton, 1999). Early experiments dealt with extracellular products (ECP) of *Photobacterium damsela subsp. piscicida* and their importance in vaccine formulation (Magarinos et al., 1992). Another early study of two immersion vaccines in sea bream, a whole-cell bacterin (WCB) and a toxoid-enriched whole-cell vaccine (WCEB), against *Photobacterium damsela subsp. piscicida* was not successful and none of the formulations confer cross-protection against serotypes 01 and 02 of *Vibrio anguillarum.* (Magarinos et al, 1994a). A controversial study of the same group suggested immunity development against pasteurellosis in very small sea bream larvae (50mg) following immersion with a toxoid-enriched whole-cell bacterin (Magarinos et al, 1999). In another study in 57- and 69-days old gilthead sea bream Artemia nauplii were used as an antigen delivery system for oral vaccination with a *Vibrio anguillarum bacteria* indicating that oral vaccination with bio encapsulated vaccines is very promising when applied at the right age. Application at too young age seems to induce immunosuppression, instead of immunisation (Joosten et al., 1995) *Photobacterium damsela subsp. piscicida* cells cultured under iron-limited conditions demonstrated an immunogenic low molecular weight lipopolysaccharide (Bakopoulos et al., 1997b). In another more recent comparative study washed cells of the above pathogen enriched with extracellular products (WC+ECP) performed better than O-antigen (O-Ag) and heat inactivated extracellular products (ECP) based ip vaccines for sea bass (Fabris et al., 1998). Extracellular products of *Photobacterium damsela subsp. piscicida* are haemolytic, cause lymphopenia, granulocytosis, increase in the number of peritoneal exudate cells, mobilization and degranulation of the eosinophilic granular cells and toxicity in gills and liver but they are not linked to typical lesions in the spleen (Noya et al.,1995a,b). Vibriosis and E.R.M vaccines have been available in Italy since 1988. Successful vaccination in sea bass but not in sea bream against *Vibrio anguillarum* (Giorgetti

et al, 1990) was confirmed in a later study (Brisinello and Fontebasso, 1991). Substitution of antimicrobial chemotherapy with preventive vaccination strategies is widely applied in aquaculture, but the need for back up solutions and available antibiotics will always be there in case of vaccination protection breakdown or new pathogen outbreaks.

4.9 CONCLUSIONS

Veterinarians and fish health specialists will continue to need a variety of efficacious, cost-effective and safe chemotherapeutants to enable prompt therapeutic intervention to battle infectious fish disease outbreaks. Many important questions are raised regarding the integration of bacterial resistance monitoring, pharmacokinetics, metabolism, clinical efficacy, drug residue depletion, target animal safety, consumer safety, and environmental impact linked to antibiotic application in Greek Mariculture. It is hoped and anticipates that this study will prove useful in providing the foundation for further and more applied antibacterial chemotherapy related research.

4.9.1 Bacterial Diseases – Antibiotic resistance

The current study indicated a range of bacterial pathogens affecting sea bass and sea bream in Greek Mariculture. It managed to describe resistance profiles and to extrapolate resistance frequencies as well as to identify farms with antibiotic application malpractice. The levels of resistant bacteria were relatively low but resistant bacteria were isolated for both OA and OTC. Multi resistant profiles were analysed. It is believed that qualitative antibiogram and MIC are very important tools in order to estimate the resistance status in an industry and further work should include this "quantified" component not only in the clinical epidemiological trials but also in the environmental impact trials. At the same time this is one of the very few studies where MIC data were correlated with achieved concentrations of Oxytetracycline and Oxolinic acid in different tissues. The practical aim of the study for the farmers was to identify if the applied treatment protocols and related achieved tissue concentrations *in vivo* were sufficient to control the bacterial pathogens of sea bass and sea bream and on the other hand which is the period

that should be allowed in order to make sure in practical terms that antibiotic concentration falls bellow the MRL level. Extensive data were provided in this field illustrating the fast metabolism in both species, the vast differences in achieved concentrations of antibiotic in a fish population and the inability to draw similarities in depletion studies based on the degree-day concept. An important issue for the containment of antibiotic resistance in the aquaculture industry is to define the problem and apply good practices and codes of conduct. Several issues should be addressed: Research has indicated that many factors are involved in the selection and spread of antimicrobial-resistant bacteria. Most important amongst these appears to be the extent of usage since the relationship between the amounts of antibacterials used and the prevalence of resistance is broadly quantitative. Thus, the control and containment of resistance is likely to be successful only if the measures employed include a reduction in the use of antimicrobials in all spheres of current application. The measures, which need to be considered for control and containment of antibiotic resistance, include: Improved prescription use, reduction of need and provision of new antimicrobials as well as education of prescribers and fish farmers. (S.S.C.A.R 1999) In order to preserve the ability to treat bacterial infections in fish, action must be taken to reduce the overall use through the implementation of preventive measures and by prudent use. In veterinary medicine, antimicrobials may be prescribed for prophylactic or therapeutic purposes. In addition, complications arise in aquaculture where disease epidemiology and treatment always refers to fish populations in different production systems. In those situations it is necessary to administer antimicrobials to the whole group even though all fish do not yet demonstrate clinical signs of infection at the time of administration, but it is likely that most of them will get the disease in the next days. In that sense the "cascade principle" for aquaculture, as mentioned, before should be reviewed and amended. Such use of antimicrobials is often referred to as metaphylactic use. The veterinary surgeon / fish pathologist

prescribing antimicrobials should have knowledge of the disease history including disease epidemiology, preventive and other measures undertaken. To achieve an optimal and prudent use of antimicrobials, guidelines for the use of antimicrobials may be established as a help to the veterinarian. Such guidelines would also support the veterinarian against demands for antimicrobials by fish farmers. These guidelines should be to achieve three goals; optimal therapeutic effect and/or protection and welfare of fish at risk, control of antimicrobial resistance and provision of practical, affordable treatment that avoids risks of residues in or damage to aquaculture products for human consumption. Emergence and spread of resistance is a serious effect of antimicrobial use in animals, so facilities to monitor and analyse regularly the prevalence and patterns of resistance should be developed as a priority. Antimicrobial susceptibility data should be quantitative and produced under strict quality assurance. Interpretation and reporting of results from different laboratories must be harmonised. More detailed knowledge about the usage of antimicrobials and the impact on epidemiology and prevalence of antimicrobial resistance in different environments will assist a greater understanding about the forces behind the development and dissemination of antimicrobial resistance. Registration of the amounts of veterinary medicines sold by each company should be available as well as the recording of indications and use of veterinary drugs and pre-medicated feeds by means of a logbook on each farm and in each veterinary practice. Such a recording system of health status and use of medicines will make it not only feasible to monitor the usage of antimicrobials on a farm and by prescription, but also the indications for antimicrobial usage and efficacy of antimicrobial therapy under practice conditions. It is understood that such studies are undertaken by pharmaceutical companies but we believe that they should be made public. All antimicrobials administered on farms should be used only as part of a comprehensive veterinary health programme. In contrast to the situation in human medicine more drastic and

effective management and other disease preventive methods can be undertaken in animal production. Batch-wise, year class-segregated production using all-in/all-out systems with biosecurity routines together with optimal nutrition, environment, management routines and vaccination strategies can greatly improve fish health and decrease the need for antimicrobials. The introduction of vaccines against furunculosis in fish farming in Norway has eliminated an alarmingly large use of antimicrobials. Recent research has been devoted mainly in the field of Probiotics as means to prevent colonisation of the intestine by bacterial pathogens mainly Vibrios. It is difficult to find or create truly novel agents, which are patentable. On the other hand there may be a finite number of appropriate targets in bacteria. Finally it is commercially unattractive to invest in research having little chance of producing a return. The cost of research, development and testing of a novel antimicrobial is now probably in excess of US$350 million and the time required for effective marketing is at least 6-7 years (Cohen, 1992). As a result, the number of companies investing in antibacterial research declined even before the recent trend towards company mergers occurred. Moreover, there is the risk that a costly new antimicrobial drug may well become obsolete within a few years, reducing the economic returns that can be expected to a level that is insufficient to justify the investment. The ten-year industrial-property protection period for veterinary medicinal products for fish should be extended to 15 years in order to enable the pharmaceutical industry to derive full benefit from such products thus initiating renewed incentives to invest and achieve marketing authorisations for fish products. The time it takes to obtain registration and to develop the data required for the extension of a marketing authorisation to different species and to different diseases should be considerably shorter. Both centralised and decentralised procedure should be maintained providing the necessary flexibility in terms of faster launching of new products in the aquaculture market. Evaluation of the operation of the procedures for the granting of market authorisation has revealed

the need to revise, in particular, the mutual recognition procedure in order to increase the scope for cooperation between Member States. Different levels of competence in different Member States as well as different level of vigilance often leave more options for treating or preventing fish diseases in some countries while leave none in others. A simple example is that a few Member states deny acting as reference Member states in the Mutual recognition procedure. A basic degree of harmonisation should be included in the enforcement of the new legislation in order to avoid the creation of indirect trade barriers. The large number of aquaculture species and relevant pathologies complicate the procedure of extension of use or species and emphasis should be made into making this process faster at a Member state level as well as at EU level. Systematic extrapolation of MRL values between species would make new drugs available faster for minor species. The 'cascade' principle needs to be modified. In the case of absence of authorised veterinary medicinal product for a condition affecting fish, a familiar case for fish veterinarians, the veterinarian is allowed to treat that fish population under his/her direct personal responsibility on a particular fish farm, in order to avoid causing unacceptable suffering to the fish population, with a licensed product in another Member state for fish or for other food producing animal The reference however to a "small number of animals" in the legislation is not applicable for aquaculture because fish are reared in cages or tanks in large numbers and these are treated as units in terms of epidemiology, prevention or treatment of fish diseases. The licensing and application of Fish vaccines should be encouraged in order to support the European Aquaculture industry trend to limit the use of antimicrobials and to ensure high product quality and safety. New searches for drugs that interfere with virulence factors or mechanisms of resistance, or which seek to modify the molecular biology of multiple resistant pathogens are being developed. However all these "new " technology drugs should be carefully audited in terms of consumer safety especially when applied to

food animals and will take a considerable time, if ever, to reach the aquaculture industry. Aspects of antimicrobial therapy are poorly addressed in under-graduate and post-graduate education. Thus, there is scope for expanding education of health professionals both in terms of good prescribing and how to minimise antimicrobial use by preventing infection occurring. For veterinary practitioners this might include updating on organised health control programmes mentioned above.

4.9.2 Kinetics

The kinetics experiments with all their limitations and novel characteristics indicated a new practical approach for the evaluation of clinical importance of kinetics work. Population versus individual fish studies are required in order to see the shortfalls from clinical and environmental perspective. We believe that this is the first study of the population effect and the statistical interpretation, like Box Plot analysis, of the antibiotic concentration in different fish in a cage. The coupling of achieved antibiotic levels with relevant MIC breakpoint values for known pathogens associated with that species and culture conditions is a unique combination that puts everything into perspective from the veterinarian's point of view. Although kinetics that take into account only the HPLC data gave the impression of variable and relatively low levels achieved in different tissues, the association with *V.anguillarum* sensitivity data and MIC breakpoints assumed for Oxolinic acid and Oxytetracycline gave the necessary clinical explanations.

The relatively low MIC breakpoint levels were achieved in different tissues in most of the experiments performed in different temperatures and this explained the reason why those treatment protocols are effective in practice. However high subject deviation in achieved concentration should be further elaborated in future studies because it might explain occasional failures as well as reoccurring outbreaks. Included in the conclusion could be species differences, tissues

differences as well temperature differences but it is believed that all researchers must step back and really see the vast number of data that none can compare and contrast, in order to realise that we need common language, common methodology, common approaches in order to tackle this extremely broad subject. One also has to remember that this is undertaken for the welfare of animals, for the reduction of the environmental impact, for the consumer protection and for the sustainability of aquaculture industry. Therefore one must be practical, accurate and give real models that will be able to predict what is really happening in practice.

4.10 Future Work

There is a definite lack of good quality information on prevalences of antimicrobial resistance, amounts of antimicrobials used, the applied modes of use, and the outcomes of use. The first priority is therefore to establish what information is currently available and to make suggestions on how it can be improved. This will ensure comprehensive, reliable and cost-effective surveillance systems. The work started for MIC method harmonisation should continue to kinetics as well as on environmental impact methodologies. More research into factors which influence the selection of resistant microorganisms is needed, particularly at the *in vivo* and epidemiological levels. More research and epidemiological data are needed to clarify the issue of inter-species transfer of antimicrobial resistance.

It is very important to evaluate the environmental impact from the antibiotic use in terms of deposition of residues in the sediment, water column and wild fauna as well as to describe the mechanisms and the locations for the selection of resistant environmental bacteria. The population dynamics of these bacteria their impact to the natural bacterial flora and the likelihood to affect the human flora along with all the relative implications should be evaluated because there are a lot of

misconceptions and aquaculture is being blamed without any data when environmental groups extrapolate data from poultry farms and other terrestrial farmed animals with no concern on the enormous differences that the aquatic environment poses. Research is needed into ways that might improve the prescription use of antimicrobials. Clinical trials of efficacy are presently almost entirely funded by the pharmaceutical industry and have objectives largely confined to satisfying regulatory authorities. Thus trials, which seek to optimise the dose, dose interval and duration of treatment are rarely done. Evaluation of the service provided to the industry must be evaluated from an empirical to scientific level where all aspects of chemotherapy will be taken into account. Veterinary prescription although compulsory, should be re-vitalised in many countries because this the way by which at all times a scientist has the responsibility of the treatment applied. Focus is needed on methods of reducing the need for antimicrobials by lowering the prevalence of infection. Widespread implementation and enforcement of methods for reducing transmission of pathogens as well as detailed epidemiological studies are needed in Mediterranean Mariculture. Effective Vaccination strategies, immunostimulation and a range of other means should reduce the need to administer drugs in aquaculture. More studies are needed on the impact of changes of farming conditions and management practices on fish stress and welfare. Rapid diagnostic methods for bacterial infections should be encouraged. Thereby antimicrobial treatments will be aimed directly at the infectious agent and the need for broad spectrum or combined antimicrobial usage will be more limited. Immunological, molecular techniques of increased sensitivity should be made available in the field. Determination of the optimum mode of oral administration of chemoterapeutants is very important in order to avoid shortfalls. Optimisation of administration will be to vary the feed regime, antibiotic concentrations, and feed type. Optimised feed regimes will be used subsequently to treat outbreaks of disease. For hatcheries vehicle drug delivery

studies should continue in order to increase affectivity and decrease environmental impact. Investigation of optimum uptake and residue kinetics will require incorporation of population distribution studies where current practice is audited and then suggestions are made to ensure increased clinical relevance and efficacy. The aim is to provide consumers with healthy safe fish of good quality. Determination of withdrawal times is important for a fish farmer in practical terms for sales scheduling. The regulatory authorities have to decide how to manage in this task in practical terms and what the MRL level means in the daily practice of a salmon or a sea bass farmer. A Code of Practice of the Greek Mariculture has been developed and adopted by Federation of the Greek Maricultures in order to transform relevant issues from this study as well as views from the literature into practical guidelines for Greek sea bass and sea bream farmers to ensure the production of high quality, nutritious and safe final product This code was the basis for an intense effort of Greek Mariculture industry that is expected to lead in September 2003 to the certification of the Greek sea bass and sea bream by AGROCERT the Greek Ministry's of Agriculture competent authority for certification of all agricultural products (Christofilogiannis 2002a). However continuous research should be in progress in order to achieve a "global perspective" of issues and factors mentioned above, to understand better the dynamics of antimicrobial chemotherapy in aquaculture and to transform those into practical advice for the fish farmers in the field.

ANNEX 1

BACTERIAL STRAIN INDEX

Fish Farm 1

Strain: RP1

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
-	+	-	white / steaky	-	-
API20E Profile					
1007004					
Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	P
21.1	32.1	46	38	22	S
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
10	0.15				

Strain: RP2

Species: Dentex Dentex					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
+	-	+	small white	-	oxidative
API20E Profile					
Antibiogram (Zones of inhibition mm)					OD=0.249
OTC	OA	SXT	FR	O129	P
36.6	8.78	35.64	24.56	R	15,24
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	2.5				

Strain: RP3

Species: Mugil cephalus					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
cocci	-	-	yellow	green	Oxidative
API20E Profile					
Antibiogram (Zones of inhibition mm)					OD=0.245
OTC	OA	SXT	FR	O129	P
34	6	S	S	R	48
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.15	80				

Strain: RP4

Species: Dentex Dentex					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
cocci	-	-	yellow	-	No reaction
API20E Profile					
Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	P
32	12	36	29	R	21
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	5				

Fish Farm 2

Strain: Argo1

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Cocci	-	-	Red	-	Fermentative
API20E Profile					
Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	P
34	6	39	6	16	
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	80				

Strain:Argo2

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
cocci	-	-	yellow	green	(-/-)
API20E Profile					
Antibiogram (Zones of inhibition mm)					OD=0.249
OTC	OA	SXT	FR	O129	P
S	R	S	R	S	R
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	40				

Strain: Argo3

Species: Gilthead Sea bream					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
cocci	-	-	yellow	green	(-/-)
API20E Profile					
Antibiogram (Zones of inhibition mm)					OD=0.267
OTC	OA	SXT	FR	O129	P
31.91	6	27.06	23.4	R	21.87
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	5				

Fish Farm 3

Strain: SF1

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
4144525					
Antibiogram (Zones of inhibition mm)					OD=0.280
OTC	OA	SXT	FR	O129	P
32	25	32	30	R	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	0.15				

Strain:SF2

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	Yellow	Fermentative
API20E Profile					
3047524 Vibrio anguillarum 1b					
Antibiogram (Zones of inhibition mm)					OD=0.245
OTC	OA	SXT	FR	O129	P
6	33	26	31	S	10
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
160	0.075				

Strain:SF3

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Long bacilli	+	+	yellow	Yellow	Fermentative
API20E Profile					
3047524 Vibrio anguillarum 1b					
Antibiogram (Zones of inhibition mm)					OD=0.265
OTC	OA	SXT	FR	O129	P
35	30	33	22	S	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	0.075				

Fish Farm 4

Strain: NE1

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
Long bacilli	**+**	**+**	**yellow**	**green**	**Fermentative**
API20E Profile					
3047524 Vibrio anguillarum 1b					
Antibiogram (Zones of inhibition mm)					**OD=0.249**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
34.44	**32.49**	**35.72**	**32.44**	**29.56**	**6**
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.3	**0.3**				

Fish Farm 5

Strain: Deep1					
Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1					
Antibiogram (Zones of inhibition mm)					OD=0.251
OTC	OA	SXT	FR	O129	P
30.55	30.41	33.98	24.63	27.94	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
1.25	1.25				

Fish Farm 6

Strain: Ast1

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	-	yellow	yellow	Fermentative
API20E Profile					
3044125 V.fluvialis					
Antibiogram (Zones of inhibition mm)					OD=0.240
OTC	OA	SXT	FR	0/129	NV
36	27	34	34	24	28
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.075				

Strain:Ast2

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	whitish / green	Fermentative
API20E Profile					
2200004 81% Pseudomonas fluorescens					
Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	P
35	32.1	23	26	26	22
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
0.3	0.3				

Strain: Ast3

Species: Gilthead Sea bream					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	-	-	yellow sticky	-	(-/-)
API20E Profile					
OOO2OOO					
Antibiogram (Zones of inhibition mm)					OD=0.256
OTC	OA	SXT	FR	O129	P
32.09	6	32.81	16	6	34.64
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	80				

Fish Farm 7

Strain: Ovr1

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
4047520 V.alginolyticus					
Antibiogram (Zones of inhibition mm)					OD=0.244
OTC	OA	SXT	FR	O129	P
38	36	32	30	26	6
AMP	NV				
8	25				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
0.3	0.3				

Strain:Ovr2

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	green	Fermentative
API20E Profile					
1047724					
Antibiogram (Zones of inhibition mm)					OD=0.23
OTC	OA	SXT	FR	O129	P
26	12	6	6	R	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.075	2.5				

Strain: Ovr3

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	yellow *	Fermentative
API20E Profile					
2000104					
Antibiogram (Zones of inhibition mm)					OD=0.252
OTC	OA	SXT	FR	O129	P
23.97	14.83	20.22	6	R	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
0.3	0.6				

Strain: Ovr4

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
4046505					
Antibiogram (Zones of inhibition mm)					OD=0.264
OTC	OA	SXT	FR	O129	P
24	18	24	20.5	R	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.625				

Strain:Ovr5					
Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	green *	Fermentative
API20E Profile					
1047524 V.anguillarum 2					
Antibiogram (Zones of inhibition mm)					OD=0.222
OTC	OA	SXT	FR	O129	P
26	15	25	19	S	10
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.075	0.625				

Strain: Ovr6					
Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1					
Antibiogram (Zones of inhibition mm)					OD=0.265
OTC	OA	SXT	FR	O129	P
32.42	31.76	34.08	27.9	25.45	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.15				

Strain: Ovr7					
Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1					
Antibiogram (Zones of inhibition mm)					OD:0.247
OTC	OA	SXT	FR	O129	P
26	19	22	R	R	
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	1.25				

Fish Farm 8

Strain: Kal1					
Species: Gilthead Sea bream					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Cocci	-	-	yellow	yellow	Oxidative
API20E Profile					

Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	P
34.46	7	33.3	26.26	R	27.59
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	5				

Strain:Kal2					
Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
1047524 V.anguillarum 2b					

Antibiogram (Zones of inhibition mm)					OD=0.257
OTC	OA	SXT	FR	O129	AMP
35	6	30	15	18	30
NV					
26					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	80				

Strain: Kal3					
Species: Puntazo puntazo					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yel. cream. egg	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1					

Antibiogram (Zones of inhibition mm)					OD=0.248
OTC	OA	SXT	FR	O129	AMP
25	11	23	22	R	R
NV					
R					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
1.25	1.25				

Strain: Kal4					
Species: Gilthead Sea bream juveniles					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	small/round/shiny	green	Fermentative
API20E Profile					
6005004 99.5% Listonella damsela					
Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	AMP
33	30.5	23	26	14	7
NV					
11					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	0.625				

Strain:Kal5					
Species: Puntazzo puntazzo					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	whitish	green	Fermentative
API20E Profile					
6005004 99.5% Listonella damsela					
Antibiogram (Zones of inhibition mm)					OD:0.252
OTC	OA	SXT	FR	O129	NV
30	24	30	22	S	
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.3				

Strain: Kal6					
Species: Puntazzo puntazzo					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	swarming	yellow	Fermentative
API20E Profile					
4144525 (according to the ALO test -++ : V.alginolyticus)					
Antibiogram (Zones of inhibition mm)					OD: 0.254
OTC	OA	SXT	FR	0/129	
34	20	23	24	R	
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.3				

Strain: Kal7					
Species: Puntazzo puntazzo 20-30g					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
4154525					
Antibiogram (Zones of inhibition mm)					OD=0.253
OTC	OA	SXT	FR	O129	P
26	12	21	19	6	6
AMP	NV				
6	12				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
0.625	0.625				

Strain:Kal8					
Species: Sea bass 350g					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
4146525 V.alginolyticus					
Antibiogram (Zones of inhibition mm)					OD=0.240
OTC	OA	SXT	FR	O129	P
36	23	38	25		6
AMP	NV				
6	14				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	0.625				

Strain: Kal9					
Species: Puntazo puntazo					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	green	Fermentative
API20E Profile					
3047524 V.anguillarum 1b					
Antibiogram (Zones of inhibition mm)					OD=0.258
OTC	OA	SXT	FR	O129	P
28	26	25	22	20	6
NV	AMP				
14	10				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.15	0.075				

Strain: Kal10

Species: Sea bass 150g					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	swarming	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1b					
Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	P
30	37,5	5	28	36	6
AMP	NV				
6	28				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
0.15	0.075				

Strain:Kal11

Species: Sea bass 350g					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+		yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1b					
Antibiogram (Zones of inhibition mm)					OD:0.252
OTC	OA	SXT	FR	O129	NV
34	30	30	32	25	28
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.15				

Strain:Kal12

Species: Sea bass 350g					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+		yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1b					
Antibiogram (Zones of inhibition mm)					OD:0.253
OTC	OA	SXT	FR	O129	NV
35	34	32	31	26	22
P	AMP				
6	6				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.15				

Fish Farm 9

Strain: PL1

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
cocci	-	-	yellow	-	no reaction
API20E Profile					

Antibiogram (Zones of inhibition mm)					OD:0.254
OTC	OA	SXT	FR	O129	P
30	6	24	6	R	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
1.25	5				

Strain: PL2

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
1047524 V.anguillarum 2					

Antibiogram (Zones of inhibition mm)					OD:0.254
OTC	OA	SXT	FR	O129	P
43.74	43.72	43.78	42.06		
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				

Strain: PL3

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum					

Antibiogram (Zones of inhibition mm)					OD:0.256
OTC	OA	SXT	FR	O129	P
38.30	40.7	36.9	34.22	20.12	
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
0.075	0.075				

Strain:PL4

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli			whitish	yellow	Fermentative
API20E Profile					
3047524 Vibrio anguillarum 1b					

Antibiogram (Zones of inhibition mm)					OD=
OTC	OA	SXT	FR	O129	P
35	36	35	33	28	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.15				

Fish Farm 10

Strain: AGR1

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	green	Fermentative
API20E Profile					
OOO3OO4 (according to ALO test - - - : V.ordalii)					
Antibiogram (Zones of inhibition mm)					OD=0.26
OTC	OA	SXT	FR	O129	P
34	6	38	23	R	38
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	40				

Strain:AGR2

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	green	Fermentative
API20E Profile					
OOO3OO4 (according to ALO test - - - : V.ordalii)					
Antibiogram (Zones of inhibition mm)					OD=0.258
OTC	OA	SXT	FR	O129	P
38	6	38	21	R	33
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.075	40				

Fish Farm 11

Strain: Petr2

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	Yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1					
Antibiogram (Zones of inhibition mm)					OD=0.265
OTC	OA	SXT	FR	O129	P
33.8	32.22	31.64	26.54	27.52	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.075				

Strain:Petr3

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	Yellow	Fermentative
API20E Profile					
1047524 V.anguillarum 1b					
Antibiogram (Zones of inhibition mm)					OD=0.249
OTC	OA	SXT	FR	O129	P
30.27	39.3	35.78	36.42	28.84	13.12
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.625	0.3				

Fish Farm 12

Strain: Chios1

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
Bacilli	**+**	**+**	**yellow**	**Yellow**	**Fermentative**
API20E Profile					
3047524 V.anguillarum 1					
Antibiogram (Zones of inhibition mm)					**OD=0.21**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
37	**35**	**32**	**34**	**S**	**6**
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
1.25	**0.075**				

Fish Farm 13

Strain:Nous1

Species: Sea bass juveniles					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
-	+	+	swarming	Yellow	Fermentative
API20E Profile					
4347525 V.alginolyticus					
Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	NV
28	21.5	28	24	7	20
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.3				

Strain:Nous2

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	Yellow	Fermentative
API20E Profile					
4146525 V.alginolyticus					
Antibiogram (Zones of inhibition mm)					OD=0.253
OTC	OA	SXT	FR	O129	NV
32	28	30	25	7	14
AMP					
6					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.3				

Strain:Nous3

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	swarming	Yellow	Fermentative
API20E Profile					
4146525 V.alginolyticus					
Antibiogram (Zones of inhibition mm)					OD=0.241
OTC	OA	SXT	FR	O129	NV
29	19	25	20	6	13
AMP					
11					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
0.3	0.3				

Strain:Nous4					
Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
-	+	+	swarming	Yellow	Fermentative
API20E Profile					
4114735 (ALO test: - + +)					
Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	NV
25	27	25	18	R	
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.3				

Strain:Nous5					
Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	Yellow	(+/+)
API20E Profile					
6144525					
Antibiogram (Zones of inhibition mm)					OD=
OTC	OA	SXT	FR	O129	NV
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.625				

Strain:Nous6					
Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
short Bacilli	+	+		Green	(+/+)
API20E Profile					
4146505					
Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	NV
26	18	24	19	R	10
AMP	NV				
6	10				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
1.25	5				

(ALO test: - + + & SAC - & TCBS: green = V.parahaemolyticus)

Strain:Nous7

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
short bacilli	+	-	swarming	-	(-/-)
API20E Profile					
2004004 Pasteurella spp.					
Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	NV
		6			
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
20	1.25				

Strain:Nous8

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	-	yellow	-	(-/-)
API20E Profile					
2004004 Pasteurella spp.					
Antibiogram (Zones of inhibition mm)					OD=0.253
OTC	OA	SXT	FR	O129	NV
8	10	40	30	6	10
AMP					
38					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
40	1.25				

Fish Farm 14

Strain:Fido1					
Species: Gilthead Sea beam juveniles					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
short bacilli	+	+	yellow	Yellow	Fermentative
API20E Profile					
4154525					
Antibiogram (Zones of inhibition mm)					**OD=0.258**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
28.83	19.52	24.21	19.29	15.16	6
Minimum Inhibitory Concentration (ppb)					
MIC OTC	**MIC OA**				
0.3	0.625				

(ALO test:- + + V.alginolyticus)

Strain:Fido2					
Species: Gilthead Sea bream juveniles					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
Long Bacilli	+	+	yellow	Yellow	Fermentative
API20E Profile					
OO4O777					
Antibiogram (Zones of inhibition mm)					**OD=0.246**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
29.25	24.03	28.81	23.61	12.65	10.12
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC OA**				
0.15	0.3				

Strain:Fido3					
Species: Gilthead Sea bream juveniles					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
Bacilli	+	-	yellow	Yellow	Fermentative
API20E Profile					
5144565					
Antibiogram (Zones of inhibition mm)					**OD=0.258**
OTC	**OA**	**SXT**	**FR**	**O129**	**NV**
27.59	18.93	27.4	20.88	15.16	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC OA**				
0.3	0.3				

Strain:Fido4					
Species: Gilthead Sea bream juveniles					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
Bacilli	+	+	yellow	Green	Fermentative
API20E Profile					
5046305					
Antibiogram (Zones of inhibition mm)					**OD=0.250**
OTC	**OA**	**SXT**	**FR**	**O129**	**NV**
7	16	R	17	R	R
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC OA**				
40	0.3				

Fish Farm 15

Strain:Prap1

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	yellow	Yellow	Fermentative
API20E Profile					
1047524 V.anguillarum 2					
Antibiogram (Zones of inhibition mm)					OD=0.246
OTC	OA	SXT	FR	O129	P
34	33	28	25	S	7
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.15	0.075				

Strain:Prap2

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	-	yellow	green	Fermentative
API20E Profile					
4046105 V.parahaemolyticus 95.5%					
Antibiogram (Zones of inhibition mm)					OD=0.252
OTC	OA	SXT	FR	O129	P
26	18	24	16	R	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
1.25					

Strain:Prap3

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	Yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1b					
Antibiogram (Zones of inhibition mm)					OD=0.26
OTC	OA	SXT	FR	O129	P
35	31	31	26	R	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
1.25	1.25				

Strain:Prap4

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1b					
Antibiogram (Zones of inhibition mm)					OD=0.254
OTC	OA	SXT	FR	O129	NV
28	33.5	29	27	22	20.5
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
2.5	0.15				

Strain:Prap5

Species: sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	Yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1b					
Antibiogram (Zones of inhibition mm)					OD=0.240
OTC	OA	SXT	FR	O129	NV
38	35	32	29	22	19
AMP					
8					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.075				

Fish Farm 16

Strain:Eyr1					
Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
cocci	**-**	**-**	**whitish**	**-**	**(-/-)**
API20E Profile					

Antibiogram (Zones of inhibition mm)					OD=0.25
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
36	**6**	**35**	**26**	**R**	**28**
Minimum Inhibitory Concentration					
MIC OTC	**MIC O.A**				
0.625	**5**				

Fish Farm 17

Strain:Ocean1

Species: Sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
bacilli	+	+	brown pigment	Yellow	Fermentative
API20E Profile					
4144525					
Antibiogram (Zones of inhibition mm)					OD=0.258
OTC	OA	SXT	FR	O129	AMP
26	18	26	24	R	6
NV					
R					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
0.3	0.625				

Strain:Ocean2

Species: sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
Bacilli	+	+	yellow	Green	Fermentative
API20E Profile					
5046105 V.Vulnificus					
Antibiogram (Zones of inhibition mm)					OD=0.257
OTC	OA	SXT	FR	O129	AMP
28	16	25	24	R	R
NV					
R					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC O.A				
1.25	0.3				

Strain:Ocean3

Species: sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
coccobacilli	+	+	swarming	yellow	Fermentative
API20E Profile					
4047125 V.alginolyticus					
Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	OA	SXT	FR	O129	NV
22	15	21	9	R	11
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
0.625	0.625				

Fish Farm 18

Strain:Zant1

Species: Gilhead Sea bream					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	-	-	yellow	green	Fermentative
API20E Profile					
7005100					

Antibiogram (Zones of inhibition mm)					OD=0.255
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
28	33	18	24	12	
NV					
8.5					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC OA**				
0.3	0.075				

Strain:Zant2

Species: Gilhead Sea bream					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
Bacilli	+	-	whit. yellow	Yellow	Fermentative
API20E Profile					
1004124 Pasteurella spp.					

Antibiogram (Zones of inhibition mm)					OD=
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
30	18	36	22	23	
AMP	**NV**				
13	12				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC OA**				
1.25	0.625				

Strain:Zant3

Species: Gilhead Sea bream					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
Bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
OOO4125 V.ordalii					

Antibiogram (Zones of inhibition mm)					OD=
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
40	46	S	32	36	8
AMP	**NV**				
15	20				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC OA**				
0.3	0.075				

Strain:Zant4

Species: Gilhead Sea bream					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
-	-	-	Yellow	-	Fermentative
API20E Profile					
OOO4OOO					

Antibiogram (Zones of inhibition mm)					OD=0.250
OTC	**OA**	**SXT**	**FR**	**O129**	**AMP**
R	R	R	R	R	R
NV					
R					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC OA**				
5	80				

Fish Farm 19

Strain:Ler1					
Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	**+**	**+**	**yellow**	**Yellow**	**Fermentative**
API20E Profile					
1047524 V.anguillarum 2					
Antibiogram (Zones of inhibition mm)					**OD=0.258**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
31.12	**30.72**	**31.92**	**29.22**	**26.54**	**6**
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.3	**0.075**				

Fish Farm 20

Strain:Myt1

Species: sea bass					
Gram	Oxidase	Motility	Colony	Growth in TCBS	O/F
-	+	+	yellow	Yellow	Fermentative
API20E Profile					
3047524 V.anguillarum1					
Antibiogram (Zones of inhibition mm)					OD=0.253
OTC	OA	SXT	FR	O129	P
30		26	21	6	6
AMP	NV				
6	12				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	MIC OA				
0.075	0.075				

Fish Farm 21

Strain: Makr1

Species:Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
Bacilli	+	weak	yellow	-	Fermentative
API20E Profile					
1003004					
Antibiogram (Zones of inhibition mm)					**OD=0.256**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
18.05	27.1	42	28	16	S
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
5	0.3				

Strain:Makr2

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum1					
Antibiogram (Zones of inhibition mm)					**OD=0.252**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
30	32	31.5	32	28	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.3	0.075				

Strain:Makr3

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
short bacilli	+	+	yellow	-	Fermentative
API20E Profile					
3042524					
Antibiogram (Zones of inhibition mm)					**OD=0.252**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
25.7	37.42	31.87	32.6	R	9.40
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.3	0.075				

Strain:Makr4

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	+	weak	yellow	yellow	
API20E Profile					
3047524 V.anguillarum 1 (Leuven)					
Antibiogram (Zones of inhibition mm)					**OD=0.250**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
17.5	30	42	32	18	S
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.625	0.075				

Strain:Makr5

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
-	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1					
Antibiogram (Zones of inhibition mm)					**OD:0.247**
OTC	**OA**	**SXT**	**FR**	**O129**	**NV**
37	38.5	32	33	28	23
P					
6					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.625	0.075				

Strain: Makr6

Species: Sea bass juveniles					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
-	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1b					
Antibiogram (Zones of inhibition mm)					**OD:0.254**
OTC	**OA**	**SXT**	**FR**	**0/129**	**P**
33	34	33.5	33	26	7
NV					
24					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.625	0.075				

Strain:Makr7

Species: Gilthead Sea bream juveniles					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	+	+	yellow	Green	Fermentative
API20E Profile					
6005004 99.5% V.damsela					
Antibiogram (Zones of inhibition mm)					**OD=0.250**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
26	30	25	22.5	24	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.3	0.075				

Strain:Makr8

Species: Sea bass (400g)					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
short bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1					
Antibiogram (Zones of inhibition mm)					**OD:0.228**
OTC	**OA**	**SXT**	**FR**	**O129**	**NV**
34.34	35.26	34.16	36.18	29.12	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.625	0.075				

Strain: Makr10

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
-	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1b					
Antibiogram (Zones of inhibition mm)					**OD:0.247**
OTC	**OA**	**SXT**	**FR**	**0/129**	**NV**
33	36	30	30	26	23
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.075	0.075				

Fish Farm 22

Strain:Kant1

Species:Sea bass juveniles					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
Bacilli	+	+	whitish sticky	-	-
API20E Profile					
1003004					
Antibiogram (Zones of inhibition mm)					**OD=0.248**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
22.9	23.48	41.94	32.32	11.7	45.5
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
1.25	0.3				

Strain:Kant2

Species:Sea bass juveniles					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	+	+	yellow	-	-
API20E Profile					
1007004					
Antibiogram (Zones of inhibition mm)					**OD=0.259**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
19.46	30.94	46	30.78	16.7	
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
2.5	0.15				

Strain:Kant3

Species: Sea bass juveniles					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	+	+	yellow	-	(-/-)
API20E Profile					
3003004					
Antibiogram (Zones of inhibition mm)					**OD=0.254**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
16.93	6	35.54	11.4	R	44
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.625	10				

Strain:Kant4

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
thin bacilli	+	+	yellow	-	(-/-)
API20E Profile					
OOOOOO4					
Antibiogram (Zones of inhibition mm)					**OD=0.242**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
33.28	38.78	27	6	6	
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
1.25	80				

Strain:Kant5

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
-	+	+	yellow	yellow	Fermentative
API20E Profile					
4146525 V.alginolyticus					
Antibiogram (Zones of inhibition mm)					**OD:**
OTC	**OA**	**SXT**	**FR**	**O129**	**NV**
28	24	25	20	6	22
P					
6					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.3	0.625				

Strain: Kant6

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth In TCBS**	**O/F**
-	+	+	yellow	yellow	Fermentative
API20E Profile					
4366525					
Antibiogram (Zones of inhibition mm)					**OD:0.240**
OTC	**OA**	**SXT**	**FR**	**0/129**	**P**
34.88	10.84	29.44	27.45	8	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.15	0.3				

Strain:Kant7					
Species:					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
4144525					
Antibiogram (Zones of inhibition mm)					**OD=0.256**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
31.18	23.78	28.39	23.21	16.66	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.3	0.625				

Strain:Kant8					
Species:Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1					
Antibiogram (Zones of inhibition mm)					**OD:0.255**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
36.4	34.22	30.28	32.05	31.59	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.3	0.3				

Strain: Kant9					
Species:Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
-	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1					
Antibiogram (Zones of inhibition mm)					**OD:0.243**
OTC	**OA**	**SXT**	**FR**	**0/129**	**P**
35.83	32.94	32.11	28.5	26.73	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.3	0.15				

Strain:Kant10

Species: sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
cocci	-	-	yellow	Yellow	Fermentative
API20E Profile					
Antibiogram (Zones of inhibition mm)					**OD=0.248**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
21.73	10.72	6	13.1		16.85
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.625	5				

Strain:Kant11

Species: sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
cocci	-	-	Yellow	Yellow (weak)	(-/-)
API20E Profile					
Antibiogram (Zones of inhibition mm)					**OD=0.259**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
30.12	6	36.46	6	6	
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
20	80				

Strain:Kant12

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	+	+	yellow	Yellow	Fermentative
API20E Profile					
3047524 V.anguillarum1					
Antibiogram (Zones of inhibition mm)					**OD=0.253**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
34.54	35.03	34.4	30.51	27.58	
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.625	0.15				

Strain:Kant13					
Species: sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
cocci	+	-	Orange	-	-
API20E Profile					
Antibiogram (Zones of inhibition mm)					**OD:**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
33.38	28.16	39.82	22.90	28.77	10
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC OA**				
0.3	0.3				

Strain: Kant14					
Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
-	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1					
Antibiogram (Zones of inhibition mm)					**OD:0.28**
OTC	**OA**	**SXT**	**FR**	**0/129**	**P**
36.58	38.57	31.85		25.19	
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
2.5	10				

Strain:Kant15					
Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
short bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
3047524 V.anguillarum 1					
Antibiogram (Zones of inhibition mm)					**OD=0.242**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
34.98	34.84	33.12	31.94	27.04	6
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.3	0.075				

Strain:Kant16

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
cocci	+	+	yel. swarm.	yellow	Fermentative
API20E Profile					
4174525					
Antibiogram (Zones of inhibition mm)					**OD:0.253**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
21	8	13	9	6	6
AMP					
6					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.3	0.625				

Strain: Kant17

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	+	+	yellow	yellow	-
API 20E profile					
1003004					
	Antibiogram (Zones of inhibition mm)				**OD:0.257**
OTC	**OA**	**SXT**	**FR**	**0/129**	**P**
5	34	46	26		20
AMP	**NV**				
6	24				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
10	0.15				

Strain: Kant19

Species: Sea bass					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	+	+	small white	yellow	Fermentative
API20E Profile					
4144525					
Antibiogram (Zones of inhibition mm)					**OD:0.254**
OTC	**OA**	**SXT**	**FR**	**0/129**	**P**
38	26	36	33	31	25
AMP	**NV**				
26	22				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
0.625	0.625				

Fish Farm 23

Strain:Vasni1

Species: Sea bream					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
-	+	+	yellow	Yellow	Fermentative
API20E Profile					
2005024 Pasteurella spp.					
Antibiogram (Zones of inhibition mm)					**OD=0.251**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
28	27	S	27	10	24
AMP	NV				
34	S				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC O.A**				
2.5	0.3				

Fish Farm 24

Strain:AqH1

Species: Gilhead Sea bream juveniles					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
bacilli	+	+	egg like	yellow	Fermentative
API20E Profile					
4146525 V.alginolyticus					
Antibiogram (Zones of inhibition mm)					**OD=0.260**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
24	12	19	8	6	6
AMP					
6					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC OA**				
0.6	0.6				

Strain:AqH2

Species: Gilhead Sea bream juveniles					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
Bacilli	+	+	creamy	Yellow	Fermentative
API20E Profile					
6544164					
Antibiogram (Zones of inhibition mm)					**OD=0.253**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
6	30	6	22	6	8
AMP	**NV**				
18	8				
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC OA**				
80	0.6				

Strain:AqH3

Species: Gilhead Sea bream					
Gram	**Oxidase**	**Motility**	**Colony**	**Growth in TCBS**	**O/F**
Bacilli	+	+	yellow	yellow	Fermentative
API20E Profile					
5446025					
Antibiogram (Zones of inhibition mm)					**OD=**
OTC	**OA**	**SXT**	**FR**	**O129**	**P**
24	15	20	20	12	6
AMP					
6					
Minimum Inhibitory Concentration (ppm)					
MIC OTC	**MIC OA**				
0.6	0.6				

REFERENCES

Abedini, S., Namdari, R., Law, F.C.P. (1998) Comparative pharmacokinetics and bioavailability of oxytetracycline in rainbow trout and chinook salmon. Aquaculture, 162 (1-2): 23-32.

Abelli, L., Picchietti, S., Romano, N., Mastrolia, L., Scapigliati, G. (1997) Immunohistochemistry of gut-associated lymphoid tissue of the sea bass *Dicentrarchus labrax* (L.). Fish & Shellfish Immunology 7 (4): 235-245.

Adams, C.A., Austin, B., Meaden, P.G., McIntosh, D. (1998) Molecular characterization of plasmid-mediated oxytetracycline resistance in *Aeromonas salmonicida*. Applied and Environmental Microbiology 64 (11): 4194-4201.

Albuquerque, A., Baptista, T., Dinis, M.T. (1999) Immunostimulants utilization in aquaculture and pasteurellosis diagnosis. Revista de Biologia 17 (1-4): 327-338.

Alderman, D.J., Rosenthal H., Smith, P., Stewart, J. and Weston, D. (1994) Chemicals used in Mariculture. Technical Report, ICES Working Group on the Impact of mariculture. ICES Cooperative Research Report 202. International Council for the Exploration of the Sea, Copenhagen.

Alderman, D.J. (1998) Fisheries chemotherapy: a review. Recent advances in aquaculture. Muir, J.F., Roberts, R.J. (eds.). Croom Helm, London, United Kingdom and Timber Press, Portland, Oregon (3): 1-61.

Alderman, D.J. and Hastings, T.S. (1998) Antibiotic use in aquaculture: development of antibiotic resistance -potential for consumer health risks in Centre for Environment, Fisheries and Aquaculture Science, Weymouth Laboratory, Weymouth, UK 2 FRS Marine Laboratory, Victoria Road, Torrey, Aberdeen, UK

Alderman D.J., Smith, P. (2001) Development of draft protocols of standard reference methods for antimicrobial agent susceptibility testing of bacteria associated with fish diseases. Aquaculture 196: 211-243.

Andresen, A.T., Rasmussen, K.E. (1990) Automated on-line dialysis and column-switching HPLC determination of flumequine and oxolinic acid in fish liver. Journal of Liquid Chromatography 13 (20): 4051-4065.

Angelidis, P., Baudin-Laurencin, F., Youinou, P. (1998) Effects of temperature on chemiluminescence of phagocytes from sea bass, *Dicentrarchus labrax* L. Journal of fish diseases 11 (4): 281-288.

Angles, M.L., Marshall, K.C. and Goodman, A.E. (1993) Plasmid transfer between marine bacteria in the aqueous phase and biofilms in reactor microcosm. Applied and Environmental Microbiology 59: 843-850.

Angulo, L., Lopez, J.E., Lema, C., Vicente, J.A. (1992) *Vibrio pelagius* associated with mortalities in farmed turbot, *Scophthalmus maximus*. Thalassas. Santiago de Compostela 10: 129-133.

Aoki, T. and Kitao, T. (1981) Drug resistance and transferable R plasmids in *Edwardsiella tarda* from fish culture ponds. Fish Pathology 15: 277-281.

Aoki, T., Kitao, T. and Kawano, K. (1981) Changes in drug resistance of *Vibrio anguillarum* in cultured ayu (*Plecoglossus altivelis)* Temminck and Schlegel, in Japan. Journal of Fish Diseases 4: 223-230.

Aoki, T., Takesmita, S., Kitao, T. (1983) The susceptibility of *Aeromonas salmonicida* strains isolated in cultured and wild salmonids to various chemotherapeutans. Bull. Jap. Soc. Sci. Fish. 49: 17-22.

Aoki, T., Kitao, T., Watanabe S. and Takeshita, S. (1984) Drug resistance and R plasmids in *Vibrio anguillarum* isolated in cultured ayu *(Plecoglossus altivelis).* Microbiology and Immunology 28:1-9.

Aoki, T. and Kitao, T. (1985) Detection of transferable R plasmids in strains of the fish-pathogenic bacterium *Pasteurella piscicida*. Journal of Fish Diseases 8: 345-350.

Aoki,T., Kanazawa, T., Kitao, T. (1985) Epidemiological surveillance of drug resistant *Vibrio anguillarum* strains. Fish Pathology 20: 199-208.

Aoki, T. (1988) Drug-resistant plasmids from fish pathogens. Microbiological Sciences 5: 219-223.

Aoki, T. (1992) Chemotherapy and drug resistance in fish farms in Japan. Diseases in Asian Aquaculture. Shariff, M., Subasinghe, R.P., Arthur, J.R. (Eds.). Proceedings of the first symposium on diseases in Asian Aquaculture, 26-29 Nov. 1990. Asian Fisheries Society, Manila, Philippines, pp. 519-529.

Aoki, T. (1997) Resistance plasmids and the risk of transfer. Furunculosis. Multidisciplinary Fish Disease Research (edited by Bernoth E.M.). pp. 433-440. London: Academic Press.

Aquamedia (2002): Fedearation of European Aquaculture Producers official website: www.aquamedia.org

Aquigrup, Madrid (Spain) (1980) Vibriosis in Spain. Its importance for marine fish polycultures. Aspects of brackishwater fish and crustacean culture in the Mediterranean. FAO; Rome (Italy), 1980., pp. 123-130., Stud. Rev. GFCM/Etud. Rev. CGPM,, (no. 57) Sainikov, N.E. (ed.).

Arai, T., Yao-Sugawara, K. and Aoki, T. (1980) The stability of R plasmids in Vibrio *Keio. J. Med. 29:.125-132.*

Austin, B., Morgan, D.A. and Alderman, D.J. (1981) Comparison of antimicrobial agents for control of vibriosis in marine fish. Aquaculture 26: 1-12.

Austin, B., Johnson, C., and Alderman, D.J. (1982) Evaluation of substituted quinolones for the control of vibriosis in turbot *(Scophthalmus maximus).* Aquaculture 29: 227-239.

Austin, B., Rayment, J. and Alderman, D.J. (1983) Control of furunculosis by oxolinic acid. Aquaculture 31: 01-108.

Austin, B. (1984) The future of bacterial fish vaccines. Vaccine 2: 249-254.

Austin, B. (1985a) Chemotherapy of Bacterial Fish Diseases. Fish and Shellfish Pathology. Ellis, A.E. (ed.) Academic Press, London, pp. 19-26.

Austin, B. (1985b) Antibiotic pollution from fish farms: effects on aquatic microflora. Microbiol. Sciences 2: 113-117.

Austin, B. and Austin, D.A. (1987) Bacterial fish pathogens: Disease in farmed and wild fish. Ellis Horwood Books Aquaculture Fish Support Chichester (UK) 364 pp.

Bakhrouf, A., Ben Ouada, H., Oueslati, R. (1995) Sea bass *Dicentrachus labrax* vibriosis treatment in a pisciculture area, in Monastir, Tunisia. Marine Life *5*(2): 47-54.

Bakopoulos, V., Peric, Z., Rodger, H., Adams, A., Richards, R. (1997a) First report of fish pasteurellosis from Malta. Journal of Aquatic Animal Health 9(1): 26-33.

Bakopoulos, V., Volpatti, D., Adams, A., Galeotti, M., Richards, R. (1997b) Qualitative differences in the immune response of rabbit, mouse and sea bass, *Dicentrarchus labrax*, L. to *Photobacterium damsela subsp. piscicida,* the causative agent of fish pasteurellosis. Fish & Shellfish Immunology 7(3): 161-174.

Balebona, M.C., Sedano, J., Bustamante, C., Zorrilla, I., Morinigo, M.A., Borrego, J.J. (1993) Bacterial pathology related to culture of gilt head bream (*Sparus aurata*) in the south of Spain. Israeli Journal of Aquaculture/Bamidgeh 44(4): 140-141.

Balebona, M.C., Morinigo, M.A., Faris, A., Krovacek, K., Maansson, I., Bordas, M.A., Borrego, J.J. (1995) Influence of salinity and pH on the adhesion of pathogenic Vibrio strains to *Sparus aurata* skin mucus. Aquaculture 132(1-2): 113-120.

Balebona M.C., Bordas M.A., Zorrilla, I., Morinigo, M.A., Borrego, J.J., (1998) Pathogenicity of *Vibrio alginolyticus* for cultured gilt-head sea bream (*Sparus aurata* L.). Applied and Environmental Microbiology 64(11): 4269-4275.

Barker G.A. and Alvarez, R.S. (1993) Chemotherapy of rainbow trout and its effects on environmental bacteria. EAFP, 6th international conference "Diseases of fish and shellfish", Brest, France, 1993

Barker, G.A., Smith, S.N., Bromage, N.R. (1990) Effect of oxolinic acid on bacterial flora and hatching success rate of rainbow trout, *Oncorhynchus mykiss*, eggs. Aquaculture, 91(3-4): 205-222.

Barker, G.A. (1994) Detection of 4-quinolone residues in rainbow trout (*Oncorhynchus mykiss Walbaum*) muscle using a bio-assay. Aquaculture, 127: 83-90.

Barnes, A.C., Lewin, C.S., Amyes, S.G.B., Hastings, T.S. (1990) In vitro activities of 4-quinolone antimicrobials against *Aeromonas salmonicida.* Ices Council Meeting 1990 (Collected Papers), Ices, Copenhagen (Denmark), 6 pp.

Barnes, A.C., Lewin, C.S., Hastings, T.S. and Amyes, S.G.B. (1990a) In vitro activities of 4-quinolones against the fish pathogen *Aeromonas salmonicida.* Antimicrobial Agents and Chemotherapy 34: 1819-1820.

Barnes, A.C., Lewin, C.S., Hastings, T.S. and Amyes, S.G.B. (1990b) Cross resistance between oxytetracycline and oxolinic acid in *Aeromonas salmonicida* associated with alterations in outer membrane proteins. FEMS Microbiology Letters 72: 337-340.

Barnes, A.C., Amyes, S.G.B., Hastings, T.S. and Lewin, C.S. (1991a) Fluoroquinolones display rapid bactericidal activity and low mutation frequencies against *Aeromonas salmonicida.* Journal of Fish Diseases 14: 661-667.

Barnes, A.C., Lewin, C.S., Hastings, T.S. and Amyes, S.G.B. (1991b) In vitro susceptibility of the fish pathogen *Aeromonas salmonicida* to flumequine. Antimicrobial Agents and Chemotherapy 35: 2634-2635.

Barnes, A.C., Lewin, C.S., Hastings, T.S. and Amyes, S.G.B (1992a) Bactericidal activity of 4-quinolones, including flumequine, against *Aeromonas salmonicida.* (Eds. Michel, C. and Alderman, D.J.). Chemotherapy in aquaculture: from theory to reality. Office International des Epizooties, Paris. p.p 326-332.

Barnes, A.C., Lewin, C.S., Hastings, T.S., and Amyes, S.G.B. (1992b) Alterations in outer membrane proteins identified in clinical isolate of *Aeromonas salmonicida* subsp. *salmonicida*. Journal of Fish Diseases 15: 279-282.

Barnes, A.C., Hastings, T.S. and Amyes, S.G.B. (1995) Aquaculture antibacterials are antagonised by seawater cations. Journal of Fish Diseases, 18: 463-465.

Bartlett, J.G. (1992) Table of antimicrobial agents. (Eds. Gorbach, S.L., Bartlett, J.G., Blacklow, N.R.) Infectious Diseases. W.B. Saunders Company, London, pp. 358-370.

Baumann, P. and Baumann, L. (1981) The marine gram-negative bacteria. (Eds. Starr, Stolp, H., Truper, H.G., Balows, A. and Schlegel H.G.) The Prokaryotes. Springer-Verlag, Berlin. Vol. II, pp. 1302-1332.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M. (1996) Antibiotic sensitivity testing by standardised single disc method. Am. J. Clin. Pathol. 45: 493-496.

Bernardet, J.F., Kerouault, B., Michel, C. (1994) Comparative study on *Flexibacter maritimus* strains isolated from farmed sea bass (*Dicentrarchus labrax*). France in Fish Pathology 29(2): 105-111.

Bernoth, E.M. (1991) Possible Hazards due to fish drugs. Bulletin of European Association of Fish Pathology 11(1): 17

Berthe, F.C.J., Michel, C., Bernardet, J-F. (1995) Identification of *Pseudomonas anguilliseptica* isolated from several fish species in France. Diseases of Aquatic Organisms 21(2): 151-155.

Bjorklund, H.V. (1988) Determination of oxytetracycline in fish by high performance liquid chromatography. Journal of Chromatography, 432: 381-387.

Bjorklund, H.V. (1990) Analysis of oxolinic acid in fish by high-performance liquid chromatography. Journal of Chromatography, 530: 75-82.

Bjorklund, H.V., Bylund, G. (1990) Temperature-related absorption and excretion of oxytetracycline in rainbow trout (*Salmo gairdneri R.*). Aquaculture, 84(3-4): 363-372.

Bjorklund, H., Bondestam, I. and Bylund, G. (1990) Residues of Oxytetracycline in wild fish and sediments from fish farms. Aquaculture, 86: 359-367.

Bjorklund, H.V., Bondestan, J., Bylund, G. (1990) Residues of oxytetracycline in wild fish and sediments from fish farms. Aquaculture, 86: 359-367.

Bjorklund, H.V. (1991) Oxytetracycline and oxolinic acid as antibacterials in aquaculture analysis, pharmacokinetics and environmental impacts. Thesis for academic dissertation, Abo Akademi University.

Bjorklund, H.V. and Bylund, G. (1991) Comparative pharmacokinetics and bioavailability of oxolinic acid and oxytetracycline in rainbow trout (*Oncorhynchus mykiss*). Xenobiotica 21: 1511-1520.

Bjorklund, H.V., Raabergh, C.M.I., Bylund, G. (1991) Residues of oxolinic acid and oxytetracycline in fish and sediments from fish farms. Aquaculture, 97(1): 85-96.

Bjorklund, H.V., Eriksson, A. and Bylund, G. (1992) Temperature related absorption and excretion of oxolinic acid in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 102: 17-27.

Björnerot, L (2001) Review of the Community animal health /zoosanitary legislation concerning aquatic animals and products in 10nth International Symposium on Nutrition and Feeding of Fish – Feeding for Quality – Rhodes 2-7 June 2002.

Black, W.D., Ferguson, H.W., Byrne, P. and Claxton, M.J. (1991) Pharmacokinetic and tissue distribution study of oxytetracycline in rainbow trout following bolus intravenous administration. J. vet. Pharmacal. Therap. *14: 351-358.*

Blanch, A.R., Pinto, R.M., Jofre, J.T. (1990) Isolation and characterization of an *Edwardsiella sp.* strain, causative agent of mortalities in sea bass (Dicentrarchus labrax). Aquaculture, 88(3-4): 213-222.

Bordas, M.A., Balebona, M.C., Rodriguez-Maroto, J.M., Borrego, J.J., Morinigo, M.A, (1998) Chemotaxis of pathogenic Vibrio strains towards mucus surfaces of gilt-head sea bream (*Sparus aurata L.*). Applied and Environmental Microbiology 64(4): 1573-1575.

Bories, G.S.F., Peleran, J.C. and Wal, J.M. (1983) Liquid chromatographic determination and mass spectrometric confirmation of chloramphenicol residues in animal tissues. J. Assoc. Off. Chem. 66 (6): 1521-1525.

Boumaud, C.A.-F., Romestand, B., Bouix, G. (1992) Isolation and partial characterization of IgM-like seabass. Aquaculture 132(1-2): 53-58.

Bovo, G., Borghesan, F., Comuzzi, M., Ceschia, G., Giorgetti, G. (1994) "Winter Disease" in reared sea bream: Preliminary observations. III Convegno Nazionale Societa' italiana di patologica ittica., 1995. Bollettino Societa Italiana di patologia ittica 7(17): 2-11.

Bowser, P.R., Schachte, J.H. Jr., Wooster, G.A., Babish, J.G. (1990) Experimental treatment of *Aeromonas salmonicida* infections with enrofloxacin and oxolinic acid: Field trails. Journal of Aquatic Animal Health 2(3): 198-203.

Bowser, P.R. and Babish J.G. (1991) Clinical pharmacology and efficacy of fluoroquinolones. Fish. Annual Review of Fish Diseases 63-66.

Braaten, B. (1991) Impact of pollution from aquaculture in six Nordic countries. Release of nutrients, effects, and wastewater treatment. Aquaculture and the Environment., 1992. Special Publication, European Aquaculture Society 16: 79-101.

Brazil, G., Gurley, D., Gannon, F. and Smith, P. (1986) Persistence and acquisition of antibiotic resistance plasmids in *Aeromonas salmonicida*. Levy, S.B. and Novick, R.P.

(Eds.). Antibiotic resistance genes: ecology, transfer and expression. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY, pp. 107-114.

Breuil, G., Billard, R., Pauw, N.D.E. (comps) (1989) Bacterial diseases of reared sea bass (*Dicentrarchus labrax*) in France. Aquaculture Europe '89. Short communications and abstracts of review papers, films/slideshows and poster papers, presented at the International Aquaculture Conference held in Bordeaux, France, 2-4 October 1989, Special Publication, European Aquaculture Society 10: 277.

Brisinello, W., Fontebasso, S. (1991) First report on vaccination against bacterial diseases in Italian fish farms. Bollettino Societa Italiana di Patologia Ittica 5: 56-61.

British Society for Antimicrobial Chemotherapy (1991) A guide to sensitivity testing: Report of the working party on antibiotic sensitivity testing. Journal of Antimicrobial Chemotherapy, 27(suppl. D.): 1-47.

Brown, L.L., Albright, L.J., Evelyn, T.P.T. (1990) Control of vertical transmission of Renibacterium salmoninarum by injection of antibiotics into maturing female coho salmon Oncorhynchus kisutch. Diseases of Aquatic Organisms 9(2): 127-131.

Bruno, D.W. (1989) An investigation into oxytetracycline residues in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 12: 77-86.

Bryan, L.E. (1989) Two forms of antimicrobial resistance: bacterial persistence and positive function resistance. Journal of Antimicrobial Chemotherapy 23: 817-823.

Buchal, M.A., Langdon, C.J. (1998) Evaluation of lipid spray beads for the delivery of water-soluble materials to a marine suspension-feeder, the Manila clam *Tapes philippinarum* (Deshayes 1853). Aquaculture Nutrition 4(4): 263-274.

Buck, J.D. (1974) Effects of medium composition on the recovery of bacteria from seawater. Journal of Experimental Marine Biology and Ecology 15: 25-34.

Buck, J.D. (1979) The plate count in aquatic microbiology. (Costerton, J.W. and Colwell, R. eds.) Native aquatic bacteria: Enumeration activity and ecology. American Society for Testing Materials, Philadelphia, pp. 19-28.

Bumguardner, B.W., King, T.L. (1996) Toxicity of oxytetracycline and calcein to juvenile striped bass. Transactions of the American Fisheries Society 125(1): 143-145.

Burns, B.G., Riet, J.M. Van De., Landry, J.G., Gilgan, M.W. (1996) Liquid chromatographic determination of oxytetracycline content in fish tissue. Canadian technical report of fisheries and aquatic sciences/Rapport technique canadien des sciences halieutiques et aquatiques. Imprint varies 34 p.p.

Capone, D.G., Weston, D.P., Miller, V., Shoemaker, S. (1996) Antibacterial residues in marine sediments and invertebrates following chemotherapy in aquaculture. Aquaculture, 145: 55-75.

Carss, D.N. (1990) Concentrations of wild and escaped fishes immediately adjacent to fish farm cages. Aquaculture 90: 29-40.

Castro, R., Couso, N., Obach, A., Lamas, J. (1999) Effect of different beta -glucans on the respiratory burst of turbot (*Psetta maxima*) and gilthead seabream (*Sparus aurata*) phagocytes. Fish & Shellfish Immunology 9(7): 529-541.

Ceschia, G., Giorgetti, G., Bovo, G.G., Danielis, L. (1992) Results of in vitro sensitivity of the main bacteria agents isolated from fish farmed in Italy. Bollettino Societa Italiana di Patologia Ittica 5(11): 21-29.

Choo, P.S. (1997) Oxytetracycline residues in muscle of red tilapia medicated orally and cultured in brackish water. Asian fisheries science. Metro Manila 10(1): 75-81.

Christofilogiannis, P. (1992) Effects of antibiotics application in Aquaculture: Antibiotic Resistance transfer among environmental bacteria under commercial fish farm conditions. MSc Thesis University of Plymouth

Christofilogiannis, P. (1993) The veterinary approach to sea bass and sea bream. Aquaculture for Veterinarians: Fish Husbandry and Medicine, Brown L. (ed.). Pergamon Press 1993.

Christofilogiannis, P., Giagnisi, M., Alexis, M., Karanikolas, K. (1994) Histological changes in fish suffering from the "Winter syndrome". 1st International Symposium on Marine Fish Culture, Training Center of the Agricultural Bank of Greece, December 1-3 1994, Athens.

Christofilogiannis, P., Alexis, M., Richards, R. (1995) Preliminary studies on the levels of oxolinic acid in the muscle of sea bream (*Sparus auratus*) after oral treatment. European Association of Fish Pathologists. Seventh International Conference: Diseases of Fish and Shellfish. Palma de Mallorca 10 - 15 September 1995.

Christofilogiannis, P. (1996) Interpretation of the Laboratory Results: A Training Manual for Aquaculture Industry. Fish Health Management in sea bass and sea bream farming, National Aquaculture Centre, Malta 9-12 October 1996.

Christofilogiannis, P., Yiagnisis, M., Rigos, G., Andriopoulou, A., Koutsodimou, M., Anastasopoulou, G., Alexis, M., Richards, R.H. (1997) Study of the antibiotic resistance of fish bacterial pathogens reported in Greek Maricultures during the period 1994 – 1996. 5th Panellinion Symposium in Oceanography and Fisheries, Kavala,

Greece 15-18 of April 1997. Proceedings. Fisheries, Aquaculture, Inland Waters, NCMR Association of Employees, 2: 177-179.

Christofilogiannis, P., Rigos, G., Giagnisi, M., Andriopoulou, A., Koutsodimou, M., Anastasopoulou, G., Alexis, M., Richards, R.H. (1997a) National Centre for Marine Research, Fish Nutrition & Fish Diseases Laboratory: Histopathological diagnostic findings of fish diseases reported in Greek Maricultures during the period 1995 – 1996. 5th Panellinion Symposium in Oceanography and Fisheries, Kavala, Greece 15-18 of April 1997.

Christofilogiannis, P., Alexi, M.N., Richards, R.H. (1997b) Antibiotics use in Greek maricultures: Study of the Oxolinic acid retention time in gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*), at high temperatures after oral treatment. 5th Hellenic Symposium on Oceanography and Fisheries. Kavala, Greece, April 15-18, 1997. Proceedings. Fisheries, Aquaculture, Inland Waters. 2: 181-184.

Christofilogiannis, P., Alexis, M., Richards, R.H. (1997c) Pharmacokinetics of Oxolinic acid in the muscle of sea bass (*Dicentrarchus labrax*) after oral treatment, at high temperature. Martinique 97, Island and Tropical Aquaculture, Les Trois-Illets, Martinique, F.W.I May 4-9, 1997.

Christofilogiannis, P., Alexis, M., Richards, R.H. (1999) Comparative Kinetics of oxytetracycline and oxolinic acid in Sea bass (*Dicentrarchus labrax*) and Sea bream (*Sparus aurata*) kept at two water temperatures. European Association of Fish Pathologists Ninth International Conference: Diseases of Fish and Shellfish. Rhodos Palace Convention Centre, 19 -24th of September 1999, Rhodes, Greece.

Christofilogiannis, P. (2000) Codes of Practice in Southern Europe. Maraqua 2nd Workshop Implications of Current and Proposed International Directives Conventions and National Initiatives IMBC - Heraklion Crete 20-22 March 2000.

Christofilogiannis, P. (2001) Current inoculation methods in MIC determination. Aquaculture, 196: 297-302.

Christofilogiannis, P. (2002) Main Sanitary Problems in European Fish Farming and needs: The Producers Point of View. Fish Health Workshop EAS - Aquaculture Europe conference. Trieste, Italy, October 16-19, 2002.

Christofilogiannis, P. (2002) Product Quality, Safety and Public Image-Self regulation EAS. Aquaculture Europe conference, Trieste, Italy, October 16-19, 2002.

Coeurdacher, J.L., Pepin, J.F., Fauvel, C., Legall, P., Bourmaud, A.F., Romestand, B. (1997) Alterations in total protein, IgM and specific antibody activity of male and female sea bass in Fish & Shellfish Immunology. 7(3): 151-160.

Cohen, M.L. (1992) Epidemiology of drug-resistance: Implications for a post antimicrobial era. Science, 257: 1050-1055.

Colorni, A. (1990) Pathology of marine warm water finfish in Israel: Problems and research. Advances in Tropical Aquaculture: Workshop held in Tahiti, French Polynesia, February 20 - March 4 1989. Actes de colloques. Institut Francais de Recherche pour l'Exploitation de la Mer. Brest. (9): 133-142.

Colorni, A., Paperna, I., Gordin, H. (1981) Bacterial Infections in Gilt-Head Sea Bream in *Sparus aurata* Cultured at Elat. Aquaculture, 23(1-4): 257-267.

Colorni, A. (1992) A systemic mycobacteriosis in the European sea bass *Dicentrarchus labrax* cultured in Eilat (Red Sea). Israeli Journal of Aquaculture/Bamidgeh 44(3): 75-81.

Colorni, A., Ankaous, M., Diamant, A., Knibb, W. (1993) Detection of mycobacteriosis in fish using the polymerase chain reaction technique. Bulletin of European Association of Fish Pathology, 13: 195-198.

Colorni, A., Avtalion, R., Knibb, W., Berger, E., Colorni, B., Timan, B. (1998) Histopathology of sea bass (*Dicentrarchus labrax*) experimentally infected with Mycobacterium marinum and treated with streptomycin and garlic (*Allium sativum*) extract. Aquaculture 160(1-2): 1-17.

Combes, B., Whalley, P.J. and Adams, R.H. (1972) Clinical manifestations of tetracycline toxicity. Bruno OTC residues 1989, JFD 12: 77-86.

Commission Decision 1999/567/EC (OJ L 216, 14.8.1999, p. 13)

Commission Decision 1999/766/EC (OJ L 302, 25.11.1999, p. 23)

Commission Decision 2000/574/EC (OJ L 240, 23.9.2000, p. 26)

Commission Decision 2001/183/EC (OJ L 67, 9.3.2001, p. 65)

Commission Decision 2001/288/EC (OJ L 99, 10.4.2001, p. 11)

Commission Decision 2001/293/EC (OJL 100, 11.4.2001, p. 30)

Commission Decision 2001/312/EC (OJ L 109, 19.4.2001, p. 66)

Commission Decision 2001/313/EC (OJ L 109, 19.4.2001, p. 67)

Commission Decision 2001/494/EC (OJ L 180, 3.7.2001, p. 34)

Commission Decision 93/169/EEC (OJ L 71, 24.3.1993, p. 16)

Commission Decision 93/22/EEC (OJ L 16, 25.1.1993, p. 8)

Commission Decision 93/55/EEC (OJ L 14, 22.1.1993, p. 24)

Commission Decision 94/306/EC (OJ L 133, 28.5.1994, p. 51)

Commission Decision 95/352/EC (OJ L 204, 30.8.1995, p. 13)

Commission Decision 96/490/EC (OJ L 202, 10.8.1996, p. 21)

Commission Decision 98/24/EC (OJ L 8, 14.1.1998, p. 26)

Council Directive 2001/572/EC (OJ L 203, 28.7.2001, p. 16)

Council Directive 2000/27/EC (OJ L 114, 13.5.2000, p. 28)

Council Directive 91/67/EEC (OJ L 46, 19.2.1991, p. 1)

Council Directive 93/53/EC (OJ L 175, 19.7.1993, p. 23)

Council Directive 95/70/EEC (OJ L 332, 30.12.1995, p. 33)

Council Directive 98/45/EC (OJ L 189, 3.7.1998, p. 12)

Communities, No L 214/1. 18 August. Council of the European Communities (1996) Council Directive no. 96/23/EC on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/E C and Decisions 89/187/EEC and 91/664/EEC. Official Journal of the European Communities, No. L 125/10, 29 April.

Colwell, R.R., Grimes, D.J. (1984) Vibrion diseases of marine fish populations. Helgolander Meeresuntersuchungen 37: 265-287.

Conte, F.P. (1969) Salt secretion. Fish physiology. Hoar, W.S., Randall, D.J. (Eds.). Academic Press, New York: 241-292.

Contessi, B., Volpatti, D., Gusmani, L., Bovo, G., Maltese, C., Mutinelli, F., Borghesan, F., Gennari, L., Sarli, G., Galeotti, M. (2000) "Winter Disease" in farmed Gilthead Sea Bream: immunological aspects. Bollettino Societa Italiana di Patologia Ittica, 12(28): 2-16.

Cougher, J.P., and Stewart, G.J. (1989) Genetic exchange in the environment. Ant. Leeuwenhoek 55: 15-22.

Cox, W.R., Rainnie, D.J. (1991) Oxytetracycline for the treatment of vibriosis in Atlantic salmon. 8. Annual Meeting of the Aquaculture Association Of Canada, Bull. Aquacult. Assoc. Canada, 91(3): 50-52.

Coyne R., Hiney, M. and Smith, P. (1993) Factors influencing the persistence of OTC in the sediments under fish cages. EAFP, 6th international conference "Diseases of fish and shellfish", Brest, France

Coyne, R., Hiney, M., O'Connor, B., Kerry, J., Cazabon, D. and Smith, P. (1994) Concentration and persistence of oxytetracycline in sediments under a marine salmon farm. Aquaculture 123, 31-42.

Coyne, R., Hiney, M. and Smith, P. (1994a). Evidence associating overfeeding on a salmon farm with a prolonged half-life of oxytetracycline in under cage sediments. Bulletin of the European Association of Fish Pathologists 14:207-210.

Coyne, R., Hiney, M. and Smith, P. (1997) Transient presence of Oxytetracycline in blue mussels *(M.vtilus edulis)* following its therapeutic use at a marine salmon farm. Aquaculture, 149,175-181.

Cravedi, J.P., Choubert, G. and Delous, G. (1987) Digestibility of chloramphenicol, oxolinic acid and oxytetracycline in rainbow trout and influence of these antibiotics on lipid digestibility. Aquaculture 60, 133-141.

Cruz e Silva, M.P., Freitas, M.S., Orge, M.L. (1997) Coinfection by monogenetic trematodes of the genus *Microtyle* V. Beneden & Hesse 1863, *Lamellodiscus ignoratus Palombi*, 1943, the protozoan *Trichodina sp.Ehrenberg*, 1838 and the presence of epitheliocyctic, *Vibrio algynoliticus* and *V. Vulnificus* in cultured seabream (*Sparus aurata L.*) in Portugal. Bulletin of the European Association of Fish Pathologists. Weymouth, 17(2): 40-42.

Curtis, L.R., Kemp, C.J., Svec, A.V. (1986) Biliary excretion C-taurocholate by rainbow trout is stimulated at warmer acclimation temperature. Comparative Biochemistry and Physioliology 84C, 87-90.

Cusack, R.R., Groman, D.B., MacKinnon, A.M., Kibenge, F.S.B., Wadowska, D. Brown, N. (2001) Pathology associated with an aquareovirus in captive juvenile Atlantic halibut *Hippoglossus hippoglossus* and an experimental treatment strategy for a concurrent bacterial infection. Diseases of Aquatic Organisms, 44(1): 7-16.

Dalsgaard, I., Bjerregaard, J. (1991) Enrofloxacin as an antibiotic in fish. Proceedings of the 5th congress of the European Association for Veterinary Pharmacology and Toxicology. pp. 300-301.

Dalsgaard, I., Madsen, L. (2000) Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. Journal of Fish Diseases, 23(3):199-209.

Datta, N. (1971) R factors in *E.coli.* Annales New York Academic Science 182: 59-64.

Dauble, D.D. and Curtis, L.R. (1989) Rapid branchial excretion of dietary quinoline by rainbow trout (*Salmo gairdneri*). Canadian Journal of Fisheries and Aquatic Sciences 46: 705-713.

Dec, C., Angelidis, P., Baudin-Launcerin, F. (1990) Effects of oral vaccination against vibriosis in turbot *scopthalmus maximus* (L.) and sea bass *Dicentrarchus labrax.* Journal of Fish Diseases, 13: 369-376.

De Paola, A., Flynn, P.A., McPearson, R.M. and Levy, S.B. (1988) Phenotypic and genotypic characterization of tetracycline-and oxytetracycline-resistant *Aeromonas hydrophilla from* cultured Channel catfish (*Ictalurus punctatus*) and their environments. Applied Enironmental Microbiology 54: 1861-1863.

DeGrandis, S.A. and Stevenson, R.M.W. (1985) Antimicrobial susceptibility patterns and R plasmidmediated resistance in the fish pathogen *Yersinia ruckeri.* Antimicrabial Agents and Chemotherapy, 27: 938-942.

Degroodt, J.M., Wyhowski de Bukanski, B., Srebrnik, S. (1994) Oxolinic acid and flumequine in fish tissues: Validation of an HPLC method; Analysis of medicated fish and commercial fish samples. Joumal of Liquid Chromatography, 17(8): 1785-1794.

Del Rio-Rodriguez, R.E., Inglis, V., Millar, S.D. (1997) Survival of *Escherichia coli* in the intestine of fish. Aquaculture Research 28(4): 257-264.

Della Rocca, G., Zaghini, A., Magni, A., Zanoni, R., Zanchetta, S., Sello, M., Malvisi, J., Sanguinetti, V., Ceschia, G. (1998) Pharmacokinetics of amoxicillin in sea bream (*Sparus aurata*) after oral and intravenous administrations. 1: comparation of serum concentrations evaluated with two different analytical methods. Bollettino Societa Italiana di Patologia Ittica, 10(24): 45-50.

Deniz, M.S., Real, F., Acosta, B., Acosta, F., Oros, J. (1993) *Vibrio harveyi* as an opportunistic pathogen on gilthead seabream (*Sparus aurata*). Actas Del IV Congreso Nacional de Acuicultura, Centro de investigaciones marinas, Pontevedra (Spain), 1993, pp. 593-598.

Diamant, A., Banet, A., Ucko, M., Colorni, A., Knibb, W., Kvitt, H. (2000) Mycobacteriosis in wild rabbitfish *Siganus rivulatus* associated with cage farming in the Gulf of Eilat, Red Sea. Diseases of Aquatic Organisms, 39(3): 211-219.

Domenech, A., Fernandez-Garayzabal, J.F., Lawson, P., Garcia, J.A., Cutuli, M.T., Blanco, M., Gibello, A., Moreno, M.A., Collins, M.D., Dominguez, L. (1997) "Winter disease" outbreak in sea-bream (*Sparus aurata*) associated with *Pseudomonas anguilliseptica* infection. Aquaculture, 156(3-4): 321-330.

Domenech, A., Fernandez-Garayzabal, J.F., Garcia, J.A., Cutuli, M.T., Blanco, M., Gibello, A., Moreno, M.A., Dominguez, L. (1999) Association of *Pseudomonas anguilliseptica* infection with "winter disease" in sea bream, *Sparus aurata* L. Journal of Fish Diseases 22(1): 69-71.

Dos Santos, N.M.S., Taverne, N., Taverne-Thiele, A.J., De Sousa, M. (1997) Characterisation of monoclonal antibodies specific for sea bass. Fish and Shellfish Immunology, 7(3): 175-191.

Doukas, V., Athanassopoulou, F., Karagouni, E., Dotsika, E. (1998) *Aeromonas hydrophila* infection in cultured sea bass, *Dicentrarchus labrax* L., and *Puntazzo puntazzo* Cuvier from the Aegean Sea. Journal of Fish Diseases, 21(4): 317-320.

Dowling, H.F. and Lepper, M.H. (1964) Hepatic reactions to tetracyclines. Journal of the American Medical Association 188: 307-309.

Drlica, K. and Coughlin, S. (1989) Inhibitors of DNA gyrase. Pharmacology and Therapeutics 44: 107-122.

Du, W.X., Marshall, M.R., Wheeler, W.B., Mathews, M., Gatlin, D., Rawles, S.D. (1995) Oxytetracycline, sulfadimethoxine, and ormetoprim residues in channel catfish by HPLC. Journal of Food Science, 60(6): 1220-1224.

Du, W.X., Marshall, M.R., Xu, D.H., Santerre, C.R., Wei, C.I. (1997) Retention of oxytetracycline residues in cooked channel catfish fillets. Journal of Food Science, 62(1): 119-122.

Duis, K., Inglis, V., Beveridge, M.C.M., Hammer, C. (1994) Leaching of four different antibacterials from oil- and alginate-coated fish-feed pellets. Aquaculture and water resource management: An International Symposium held at the University of Stirling, 21-25 JUNE 1994. Aquaculture Research, 26(8): 549-556.

DuPont H., Steele J.H. (1987) Use of Antimicrobial Agents in Animal Feeds: Implications for Human Health. Reviews of Infectious Diseases, 9(3): 447-460.

Easter M.C. and Gibson D.M. (1989) Determination of microorganisms by electrical measurements. Rapid methods in food microbiology, 26 M.R. Adams & CFA Hope ed., Elsevier, pp. 57-100.

Egidius, E., Andersen, K. (1979) The use of Furanace against vibriosis in rainbow trout *Salmo gairdneri* Richardson in salt water. Journal of Fish diseases, 2: 79-80.

Elema, M.O., Hoff, K.A., Kristensen, H.G. (1996) Bioavailability of oxytetracycline from medicated feed administered to Atlantic salmon (*Salmo salar* L.) in seawater. Aquaculture, 143(1): 7-14.

Ellis, A.E., Roberts, R.J., Tytler, P. (1978) The Anatomy and Physiology of Teleost. (Roberts, R.J. Ed.), Fish Physiology, Balliere Tindall, London, pp. 13-54.

Ellis A.E. (1991) Tissue residues of chemotherapeutants in fish. Bulletin of European Association of Fish Pathologists 11(1), 22.

Endo, T., Ogoshima, K., Hayasaki, S., Kaneko, S. and Ohshima, S. (1973a) Application of oxolinic acid as a chemotherapeutic agent against infectious diseases of fish. I. Antibacterial activity, chemotherapeutic effect and pharmacokinetic effect of oxolinic acid in fish. Bulletin of the Japanese Society of Science and Fisheries 39: 165-171.

Endo, T., Sakuma, M., Tanaka, H., Ogishima., K. and Hara, T. (1973b) Application of oxolinic acid as a chemotherapeutic agent against infectious diseases of fish. I. Study of chemotherapeutic effect by whole body autobacteriography. Bulletin of the Japanese Society of Science and Fisheries 39, 173-177.

Endo, T., Onozawa, M., Hamaguchi, M. and Kusuda, R. (1987) Enhanced Bioavailability of Oxolinic Acid by Ultra-fine Size Reduction in Yellowtail. Nippon Suisan Gakkaishi 53(10): 1711-1716.

Endo, T. and Onozawa, M. (1987) Effects of pH and Temperature on the Uptake of Oxolinic Acid by Goldfish Nippon. Suisan Gakkaishi 53(4) 551-555.

Ervik, A., Samuelsen, O.B., Juell, J.E. and Sveer, H. (1994a) Reduced environmental impact of antibacterial agents applied in farms using the Liftup feed collector system or a hydroacoustic feed collector. Diseases of Aquatic Organisms 19: 101-104.

Ervik, A., Thorsen, B., Eriksen, V., Lunestad, B.T. and Samuelsen, O.B. (1994b) Impact of administering antibacterial agents on wild fish and blue mussels *Mytilus edulis* in the vicinity of fish farms. Diseases of Aquatic Organisms 18: 45-51.

Esteban, M.A., Meseguer, J. (1997) Factors influencing phagocytic response of macrophages from the sea bass. Anatomical Record, 248(4): 533-541.

Fabris, A., Mazzolini, E., Magni, A., Ceschia, G., Vismara, D., Passera, A., Giorgetti, G. (1998) Vaccination trials with *Pasteurella piscicida* different preparations and correlation with humeral immunity in sea bass (*Dicentrarchus labrax*). Bollettino Societa Italiana di Patologia Ittica, 10(24): 2-9.

Fanelli, G.M. and Nigrelli, R.F. (1963) Renal excretion of tetracycline in the aglomerular toadfish, *Opsanus tau*. Proceedings, Experimental Biology and Medicine 114, 582-584.

Farrar, W.E. (1985) Antibiotic resistance in developing countries. J. Infect. Dis. 152: 1103-1106.

FDA (Food and Drug Administration) (1994) Advisory committees discuss fluoroquinolones. FDA Veterinarian *(luly/Aug* st):2-5.

Femardes, M.H.B. (1977) Bacterial disease of sea bass *Dicentrarchus labrax* reared in the laboratory. An approach to treatment. Aquaculture, 10: 317-322.

Fribourgh, J.H., Meyer, F.R. and Robinson, J.A. (1969) Oxytetracycline leaching from medicated fish feeds. Technical Papers of the Bureau of Sport Fisheries and Wildlife 40: 1-7.

Gauthier, M.J. (1984) Heterotrophic bacterial flora from necrosis of marine fishes: Phenolyacal analysis and resistance to heavy metals and antibiotics. Proceedings of the international symposium No. 331 on Marine Bacteriology CNRS Marseille, Bianchi, A. (ed.) Colloq. Int. CNRS: 179-183.

Genovese, L., Micale, V. (1991) Experimental infection of *Dicentrarchus labrax* with NCIMB 6 and NCIMB 1291 Vibrio strains. Bollettino Societa Italiana di Patologia Ittica 6(1-4): 1991.

Giles, J.S., Hariharan, H. and Heaney, S.B. (1991) Evaluation of six antimicrobial agents against fish pathogenic isolates of *Vibrio anguillarum*, *Vibrio ordalii* and *Aeromonas salmonicida*. Bulletin Aquaculutre Association of Canada 91: 53-55.

Ginkel, L.A. van, Rossum, H.J. van, Zoontjes, P.W., Blitterswijk, H. van, Ellen G., Heeft, E. van der, De Jong, M. and Zomer, G. (1990) Development and validation of a gas chromatographic – mass spectrometric procedure for the identification and quantification of residues of chrloramphenicol. Analytical Chimica Acta 237: 61-69.

Giorgetti, G., Ceschia, G., Perantoni, P., Zamburlini, S., Ingle, E., Bossu, M., Tibaldi, E. (1990) Evaluation of infection resistance and to growth of sea bass. Bollettino Societa Italiana di Patologia Ittica, 5: 50-55.

Giorgetti, G., Ceschia, G. (1994) Pasteurellosis in the Mediterranean area: Experiences from Italy. 3rd International marine biotechnology conference: program, abstracts and list of participants, Tromsoe University, Tromsoe (Norway) p. 63.

Giroud J.P. (1992) Incidents et accidents des antibiotiques. (Michel, C.M. and Alderman, D.J. Eds) Chemotherapy in aquaculture: from theory to reality. Office International des Epizooties, Paris, pp.143-153.

Godwin, D. and Slater, H.J. (1979) The influence of growth environment on the stability of a drug resistance plasmid in *Eschericia coli* K 12. Joumal of General Microbiology 111: 201-210.

Goodman, A.E., Hild, E., Marshall, K.C. and Hermansson, M. (1993) Conjugative plasmid transfer between bacteria under simulated marine oligotrophic conditions. Appl. Environ. Microbiol. 59: 1035-1040.

Grave, K., Engeltad, M., Soli, N.E. and Hastein, T. (1990) Utilization of antibacterial drugs in salmonid farming in Norwa during 1980-1988. Aquaculture 86: 347-358.

Griffiths, R.P., Moyer, C.L., Caldwell, B.A. and Morita, R.Y. (1990) Long-term starvation-induced loss of antibiotic resistance in bacteria. Microbial Ecology 19: 251-257.

Grisez, L., Reyniers, J., Verdonck, L., Swings, J., Ollevier, F. (1997) Dominant intestinal microflora of sea bream and sea bass larvae, from two hatcheries, during larval development. Aquaculture, 155(1-4): 391-403.

Grondel, J.L. and. Boesten, J.A.M. (1982) The influence of antibiotics on the immune system. I. Inhibition of the itogenic leukocyte response *in vitro* by oxytetracycline. Developmental and Comparative Immunology, Supplement 2: 211-216.

Grondel, J.L., Gloudemans, A. G. and Van Muiswinkel, W. B. (1985) The influence of antibiotics on the immune system II. Modulation of fish leukocyte responses in carp. Veterinary Immunology and Immunopathology 9:251-260.

Grondel, J.L., Nouws, F.M. and Van Muiswinkel, W.B. (1987) The influence of antibiotics on the immune system. Journal of fish Diseases, 10: 35-43.

Grondel, J.L., Nouws, J.F.M., De Jong, M., Schoutte, A.R. and Driessens, F. (1987a) Pharmacokinetics and tissue distribution of oxytetracycline in carp (*Cyprinus carpio* L.) following different routes of administration. Fish Diseases, 10: 153-163.

Grondel, J.L., Nouws, J.F.M., Schoutte, A.R. and Driessens, F. (1989) Comparative pharmacokinetics of oxytetracycline in rainbow trout (*Salmo gairdneri*) and African catfish (*Clarias gariepinus*). J. Vet. Pharmacol. Ther. 12: 157-162.

Grondel, J.L., Nouws, J.F.M. and Haenen, O.L.M. (1992) Fish and antibiotics: pharmacokinetics of sulfadimidine in carp *(Cyprinus carpio).* Veterinary Immunology and imunopathology, 12:281-286.

Guichard, B. (2000) The becoming of a medicated feed in the digestive tract of turbot (*Psetta maxima*, L. 1758), for the study of the excretion of an antibacterial agent in marine fish culture). Ecole Natl. veterinaire ENVT, Nantes (France), 111 pp Author's Degree: Thesis (Dr. Veterinaire); Incl. bibliogr.: 96 ref

Hansen, K.P., Lunestad, B.T. and Samuelsen, O.B. (1992a) Effects of oxytetracycline, oxolinic acid and flumequine on bacteria in an artificial marine fish farm sediment. Canadian Journal of Microbiology 38: 1307-1312.

Hansen, K.P., Lunestad, B.T. and Samuelsen, O.B. (1992b) Ecological effects of antibiotics and chemotherapeutants from fish farming. (Michel, C. and Alderman, D.J. Eds.) Chemotherapy in aquaculture: From theory to reality. Office International des Epizooties, Paris, pp. 174-178.

Haug, T., Hals, P.A. (2000) Pharmacokinetics of oxytetracycline in Arctic charr (*Salvelinus alpinus L.*) in freshwater at low temperature. Aquaculture, 186(3-4): 175-191.

Hawke, J.P., Piakas, S.M., Minton, R.V., McPhearson R.M., Sneider, T.G. and Guarino, A.M. (1987) Fish pasteurellosis of cultured striped bass *(Morone saxatilis)* in coastal Alabama. Aquaculture 65: 193-204.

Hedges, R.W., Smith, P. and Brazil, G. (1985) Resistance plasmids of Aeromonads. Journal of General Microbiology 131: 2091-2095.

Hektoen, H., Berge, J.A., Hormazabal, V. and Lunestad, B.T. (1995) Persistence of antibacterial agents in the marine sediments. Aquaculture, 133:175-184.

Herman, R.L. (1969a) Oxytetracycline in fish culture a review Bureau of Sport Fisheries and Wildlife. US Dept. Interior, Washington DC Technical Papers 31: 9.

Herman, R.L. (1969b) Oxytetracycline toxicity to trout. US Dept of the Interior, Fish and Wildlife Service: Fish Disease Leaflet 33: 3.

Herman, R.L., Collis, D. and Bullock, G.L. (1969) Oxytetracycline residues in different tissue of trout. Technical Papers of the Bureau of Sport Fisheries and Wildlife 37:1-9.

Herwing, N. (1979) Handbook of drugs and chemicals used in the treatment of fish diseases. Thomas, Springfield: 272.

Herwing, R.P. and Gray, J.P. (1997) Microbial response to antibacterial treatment in marine microcosms. Aquaculture 152: 139-154.

Herwing, R.P., Gray, J.P. and Weston, D.P. (1997) Antibacterial resistant bacteria in surficial sediments near salmon net-cage farms in Puget Sound, Washington. Aquaculture: 263-283.

Hjeltnes, B., Roberts, R.J. (1993) Vibriosis. Bacterial diseases of fish, Inglis, V., Roberts, R.J., Bromage, N.R. (eds.). New York, Halsted Press: 109-121.

Holtmannspotter, H., Their, H.P. (1982) Analysenmethod fur Ruckstande von sechs Sulfonamiden und Chloramphenicol durch Gasschromatographie an Glaskapillaren. Ditsch. Lebensmittel-Rundschau, 78 (10): 347-350.

Hoppe, H.G. (1976) Determination and properties of actively metabolising heterotrophic bacteria in the sea, investigated by means of micro-autoradiography. Marine Biology, 36: 291-302.

Hormazabal, V., Yndestad, M. (1994a) A rapid and time-effective assay for determination of oxolinic acid and flumequine in fish tissues by HPLC. Journal of Liquid Chromatography, 17(13): 2911-2917.

Hormazabal, V., Yndestad, M. (1994b) Simultaneous extraction and determination of oxolinic acid and flumequine in fish silage by HPLC. Journal of Liquid Chromatography, 17(13): 2901-2909.

Hormazabal, V., Yndestad, M. (2001) Simple assay for the determination of flumequine and oxolinic acid in fish muscle and skin by HPLC. Journal of Liquid Chromatography and Related Technologies, 24(1): 109-115.

Hoshino, Y., Horie, M., Nose, N. and Iwasaki, H. (1984) Determination of tetracyclines and macrolide residues in meats by high performance liquid chromatography. J. Food Hyg. Soc. Japan, 25: 430-435.

Huber, W.G. (1986) Allergenicity of antimicrobial drug residues. Drug residues in animals, Rico, G. (Ed). Academic press, London, pp. 33-50.

Hugh, R., Leifson, E. (1953) The taxonomic significance of fermentative versus oxidative Gram-negative bacteria. J. Bacteriol., 66: 24-26.

Hunn, J.B. (1982) Urine flow rate in freshwater salmonids: a review. Progressive Fish Culturist 44:119-126.

Husevag, B., Lunestad, B.T., Johannessen, P.J., Enger, O. and Samuelsen, O.B. (1991) Simultaneous occurrence of *Vibrio salmonicida* and antibiotic-resistant bacteria in sediments at abandoned aquaculture sites. Journal of Fish Diseases, 14: 631-640.

Husevag, B. and Lunestad, B.T. (1995) Presence of the fish pathogen *Aeromonas salmonicida* and bacteria resistant to antimicrobial agents in sediments from Norwegian fish farms. Bulletin of the European Association of Fish Pathologists, 15: 17-19.

Hustvedt, S.O., Salte, R. (1991) Distribution and elimination of oxolinic acid in rainbow trout (*Oncorhynchus mykiss Walbaum*) after a single rapid intravascular injection. Aquaculture, 92 (4): 297-303.

Hustvedt, S.O., Storebakken, T. and Salte, R. (1991) Does oral administration of oxolinic acid or oxytetracycline affect feed intake of rainbow trout? Aquaculture, 92: 109-113.

Hustvedt, S.O., Salte, R., Kvendset, O., Vassvik, V. (1991a) Bioavailability of oxolinic acid in Atlantic salmon (*Salmo salar L.*) from medicated feed. Aquaculture, 97 (4): 305-310.

Hustvedt, S.O., Salte, R., Vassvik, V. (1991b) Absorption, distribution and elimination of oxolinic acid in Atlantic salmon (*Salmo salar L.*) after various routes of administration. Aquaculture, 95(3-4):193-199.

Hustvedt, S.O., Salte, R., Vassvik, V. (1992) Combatting cold-water vibriosis in Atlantic salmon (*Salmo salar L.*) with oxolinic acid: A case report. Aquaculture, 103 (3-4):213-219.

Ikai, Y., Oka, H., Kawamura, N. and Yamada, M. (1989) Improvement of chemical analysis of antibiotics. Journal of Chromatography, 477:397-406.

Inglis, V., Frerichs, G.N., Millar, S.D. and Richards, R.H. (1991) Antibiotic resistance of *Aeromonas salmonicida* isolated from Atlantic salmon (*Salma salar* L.) in Scotland. Journal of Fish Diseases, 14, 353-358.

Inglis, V., Millar, S.D. and Richards, R.H. (1993) Resistance of *Aeromanas salmonicida* to amoxicillin. Journal of Fish Diseases, 16:389-395.

Inglis, V., Yimer, E., Bacon, E.J. and Ferguson, S. (1993a) Plasmid-mediated antibiotic resistance in *Aeromonas salmonicida* isolated from Atlantic salmon (*Salmo salar* L.) in Scotland. Journal of Fish Diseases, 16:593-600.

Inglis, V., Palmer, R., Shatwell, J.P., Branson, E.J. and Richards, R.H. (1993b) Amoxicillin concentrations in the serum of Atlantic salmon *(Salma salar* L.) during furunculosis therapy. Veterinary Record, 133: 617-621.

International Council For The Exploration Of The Sea (1987) Report of the ad hoc Study Group on "Environmental Impact of Mariculture". *CM 1987/F 2a: Sedimentation and Benthos pp. 9-11.*

International Council For The Exploration Of The Sea (1987a) Report of the ad hoc Study Group on Environmental Impacts of Mariculture" *CM 1989/F 11 Dunstaffnage Marine Laboratory, Oban, Scotland, 19.04-24.04.1989. Chemical usage in mariculture pp. 21-32*

International Council For The Exploration Of The Sea (1987b) Report of the ad hoc Study Group on "Environmental Impact of Mariculture" *CM 1987/F 2b: Chemicals in aquaculture pp. 11-15*

Intorre, L., Cecchini, S., Bertini, S., Cognetti Varriale, A.M., (2000) Pharmacokinetics of enrofloxacin in the seabass (*Dicentrarchus labrax*). Aquaculture, 182(1-2): 49-59.

Ishida, N. (1990) Comparison of tissue level of oxolinic acid in fresh and sea water fishes after the oral administration. Nippon Suisan Gakkaishi 56:281-286.

Ishida, N. (1992a) A study on the metabolic fate of oxolinic acid, a synthetic antibacterial agent, in cultured fishes. BULL. NATL. RES. INST. FISH. SCI. (JAPAN)/ CHUOSUIKEN KENPO., 4:1-58.

Ishida, N. (1992b) Tissue levels of oxolinic acid after oral or intravascular administration to freshwater and seawater rainbow trout. Aquaculture, 102:9-15.

Jacobsen, P. and Berglind, L. (1988) Persistence of oxytetracycline in sediments from fish farms. Aquaculture 70: 365-370.

Jacobsen, M.D. (1989) Withdrawal times of fresh water rainbow trout, *Salmo gairdneri Richardson*, after treatment with oxolinic acid, oxytetracycline and trimethoroprim. J. Fish Diseases, 12: 29-36.

Jawetz, E. (1987) Principles of antimicrobial drug action. Basic and Clinical pharmacology, B.G. Katzung (editor), 3rd edition, Appleton and Lange, Norwalk, Connecticut.

Jeney, G., Galeotti, M., Volpatti, D. (1994) Effect of immunostimulation on the non-specific immune response of sea bass, *Dicentrarchus labrax, L.* International Symposium on Aquatic Animal Health: Program and Abstracts., Univ. of Califomia, School of Veterinary Medicine, Davis, CA (USA), 1994, pp. p. P-76 NT.

Jobling, M. and Hjelmeland, K. (1992) Emaering og fordoyelse *Fiskens Fysiologi pp. 234-257.*John Grieg Forlag AS, Stavanger.

Johnsen, R. (1994) Effects of antibacterial agents on transformation of fatty acids in sediment from a marine fish farm site. Science of the Total Environment 152(2):143-152.

Jones, S.E. and Jago, C.F. (1993) *In situ* assessment of modification of sediment properties by burrowing invertebrates. Marine Biology 115: 133-142.

Joosten, P.H.M., Aviles-Trigueros, M., Sorgeloos, P., Rombout, J.H.W.M. (1995) Oral vaccination of juvenile carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*) with bioencapsulated *Vibrio anguillarum bacterin*. Fish & Shellfish Immunology 5(4): 289-299.

Jung, T.S., Thompson, K.D., Adams, A. (1999) A comparison of the antigenicity of Photobacterium *damsela subsp. piscicida* cultured either in vivo or in vitro. Fourth Symposium on Diseases in Asian Aquaculture: Aquatic Animal Health for Sustainability. November 22-26, 1999, Cebu International Convention Center, Waterfront Cebu City Hotel, Cebu City, Philippines. Book of abstracts.

Jung, T.S., Thompson, K.D., Morris, D.J., Adams, A., Sneddon, K. (2001) The production and characterization of monoclonal antibodies against Photobacterium *damselae ssp. piscicida* and initial observations using immunohistochemistry. Journal of Fish Diseases, 24(2): 67-77.

Kanzaki, M., Takeba, K., Murakani, F., Maruyama, T. and Matsumoto, M. (1984) An improvement of simplified detection method for residual sulfonamides in meat and fish. Ann. Rep. Tokyo Metr. Res. Lab. P.H. 35: 202-206.

Kapetanaki, M., Kerry, J., Hiney, M., O'Brien, C., Coyne, R., Smith, P. (1995) Emergence, in oxytetracycline-free marine mesocosms, of microorganisms capable of colony formation on oxytetracycline-containing media. Aquaculture, 134(3-4): 227-236.

Karakassis, I., Tsapakis, M., Hatziyanni, E. (1998) Seasonal variability in sediment profiles beneath fish cages in the Mediterranean. Mar. Ecol. Prog. 162: 243-252.

Kasuga, Y., Sugitani, A., Yamada, F., Arai, M. and Morikawa, S. (1984) Oxolinic acid residues in tissues of cultured rainbow trout and ayu fish. Journal of the Food Hygienic Society of Japan, 25: 512-516.

Kent, M.L. (1992) Diseases of seawater netpen-reared salmonid fishes in the Pacific Northwest. Canadian Special publication of Fisheries and Aquatic sciences 116:76.

Kerry, J., Hiney, M., Gilroy, D., Rudden, S. and Smith, P. (1993) Fish feed as a source of OTC resistant bacteria in the sediments under fish cages in EAFP, 6th international conference "diseases of fish and shellfish", Brest, France.

Kerry, J., Hiney, M., Coyne, R., Cazabon, D., NicGabhainn, S. and Smith, P. (1994) Frequency and distribution of resistance to oxytetracycline in microorganisms isolated from marine fish farm sediments following therapeutic use of oxytetracycline. Aquaculture 123: 43-54.

Kerry, J., Hiney, M., Coyne, R., NicGabhainn, S., Gilroy, D., Cazabon, D. and Smith, P. (1995) Fish feed as a source of oxytetracycline resistant bacteria in the sediments under fish farms. Aquaculture 131:101-113.

Kerry, J., Slattery, M., Vaughan, S. and Smith, P. (1996a) The importance of bacterial multiplication in the selection, by oxytetracycline-HCl, of oxytetracycline-resistant bacteria in marine sediment microcosms. Aquaculture 144: 103-119.

Kerry, J., Coyne, R., Gilroy, D. and Smith, P. (1996b) Spatial distribution of oxytetracycline and elevated frequencies of oxytetracycline resistance in sediments beneath a marine salmon farm following oxytetracycline therapy. Aquaculture 145: 31-39.

Kerry, J., NicGabhainn, S. and Smith, P. (1997) Changes in oxytetracycline resistance of intestinal microflora following oral administration of this agent to Atlantic Salmon (*Salmo salar* L.) smolts in a marine environment. Aquaculture 157:187-193.

Kim, M.S., Park, S.W., Huh, M.D., Jeong, H.D. (1998) Effect of temperature and bacterial infection on the absorption and elimination of oxolinic acid in Nile tilapia (*Oreochromis niloticus*). Journal of the Korean Fisheries Society. Pusan 31(5): 677-684.

Klein, B.U., Boehm, K.H. (1994) Examinations about transferable antibiotic resistance on obligate and facultative fish pathogenic bacteria. International Symposium on Aquatic Animal Health: Program and Abstracts. Univ. of California, School of Veterinary Medicine, Davis, CA (USA), 1994: 40-41.

Klein, B.U. (1995) Investigations on transferable antibiotic resistance through R-plasmids between obligate and facultative fish pathogenic bacteria. 1995, 132 pp. NT: Notes Bibliogr.: 159 ref. AB:

Kleinow, K.M., Jarboe, H.H., Shoemaker, K.E. and Greenless, K.J. (1994) Comparative pharmacokinetics and bioavailability of oxolinic acid in channel catfish *(Ictalurus punctatus)* and rainbow trout *(Oncorhynchus mykiss)*. Canadian Journal of Fisheries and Aquatic Sciences: 1205-1211.

Knibb, W.R., Colomi, A., Zlotkin, A., Ankaoua, M., Diamant, A. (1992) Detection and identification of marine mycobacteria using PCR anddirect sequencing of 16S ribosomal sequence. Israeli Journal of Aquaculture/Bamidgeh, 44(4): 141.

Knibb, W.R., Colomi, A., Ankaoua, M. (1993a) Identification and detection of a marine Mycobacterium sp. From *Dicentrarchus labrax* using direct sequencing of 16S ribosomal sequences and polymerase chain reaction. From Discovery to Commercialisation, European Aquaculture Soc., Oostende (Belgium), 1993, p. 402, Special Publication, European Aquaculture Society, 19.

Knibb, W.R., Colomi, A., Ankaoua, M., Lindell, D., Diamant, A., Gordin, H. (1993b) Detection and identification of a pathogenic marine mycobacterium from the European seabass *Dicentrarchus labrax* using polymerase chain reaction and direct sequencing of 16S rDNA sequences. Molecular Marine Biology and Biotechnology, 2(4): 225-232.

Koenings, J.P. and Lipton, J. (1986) Quantitative Determination of Oxytetracycline Uptake and Release by Juvenile Sockeye Salmon. Transactions of the American Fisheries Society 115: 621-629.

Kreutzman, H.L. (1977) The effects of chloramphenicol and oxytetracycline on hematopoiesis in the European eel (*Anguilla anguilla*). Aquaculture 10: 323-334.

Kusada, R., Itaoka, M., Kawai, K. (1988) Drug sensitivity of *Pasteurella piscisida* strains isolated from cultured Yellowtail from 1984 to 1985. Bull. Jap. Soc. Sci. Fish. 54: 1521-1526.

Kusada, R., Sugiura, H., Kawai, K. (1990) Drug sensitivity of *Pasteurella piscisida* strains isolated from cultured Yellowtail from 1986 to 1988. Bull. Jap. Soc. Sci. Fish. 56: 239-242.

Kusser, W.C., Newman, S.G. (1990) Detection of oxytetracycline residues in fish tissues using a sensitive bioassay. Journal of Fish Diseases, 13(6): 545-548.

Kusuda, R., Ninomiya, M., Hamaguchi, M., Muraoka, A. (1988) The efficacy of ribosomal vaccine prepared from *pasteurella piscicida* against pseudoturbeculosis in cultured yellowtail. Fish Pathology, 23: 191-196.

Lambert, H.P. and O'Grady, F.W.O. (1992) Antibiotics and chemotherapy. Churchill Livingstone Edinburgh, 562.

Le Breton, A.D. (1989) Heterotrophic microflora associated with an intensive rearing of two hatches of *Dicentrarchus labrax larvae*. Quantitative and qualitative studies sensitiveness to various Antibiotics. Ecole Nationale veterinaire, Toulouse: 72

Le Breton, A.D. (1999) Mediterranean finfish pathologies: present status and new developments in prophylactic methods. Bulletin of the European Association of Fish Pathologists, 19(6): 250-253.

Ledo, A., Dopazo,. C.P., Romalde, L., Toranzo, A.E. and Barja, I.L. (1987) Effectiveness of different chemotherapeutic agents for controlling bacterial fish diseases. Bulletin of the European Association of Fish Pathologists 20-22.

Lepper, M.H., Zimmerman, H., Carrol, G., Galdwell, E.R., Spies, H.W., Wolfe, C.K. and Dowling, H.F. (1951) Effect of large doses of aureomycin, terramycin and chloramphenicol on livers of mice and dogs. A.M.A. Archives of Internal Medicine 88: 284-295.

Levy, S.B. (1986) Ecology of antibiotic resistance determinants. Antibiotic resistance genes: ecology, transfer and expression. Levy, S.B. and Novick, R. (eds). Cold Spring Harbor Laboratory, New York, pp. 17-29.

Levy, S.B., Burke, J.P. and Wallace, C.K. (ed.) (1987) Antibiotic use and antibiotic resistance worldwide. Rev. Infect. Dis. 9 (Suppl.): 231-316.

Levy, S.B., Marshall, B., Schluederberg, S., Rowse, D. and Davis, J. (1988) High Frequency of Antimicrobial Resistance in Human Fecal Flora. Antimicrobial Agents and Chemotherapy, Dec. 1988: 1801-1806.

Lewin, C.S.,and Hastings T.S. (1990) In vitro activities of oxolinic acid, cyprofloxacin, and norfloxacin against *Aeromonas salmonicida*..Journal of Fish Diseases, 13:377-384.

Lewin, C.S. (1992) Mechanisms of resistance development in aquatic microorganisms. Chemotherapy in aquaculture: from theory to reality. C. Michel and D.J. Alderman, (eds). Office International des Epizooties, Paris., pp. 288-301

Lloyd, D.N. and Van der Merwe, D. (1987) The use of a diffusion test for the detection of antibiotics in the tissues of slaughter stock. Journal of the South African Veterinary association, 58: 183-186.

Locatelli, L., Wardle, R., Papadopoulou, G., Christofilogiannis, P., Yversen H.B. (1999) Good Clinical Practice (GCP) Field Trials of vaccination strategies for the prevention of Pastereullosis caused by *Photobacterium damsela* subsp. piscicida in farmed sea bass (*Dicentrarchus labrax*) and Sea bream (*Sparus auratus*). European Aquaculture Society Conference, Aquaculture Europe 99, Towards predictable quality conference 7-10th of August 1999, Trondheim, Norway.

Long, A.R., Hsieh, L.C., Malbrough, M.S., Short, C.R. and Barker, S.A. (1990) Matrix Solid Phase Dispersion Isolation and Liquid Chromatographic Determination of Sulfadimethoxine in *Catfish (Ictalurus punctatus)* Muscle Tissue. Assoc. Off Anal Chem., 73(6): 868-871.

Lopez, R.A., Pazos, F., Fouz, B., Skarmeta, A.M., Barja, J.L. (1993) Resistance to chemotherapeutic agents in bacteria associated to mortalities occurred in marine fish cultures. Actas del IV Congreso Nacional de Acuicultura, Centro de Investigaciones Marinas, Pontevedra (Spain), pp. 713-718.

Lunden, T., Miettinen, S., Loennstroem, L.G., Lilius, E.M., Bylund, G. (1998) Influence of oxytetracycline and oxolinic acid on the immune response of rainbow trout (*Oncorhynchus mykiss*). Fish & Shellfish Immunology, 8(3): 217-230.

Lunden, T., Bylund, G. (2000) The influence of in vitro and in vivo exposure to antibiotics on mitogen-induced proliferation of lymphoid cells in rainbow trout (*Oncorhynchus mykiss*). Fish & Shellfish Immunology, 10(5): 395-404.

Lunestad, B.T. and Goksoyr, J. (1990) Reduction in the antibacterial effect of oxytetracycline in sea water by complex formation with magnesium and calcium. Diseases of Aquatic Organisms 9: 67-79.

Lunestad, B.T., Samuelsen, O.B., Fjelde, S. and Ervik, A. (1995) Photostability of eight antibacterial agents in seawater. Aquaculture 134: 217-225.

Luzzana, U., Serrini, G., Moretti, V.M., Maggi, C.L., Valfre, F., Polidori, P. (1994) Effect of temperature and diet composition on residue depletion of oxytetracycline in cultured channel catfish. Analyst 119: 2757-2759.

Luzzana, U., Serrini, G., Moretti, V.M., Valfre, F. (1995) Residue depletion of oxytetracycline from muscle tissue of thermal aquaculture-reared channel catfish (*Ictalurus punctatus*) fed medicated diets with different energy content. Bollettino Societa Italiana di Patologia Ittica, 7(16): 48-55.

Mac Alister, E. and Partners (1999) Forward Study of Community Aquaculture. 3rd Thematic Conference PESCA The future of Aquaculture in Europe, Santiago De Compostella Spain, pp. 49-50.

MacDonnell, M.T., Colwell, R.R. (1985) Phylogeny of the Vibrionaceae and recommendations for two new genera, *Listonella* and *Shewanella*. Sys. Appl. Microbiol., 6: 171-182.

Madsen, L., Arnbjerg, J., Dalsgaard, I. (2001) Radiological examination of the spinal column in farmed rainbow trout *Oncorhynchus mykiss* (Walbaum): experiments with *Flavobacterium psychrophilum* and oxytetracycline. Aquaculture Research, 32(3): 235-241.

Magarinos, B., Santos, Y., Romalde, J.L., Rivas, C., Barja, J.L., Toranzo, A.E. (1992) Pathogenic activities of live cells and extracellular products of the fish pathogen *Pasteurella piscicida*. J. GEN. MICROBIOL., 138(12): 2491-2498.

Magarinos, B., Romalde, J.L., Barja, J.L., Toranzo, A.E., (1994) Evidence of a dormant but infective state of the fish pathogen *Pasteurella piscicida* in seawater and sediment. Applied and Environmental Microbiology, 60(1): 180-186.

Magarinos, B., Romalde, J.L., Santos, Y., Casal, J.F., Barja, J.L., Toranzo, A.E., (1994a) Vaccination trials on gilthead seabream (*Sparus aurata*) against Pasteurella piscicida. Aquaculture, 120(3-4): 201-208.

Magarinos, B., Pazos, F., Santos, Y., Romalde, J.L., Toranzo, A.E. (1995) Response of *Pasteurella piscicida* and *Flexibacter maritimus*. Diseases of Aquatic Organisms, 21(2): 103-108.

Magarinos, B., Romalde, J.L., Barja, J.L., Nunez, S., Toranzo, A.E. (1999) Protection of gilthead seabream against pasteurellosis at the larval stages. Bulletin of the European Association of Fish Pathologists. Weymouth, 19(4): 159-161.

Magarinos, B., Couso, N., Noya, M., Merino, P., Toranzo, A.E., Lamas, J. (2001) Effect of temperature on the development of pasteurellosis in carrier gilthead seabream (*Sparus aurata*). Aquaculture, 195(1-2): 17-21.

Magni, A., Mazzolini, E., Fabris, A., Ceschia, G., Vismara, D., Corradi, A., Passera, A., Giorgetti, G. (1998) Histopathological lesions by *Pasteurella piscicida* ECP in sea bass (*Dicentrarchus labrax*). Bollettino Societa Italiana di Patologia Ittica, 10(25): 32-39.

Malvisi, J., della Rocca, G., Anfossi, P., Giorgetti, G. (1996) Tissue distribution and residue depletion of oxytetracycline in sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) after oral administration. Aquaculture, 147(3-4): 159-168.

Maniatis, K., Morris, D.J., Adams, A., Pearson, M. (2000) Detection of *Photobacterium damsela* subspecies piscicida in fixed tissue sections using immunohistochemistry and antigen retrieval immunohistochemistry. Journal of Fish Diseases, 23(5): 343-347.

Market and Investment Consultants Ltd, Ergoplan SA and IMBC (1998). Market Research for Sea Bass and Sea Bream

Martinsen, B., Oppegaard, H., Wichstroem, R., Myhr, E. (1992) Temperature-dependent in vitro antimicrobial activity of four 4-quinolones and oxytetracycline against bacteria pathogenic to fish. Antimicrobial Agents & Chemotherapy, 36(8): 1738-1743.

Martinsen, B., Horsberg, T.E. (1994) Antimicrobial activities and pharmacokinetic properties of oxytetracycline, sulfadiazine/trimethoprim, enrofloxacin, RO 09-1168, flumequine and oxolinic acid in Atlantic salmon (*Salmo salar*). International Symposium on Aquatic Animal Health: Program and Abstracts., Univ. of California, School of Veterinary Medicine, Davis, CA (USA), 1994: 87.

Martinsen, B., Holsberg, T.E. and Burke. M. (1994) Multiple-dose pharmacokinetic and depletion studies of sarafloxacin in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 17:111-121.

Martinsen, B. and Horsberg, T.E. (1995) Comparative single-dose pharmacokinetics of four quinolones, oxolinic acid, flumequine, sarafloxacin and enrofloxacin in Atlantic salmon (*Salmo salar*) held in seawater at 10°C. Antimicrobial Agents and Chemotherapy, 39: 1059-1064.

Matsuoka, S., Kamada, S. (1995) Discharge of Pasteurella piscicida cells from experimentally infected yellowtail. Fish Pathology, 30(3): 221-225.

Mazzolini, E., Fabris, A., Ceschia, G., Vismara, D., Magni, A., Amadei, A., Passera, A., Danielis, L., Giorgetti, G. (1997) Pathogenic variability of *Pasteurella piscicida* during in vitro cultivation as a preliminary study for vaccine production. Journal of Applied Ichthyology, 14(3-4): 265-268.

McGill, A.S. and Hardy, R. (1992) Review of the methods used in the determination of antibiotics and their metabolites in farmed fish. Chemotherapy in aquaculture: from theory to reality, Michel, C.M. and Alderman, D.J. (Eds). Office International des Epizooties, Paris.

McPhearson, R.M., Depaola, A., Zywno, S.R., Motes, M.L. and Guarino, A.M. (1991) Antibiotic resistance in Gram-negative bacteria from cultured catfish and aquaculture ponds. Aquaculture 99: 203-211.

Meinertz, J.R., Gaikowski, M.P., Stehly, G.R., Gingerich, W.H., Evered, J.A. (2001) Oxytetracycline depletion from skin-on fillet tissue of coho salmon fed oxytetracycline medicated feed in freshwater at temperatures less than 9°C. Aquaculture, 198(1-2): 29-39.

Meitong, Li; Guo, Wenlin; Zhong, Feng; Zheng, Guoxing; Shen, Yalin; Zhou, Kai; Wang, Pei; Jiang, Jinfeng; mhu, Xinyuan (1997) Studies on the depletion of Oxytetracycline residues on eel (*Anguilla japonica*) tissues. Journal of fisheries of China/Shuichan Xuebao. Shanghai, 21(1): 39-43.

Michel, C. (1986) Practical value, potential dangers and methods of using antibacterial drugs. Fish. Rev. Sci. Tech. Off. Int. Epiz. 95: 659-675.

Michel, C. and Alderman, D.J. (1992) Chemotherapy in aquaculture: From theory to reality. Office International des Epizooties, Paris.

Minkoff, G., Blanch, A.R., Alsina, M., Jofre, J.T. (1992) Control of microflora associated with rotifers, *Brachionus plicatilis*. Israeli Journal of Aquaculture/ Bamidgeh, 44(4): 130.

Mirzargar, S.S., Soltani, M., Rostami, M. (2000) Assessment of residues of some antibiotics in experimental treatment of common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) using microbiological and chromatography-bioautography. Journal of the Faculty of Veterinary Medicine, University of Tehran, 55(1): 27-31.

Miyazaki, T., Nakauchi, R., Kubota, S.S. (1984) Toxicological examinations of oxolinic acid and nalidixic acid in yellowtail. Bull. Fac. Fish. Mie Univ., 11: 15-26.

Moller, H., Anders, K. (1986) Diseases and parasites of marine fishes. Kiel, Germany, Verlag Moller: 365.

Moster, G. (1986) Use of antibiotics in Norwegian aquaculture. Sogn og Fjordane Distriktchogskule, Sogndal.

Moutou, K.A., Braunbeck, T., Houlihan, D.F. (1997) Quantitative analysis of alterations in liver ultrastructure of rainbow trout *Oncorhynchus mykiss* after administration of the aquaculture antibacterials oxolinic acid and flumequine. Diseases of Aquatic Organisms, 29(1): 21-34.

Moutou, K.A., Burke, M.D., Houlihan, D.F. (1998) Hepatic P450 monooxygenase response in rainbow trout (*Oncorhynchus mykiss* (Walbaum)) administered aquaculture antibiotics. Fish Physiology and Biochemistry, 18(1): 97-106.

Moutzouris, C.I., Braunbeck, T., Mamuris, Z. (2000) Effects of oxolinic acid and flumequine on liver ultrastructure of rainbow trout (*Oncorhynchous mykiss*). 6th Hellenic Symposioum on Oceanography and Fisheries. Chios, Greece, May 23-26, 2000. Proceedings. Vol. 2: Fisheries, Inland waters, Aquaculture, 2: 355-357.

Muroga, K., Iida, M., Matsumoto, H., Nakai, T. (1986) Detection of *Vibrio anguillarum* from Waters. Bullettin of the Japanese society of Scientific Fisheries, 52(4): 641-647.

Nakajima, T., Suzuki, M., Harada, K., Inoue, M. and Mitsuchasi, S. (1983) Transmission of R-plasmids in *Vibrio anguillarum* to *Vibrio cholerae*. Microbiology and Immunology 27: 195-198.

Nakamura, S., Inoue, S., Shimizu, M., Iyobe, S. and Mitsuhashi, S. (1976) Inhibition of conjugal transfer of R-plasmids by pipemidic acid and related compounds. Antimicrobial Agents and Chemotherapy, 10: 779-785.

Nambari, R., Abedini, S., Law, F.C.P. (1996) Tissue distribution and elimination of oxytetracycline in sea water chinook and coho salmon following medicated feed treatments. Aquaculture 144: 27-38.

Namdari, R., Abedini, S., Albright, L., Law, F.C.P. (1998) Tissue Distribution and Elimination of Oxytetracycline in Sea-Pen Cultured Chinook Salmon *Oncorhyncus tshawytscha,* and Atlantic Salmon, *Salmo salar L.*, Following Medicated-Feed Treatment. Journal of Applied Aquaculture, 8(1): 39-51.

Namdari, R., Abedini, S., Law, F.C.P. (1999) A comparative tissue distribution study of oxytetracycline in rainbow trout, *Oncorhynchus mykiss* (Walbaum), and chinook salmon, *Oncorhynchus tshawytscha* (Walbaum). Aquaculture Research, 30(4): 279-286.

Neu, H.C. (1990) Penicillins. Principles and Practices of Infectious Diseases, 3rd Edition. Mandell, G.L., Dougas, R.G. and Bennet, G.E. (Eds). Churcill Livingstone, NY, pp.230-246.

Nordmo, R., Riseth, J.M.H., Varma, K.J., Sutherland, I.H., Brokken, E.S. (1998) Evaluation of florfenicol in Atlantic salmon, *Salmo salar L.*: efficacy against furunculosis due to *Aeromonas salmonicida* and cold water vibriosis due to *Vibrio salmonicida*. Journal of Fish Diseases, 21(4): 289-297.

Nordmo, R., Varma, K.J., Sutherland, I.H., Brokken, E.S. (1994) Florfenicol in Atlantic salmon, *Salmo salar L.*: Field evaluation of efficacy against furunculosis in Norway. Journal of fish diseases. Oxford, 17(3): 239-244.

Nose, N., Hoshino, Y., Kikuchi, Y., Horie, M., Saitoh, K. and Kawachi, T. (1987) Simultaneous Liquid Chromatographic Determination of Residual Synthetic Antibacterials in Cultured Fish. Assoc. Off. Anal. Chem., 70(4): 714-717.

Noya, M., Magarinos, B., Lamas, J. (1993) Histopathology associated with *Pasteurella piscicida* and its extracellular products (ECP) in seabream, *Sparus aurata*. Actas del IV Congreso Nacional de Acuicultura, Centro De Investigaciones Marinas, Pontevedra (Spain), 1993 : 599-604.

Noya, M., Magarinos, B., Lamas, J. (1995a) Interactions between peritoneal exudate cells (PECs) of gilthead seabream (*Sparus aurata*) and Pasteurella piscicida. A morphological study. Aquaculture, 131(1-2): 11-21.

Noya, M., Magarinos, B., Toranzo, A.E., Lamas, J. (1995b) Sequential pathology of experimental pasteurellosis in gilthead seabream *Sparus aurata*. A light- and electron-microscopic study. Diseases of Aquatic Organisms, 21(3): 177-186.

Nusbaum, K.E., Shotts, E.B. (1981) Action of selected antibiotics on four common bacteria associated with diseases of fish. Journal of Fish Diseases, 4: 397-404.

Nygaard, K., Lunestad, B.T., Hektoen, H., Berge, J.A., Hormazabal, V. (1992) Resistance to oxytetracycline, oxolinic acid and furazolidone in bacteria from marine sediments. Aquaculture, 104(1-2): 31-36.

O'Brien, T.F., DiGiorgio, K.C., Parsonnet, K.C., Kass, E.H. and Hopkins, J.D. (1993) Plasmid diversity in *Escherichia coli* isolated from processed poultry and poultry processors. Veterinary Microbiology 35: 243-255.

O'Grady, P., Palmer, R., Hickey, C. and Smith, P.R. (1986) Antibiotic therapy of furunculosis in freshwater and sea water. Pathology in Marine Aquaculture. Vivares, C.P., Bonami, J.R. and Jaspers, E. (eds). European Aquaculture Society, No 9, Bredene, Belgium.

O'Grady, P., Moloney, M. and Smith, P.R. (1988) Bath administration of the quinolone antibiotic flumequine to brown trout (Sa*lmo trutta*) and Atlantic salmon (*Salmo salar*). Diseases of Aquatic Organisms 4: 27-33.

Olsson, J.C., Joborn, A., Westerdahl, A., Blomberg, L., Kjelleberg, S., Conway, P.L. (1996) Is the turbot, *Scophtalmus maximus* (L.), intestine a port of entry for the fish pathogen *Vibrio anguilarum.* Journal of Fish Diseases, 19: 225-234.

Onji, Y., Uno, M., Tanigawa, K. (1984) Liquid chromatographic determination of tetracycline residues in meat and fish. J. Assoc. Off. Anal. Chem., 67: 1135-1137.

Oshima, Y., Kobayashi, K., Lumbanbatu, J.F., Ooie, K., Kawahara, Y., Imada, N. (1994) Effect of drug-metabolizing enzyme activity induced by PCB on the duration of drugs in fish. Aquaculture, 120(1-2): 25-31.

Palmer, R., Kawai, K. and Kusuda, R. (1992) In vitro activity of quinolone antibacterials against selected fish pathogens. Gyobyo Kenkyu 27: 131-142.

Paperna, I., Colorni, A., Gordin, H., Kissil, G.W. (1977) Diseaes of Sparus aurata in marine culture at Elat. Aquaculture, 10(3): 195-213.

Paperna, I.; Martinez Gonzalez, F. (1980) Check list of diseases, microbial and parasitic pathogens diagnosed from cultured marine fish in the Mediterranean. Aspects of brackishwater fish and crustacean culture in the Mediterranean., FAO; Rome (Italy), 1980, Salnikov, N.E. (ed.): (no. 57): 11-27., Stud. Rev. GFCM/Etud. Rev. CGPM.

Paperna, I. (1984) Review of diseases affecting cultured *Sparus aurata* and *Dicentrarchus labrax* . Aquaculture of Sea Bass and Sparids., Barnabe, G; Billard, R (eds): 465-482

Papoutsoglou, S.E. (1991) Impact of aquaculture on the aquatic environment in relation to applied production system. European Aquaculture Society Special Publication No 16 N. De Pauw, G. Goyce (Eds) Dublin Ireland European Aquaculture Society pp. 71-77.

Papoutsoglou, S.E. (1996) Role of management in achievement of aquaculture' s production systems aims. The role of Aquaculture in World Fisheries, Proceedings of World Fisheries Congress. Theme 6 Ed.: TG Heggberget. New Delhi: Oxford and IBH Publishing Co. (Mohan Primlary), pp. 116-120.

Papoutsoglou, S.E., Costello, M.J., Stamou, E., Tziha, G. (1996) Environmental conditions at sea-cages and ectoparasites on farmed European sea bass *Dichentrarchus labrax* (L.) and gilt-head sea bream, *Sparus aurata* L., at two farms in Greece. Aquaculture, 27: 25-34.

Papoutsoglou, S.E. (2000) Monitoring and regulation of marine aquaculture in Greece: licensing, regulatory control and monitoring guidelines and procedures. Journal of applied Ichthyology, 16: 167-171.

Phillips, I. (1986) Resistance as a cause of treatment failure. Journal of Antimicrobial Chemotherapy. 18(suppl. C): 255-260.

Pinto, R.M., Jofre, J., Bosch, A. (1993) Interferon-like activity in sea bass affected by viral erythrocytic infection. Fish & Shellfish Immunology, 3(2): 89-96.

Plakas, S.M., Mcphearson, R.M. and Guarino, A.M. (1988) Disposition and bioavailability of H-Tetracycline in the channel catfish (*Ictalurus punctatus*). Xenobiotica, 18(1): 83-93.

Poher, I., Blanc, G., Loussouam, S. (1997) Pharmacokinetics of oxolinic acid in sea-bass, *Dicentrarchus labrax* (L. 1758), after a single rapid intravascular injection. J. vet. Pharmacol. Therap., 20: 267-275.

Poher, I. and. Blanc, G. (1998) Pharmacokinetics of a discontinuous absorption process of oxolinic acid in turbot, *Scophthalmus maximus,* after a single oral administration. Xenobiotica, 28(11): 1061-1073.

Pouliquen, H., Le Bris, H. (1996) Sorption of oxolinic acid and oxytetracycline to marine sediments. Chemosphere, 33(5): 801-815.

Pouliquen, H., Le Bris, H., Buchet, V., Pinault, L. (1996) Comparative study on the contamination and decontamination of Japanese oyster *Crassostrea gigas* and blue mussel *Mytilus edulis* by oxytetracycline and oxolinic acid. Marine ecology progress series. Oldendorf, 133(1-3): 143-148.

Pretti, C., Milone, M.T.A., Cognetti-Varriale, A.M. (1999) Fish pasteurellosis: sensitivity of blotting analysis on the internal organs of experimentally infected sea bass (*Dicentrarchus labrax*). Bulletin of the European Association of Fish Pathologists. Weymouth, 19(3): 120-122.

Prickett, R.A. (1998) European sea bass and sea bream industry: Development present status and prospects for the future. Proceedings of the Special Session on Marine Fish Culture Aquaculture Canada '97., Mar 1998, no. 98-1: 21-29, Bulletin of the Aquaculture Association of Canada. St. Andrews NB.

Pritchard, J.B. and Bend, J.R. (1991) Relative roles of metabolism and renal excretory mechanisms of xenobiotic elimination by fish. Environmental Health Perspectives 90: 85-92.

Purcell, L., Dineen, T., Kerry, J., Vaughan, S., Smith, P. (1996) The biological significance of breakpoint concentrations of oxytetracycline in media for the examination of marine sediment microflora. Aquaculture, 145: 21-30.

Pye-MacSwain, J., Rainnie, D., Cawthorn, E., Johnson, G. (1994) Plasma pharmacokinetics and bioavailability of oxytetracycline in Atlantic salmon held in seawater. Proceedings, 1992 Meeting of The Aquaculture Association of Canada, 1-3 June, 1992, University Of British Columbia, Vancouver, BC., 1992, pp. 70-72, Bulletin of the Aquaculture Association of Canada. St. Andrews NB, 92(3).

Ransom, D.P., Lannan, C.N., Rohovec, J.S., Fryer, L.J. (1984) Comparison of histopathology caused by *Vibrio anguilarum* and *Vibrio ordalii* in three species of Pacific salmon. Journal of Fish Diseases, 7: 107-115.

Rasmussen, K.E., Tonnesen, F., Thanh, H.H., Rogstad, A., Aanesrud, A. (1989) Solid-phase extraction and high performance liquid chromatographic determination of flumequine and oxolinic acid in salmon plasma. Journal of Chromatography, 496: 355-364.

Rawles, S.D., Kocabas, A., Gatlin, D.M. III, Du, W.X., Wei, C.I. (1997) Dietary supplementation of Terramycin and Romet-30 does not enhance growth of channel catfish but does influence tissue residues. Journal of the World Aquaculture Society, 28(4): 392-401.

Real, F.A., Oros, J., Acosta, F., Acosta, B., Santana, P., Deniz, S. (1997) Pasteurellosis of gilthead seabream (*Sparus aurata*) in Gran Canaria Island, Spain. Bulletin of the European Association of Fish Pathologists. Weymouth, 17(5): 153-157.

Reja, A., Moreno, L., Serrano, J.M., Santiago, D., Soler, F. (1996) Concentration-time profiles of oxytetracycline in blood, kidney and liver of tench (*Tinca tinca L*) after intramuscular administration. Veterinary and Human Toxicology, 38(5): 344-347.

Richards, R.H. (1992) Constraints on drug prescription-a veterinary viewpoint. Chemotherapy in aquaculture: From theory to reality. Michel, C. and Alderman, D.J. (Eds). Office International des Epizooties, Paris, pp.88-93.

Richards, R.H., Inglis, V., Frerichs, G.N., Millar, S.D. (1992) Variation in antibiotic resistance patterns of *Aeromonas salmonicida* isolated from Antlantic salmon *Salmo salar* in Scotland. Chemotherapy in Aquaculture: from theory to reality. Michel, C.M. and Alderman, D.J. (eds.). Off. Intern. Epiz., Paris: 276-287.

Rigos, G., Alexis, M., Nengas, I. (1999) Leaching, palatability and digestibility of oxytetracycline and oxolinic acid included in diets fed to seabass *Dicentrarchus labrax L.* in Laboratory of Fish Nutrition and Pathology, National Centre of Marine Research, Aghios Kosmas, Hellinikon, 16604 Attiki, Greece. Aquaculture Research, 30(11-12): 841-847.

Rigos, G., Alexis, M., Andriopolou, A. and Nengas, I. (2001) Phapacokinetics and tissue distribution of oxytetracycline in sea bass, *Dicentrarchus labrax,* at two water temperatures. Aquaculture (In press).

Rijkers, G.T., Teunissen, A.G., Oosterom, R.V. and Van Muiswinkel, W.B. (1980) The immune system of cyprinid fish. The immunosuppressive effect of the antibiotic oxytetracycline in carp (*Cyprinus carpio* L.). Aquaculture 19: 177-189.

Rijkers, G.T., Oosterom, R.V. and Van Muiswinkel, W.B. (1981) The immune system of cyprinid fish. Oxytetracycline and the regulation of humoral immunity in carp (*Cyprinus carpio* L.). Veterinary Immunology and Immunopathology 2: 281-290.

Roberts, R.J. (1978) Fish pathology. Bailliere-Tindall, London.

Rodgers, C.J. (1991) The usage of vaccination and antimicrobial agents for control of *Yersinia ruckeri*. Journal of Fish Diseases, 14(3): 291-301.

Rodgers, C.J. (1991). Epidemiological studies of the bacterial fish pathogen *Yersinia ruckeri*. PhD Thesis, University of Plymouth.

Rodgers, C.J. and Austin, B. (1983) Oxolinic acid for control of enteric redmouth disease in rainbow trout. The Veterinary Record 112:83.

Rodgers, C.J. (2001) Resistance of *Yersinia ruckeri* to antimicrobial agents in vitro. Aquaculture, 196(3-4): 325-345.

Rogstad, A., Hormazabal, V. and Yndestad, M. (1988) Optimization of solid phase extraction of oxytetracycline from fish tissue and its determination by HPLC. Journal of Liquid Chromatography, 11(11): 2337-2347.

Rogstad, A., Hormazabal, V. and Yndestad, M. (1989) Simultaneous extraction and determination of oxolinic acid and flumequine in fish tissues by high-performance liquid chromatography. Journal of Liquid Chromatography, 12(15): 3073-3086.

Rogstad, A., Hormazabal, V., Ellingsen, O.F. and Rasmussen, K.E. (1991) Pharmacokinetic study of oxytetracycline in fish. I. Absorption, distribution and accumulation in rainbow trout in freshwater. Aquaculture. 96: 219-226.

Rogstad, A., Ellingsen, O.F. and Syvertsen, C. (1993) Pharmacokinetics and bioavailability of flumequine and oxolinic acid after various routes of administration to Atlantic salmon in seawater. Aquaculture, 110: 207-220.

Romestand, B., Dragesco, A., Breuil, G., Coste, F., Bouix, G. (1993) An ELISA technique for rapid diagnosis of vibriosis in sea bass *Dicentrarchus labrax*. Diseases of Aquatic Organisms, 15(2): 137-143.

Roque, A., Turnbull, J.F., Gomez-Gil, B. (1998) Delivery of Bioencapsulated Oxytetracycline to the Marine Shrimp *Penaeus monodon*. Journal of the World Aquaculture Society, 29(2): 249-251.

S.S.C.A.R (1999) Opinion of the Scientific Steering Committee on Antimicrobial Resistance (1999) Unit B3 - Management of scientific committees II Directorate B - Scientific Health Opinions, Consumer Policy and Consumer Health Protection Directorate-General XXIV European Commission.

Saeed, M.O. (1995) Association of *Vibrio harveyi* with mortalities in cultured marine fish in Kuwait. Aquaculture, 136(1-2): 21-29.

Sakai, D.K. (1987) Transformation of a non-pathogenic *Aeromonas salmonicida mutant* with intraspecific, interspecific and intergeneric bacterial DNA fragments in a laboratory model of river sediments. Nippon Suisan Gakkaishi 53: 1141-1149.

Salati, F., Tassi, P., Cau, A., Kusuda, R. (1997) Microbiological study on Gram-negative pathogenic bacteria isolated from diseased sea bass, *Dicentrarchus labrax Linneo* 1758 cultured in Italy. Bollettino Societa Italiana di Patologia Ittica, 9(22): 9-22.

Salte, R. (1982) Oxytetracycline residues in rainbow trout (*Salmo gairdneri*) fed a commercial medicated feed. Acta Veterinaria Scandinavica, 23: 150-152.

Salte, R. and Liesto, K. (1983) Drug withdrawal from farmed fish. Depletion of oxytetracycline, sulfadiazine and trimethroprim from muscular tissue of rainbow trout (*Salmo gairdneri*). Acta Vet. Scand., 24: 418-430.

Samuelsen, O.B., Torsvik, V., Kupka-Hansen, P., Pittman, K., Ervik, A. (1988) Organic waste and antibiotics from aquaculture. ICES C.M. 1988/F:14.

Samuelsen, O.B. (1989a) Degradation of oxytetracycline in seawater at two different temperatures and light intensities and the persistence of oxytetracycline in the sediment from a fish farm. Aquaculture, 83: 7-16.

Samuelsen, O.B. (1989b) Determination of flumequine in fish by high-performance liquid chromatography and fluorescence detection. Journal of Chromatography, 497: 355-359.

Samuelsen, O.B. (1991) The fate of antibiotics/chemotherapeutics in marine aquaculture sediments. Problems of Chemotherapy in Aquaculture, from theory to reality. OIE (Editors), Proccedings of a Symposium, 12 – 15 march 1991, at Paris, pp 87-95.

Samuelsen, O.B., Solheim, E., Lunestad, B.T. (1991) Fate and microbiological effects of furazolidone in a marine aquaculture sediment. Science of the Total Environment, 108(3): 275-283.

Samuelsen, O.B., Torsvik, V. and frvik, A. (1992a) Long range changes in oxytetracyclife concentration and bacterial resistance towards oxytetracycline in a fish farm sediment after medication. Science of the Total Environment, 114: 25-36.

Samuelsen, O.B, Lunestad, B.T., Husevag, B., Holleland, T., Ervik, A. (1992b) Residues of OA in wild fauna following medication in fish farms. Diseases of Aquatic Organisms,12: 111-119.

Samuelsen, O.B. (1994) Environmental Impacts Of Antibacterial Agents In Norwegian Aquaculture. Proceedings of the Canada-Norway Workshop on Environmental Impacts of Aquaculture., Avforsknings Instituttet, Bergen (Norway), 1994, pp. 107-113, Fisken og Havet no. 13.

Samuelsen, O.B. (1997) Efficacy of bath-administered flumequine and oxolinic acid in the treatment of vibriosis in small Atlantic halibut. Journal of Aquatic Animal Health, 9(2): 127-131. IS: ISSN 0899-7659

Samuelsen, O.B., Hjeltnes, B., Torkildsen, L. (1999) Efficacy of orally administered oxolinic acid and Vetoquinol, an oxolinic acid ester, for the treatment of furunculosis in Atlantic salmon *Salmo salar* held in seawater. Diseases of Aquatic Organisms, 37(1): 53-59.

Samuelsen, O.B., Ervik, A., Pursell, L., Smith, P. (2000) Single-dose pharmacokinetic study of oxolinic acid and vetoquinol, an oxolinic acid ester, in Atlantic salmon (*Salmo salar*) held in seawater and in vitro antibacterial activity against *Aeromonas salmonicida*. Aquaculture, 187(3-4): 213-224.

Sandaa, R.A., Torsvik, V.L. and Goksoyr, J. (1993) Transferable drug resistance in bacteria from fish-farm sediments. Canadian Journal of Microbiology 38: 1061-1065.

Sandaa, R.A. and Enger, Q. (1994) Transfer in marine sediments of naturally occurring plasmid pRAS1 encoding multiple antibiotic resistance. Applied Environmental Microbiology 60: 4234-4238.

Sarusic, G (1999), Clinical signs of the winter disease phenonomen in sea bream (*Sparus aurata*, L.) in Bulletin of the European Association of Fish Pathologists. Weymouth, 19(3): 113-119.

Sarusic, G., Bavcevic, L. (2000) Nutrition as possible ethiological (sic) aetiological agent of winter disease syndrome in sea bream (*Sparus aurata L.*) Ribarstvo. Zagreb, 58(4): 153-161.

Saye, D.J. and Miller, R.V. (1989) The aquatic environment: consideration of horizontical gene transmission in a diversified habitat. Gene transfer in the environment. Levy, S.B an Miller, R.V. (Eds). McGraw Hill, NY, pp. 223-259.

Schlotfeldt, H.J., Neumann, W., Furhman, H., Portueller, K. and Boehm, H. (1985) Remarks on increasing resistance of fish pathogenic and facultative fish pathogenic bacteria in lower Saxoy (FRG). Fish Pathology 20: 85-91.

Schultz, J.C., Adamson, J.S., Workman, W.W. and Norman, T.D. (1963) Fatal liver disease after intravenous administration of tetracycline in high dosage. New England Journal of Medicine 289: 999-1004.

Scott, P. (1993) Therapy in Aquaculture. Aquaculture for Veterinarians: Fish Husbandry and medicine. Brown, L. (ed.). 1st edn. Pergamon Press, Oxford, UK. 131-152.

Sedano, J., Zorrilla, I., Morinigo, M.A., Balebona, M.C., Vidaurreta, A., Bordas, M.A., Borrego, J.J. (1996) Microbial origin of the abdominal swelling affecting farmed larvae of gilt-head seabream, *Sparus aurata L.* Aquaculture Research, 27(5): 323-333.

Segawa, I. (1995) Determination of oxytetracycline in cultured marine fish serums by high performance liquid chromatography. Bull. Natl. Res. Inst. Aquacult. (Japan)/ Yoshokukenho, 24: 73-78.

Sera, H. and Ishida, Y. (1972) Bacterial flora in the digestive tract of marine fish-III. Classification of isolated bacteria. Bulletin of the Japanese Society of Scientific Fisheries, 38:.853-858.

Shepherd, C.J. and Bromage, N.R. (Eds) (1989) Intensive Fish Farming. BSP Professional Books, Oxford pp. 230-236.

Shoemaker, N.B., Wang, G.R. and Salyers, A.A. (1992) Evidence for natural transfer of a tetracycline resistant gene between bacteria from the human colon and bacteria from the bovine rumen. Applied Environmental Microbiology 58: 1313-1320.

Shotts, E.B., Teska, J.D. (1989) Bacterial pathogens of aquatic vertebrates. Methods for the microbiological examination of fish and shellfish, Austin, B., Austin, D.A. (eds.), New York, John Wiley & Sons: 164-186.

Siegert, K. (1985) Nachweis von Sulfonamiden und Antibiotika mit Methoden der gekopelten Gaschromatographie-Mssenspekrometrie. Fleischwirtschaft, 65: 1496-1497.

Silva, E.R., Martinez, E., Figueras, A., Barja, J.L. (1993) Microflora associated with healthy and diseased turbot (*Scophthalmus maximus*) from the farms in northwest Spain. Aquaculture, 114(3-4): 189-202.

Simpson, R.M., Suhre, F.B. and Shafer, J.W. (1985) Quantitative gas chromatographic–Mass spectometric assay of five sulfonamide residues in animal tissue. J. Assoc. Off. Anal. Chem., 68(1): 23-26.

Skjoelstrup, J., McLean, E., Nielsen, P.H., Frier, J.O. (2000) The influence of dietary oxolinic acid on fluidised bed biofilter performance in a recirculation system for rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 183(3-4): 255-268.

Smith, H.W. (1969) Transfer of antibiotic resistance from animal and human strains of *E. coli* in the alimentary tract of man. Vet. Record 85: 31-33.

Smith, J.T. (1990) Effects of physiological cation concentration of 4-quinolone absorption and potency. The 4-quinolones:Antibacterials in vitro, Crumplin, G.C. (Ed), Springer-Verlag, pp. 15-21.

Smith, P., Coyne, R., Vaughan, S., Cazabon, D., Kerry, J., Hiney, M. (1993) The measurement of antibiotic concentrations in the environment; the issues of relevance and accuracy. Book of Abstracts, Sixth International Conference of the European Association of Fish Pathologists. Brest, France: 104p.

Smith, P., Hiney, M.P. and Samuelsen, O.B. (1994) Bacterial resistance to antimicrobial agents used in fish farming: a critical evaluation of method and meaning. Annual Review of Fish Diseases 4: 273-313.

Smith, P. (1995) What do changes in the frequency of resistance to oxytetracycline in the sediments under marine fish farms mean? Bulletin of the Aquaculture Association of Canada 95: 21-25.

Smith, P., Pursell, L., McCormack, F., O'Reilly, A. and Hiney, M. (1995) On the significance of bacterial resistance to oxytetracycline in sediments under Norwegian fish farms. Bulletin of the European Association of Fish Pathologists, 15: 105-106.

Smith, P. (1996) In sediment deposition the dominant fate of oxytetracycline used in marine fish farms; a review of available evidence. Aquaculture 146: 157-169.

Smith, P. and Samuelsen, O.B. (1996) Estimates of the significance of out-washing of oxytetracycline from sediments under Atlantic salmon sea-cages. Aquaculture, 144: 17-26.

Smith, P., Niland, T., O'Domhnaill, F., O'Tuathaigh, G. and Hiney, M. (1996) Influence of marine sediments and divalent cations on the activity of oxytetracycline against *Listonella anguillarum*. Bulletin of the European Association of Fish Pathologists 16: 54-57.

Soltani, M. (1998) Review of the literature relating to the antibacterial properties of piscine skin mucus. Journal of the Faculty of Veterinary Medicine, University of Tehran, 53: 1-2.

Sorum, H., Poppe, T.T., Olsvik, O. (1988) Plasmids in *Vibrio salmonicida* isolated from salmonids with hemorrhagic syndrome (Hitra disease). Journal of Clinical Microbiology 26: 1679-1683.

Soulides, D.A., Pinck, L.A. and Alison, F.E. (1961) Antibiotics from soils.3 Further studies of antibiotics from clays. Soil Science 92: 90-93.

Spanggaard, B., Joergensen, F., Gram, L., Huss, H.H. (1993) Antibiotic resistance in bacteria isolated from three freshwater fish farms and an unpolluted stream in Denmark. Aquaculture, 115(3-4): 195-207.

Stacell, M., Huffman, D.G. (1994) Oxytetracycline-induced photosensitivity of channel catfish. Progressive Fish-Culturist, 56(3): 211-213.

Stamm, J.S., Hanson, C.W., Chu, D.T.W., Bailer, R., Vojtko, C., Fernandes, P.B. (1986) In vitro evaluation of A-56619 (difloxacin) and A-56620: new aryl-fluoroquinolones. Antimicrob. Agents Chemother., 29: 193-200.

Stamm, J.M. (1989) In vitro resistance by fish pathogens to aquaculture antibacterials including the quinolones difloxacin (A-56619) and sarafloxacin (A-56620). Journal of Aquatic Animal Health 1: 135-141.

Starliper, C.E., Cooper, R.K., Shotts, E.B. Jr. and Taylo, P.W. (1993) Plasmid-mediated Romet resistance of *Edwardsiella ictaluri*. Journal of Aquatic Animal Health 5:1-8.

Starliper, C.E., Cooper, R.K. (1998) Biochemical and conjugation studies of Romet-resistant strains of *Aeromonas salmonicida* from salmonid rearing facilities in the eastern United States. Journal of Aquatic Animal Health, 10(3): 221-229.

Stoffregen, D.A., Chako, A.J., Backman, S. and Babish, J.G. (1993) Successful therapy of furunculosis in Atlantic salmon, *Salmo salar* L., using the fluoroquinolone antimicrobial agent enrofloxacin. Journal of Fish Diseases 16: 219-228.

Stoffregen, D., Bowser, P., Babish, J. (1996) Antibacterial Chemotherapeutants for Finfish Aquaculture: A synopsis of Laboratory and field Efficacy and safety Studies in Journal of Aquatic Animal Health 8: 181-207.

Studnicka, M., Siwicki, A.K., Morand, M., Rymuszka, A., Bownik, A., Terech-Majewska, E. (2000) Modulation of nonspecific defence mechanisms and specific immune responses after suppression induced by xenobiotics. Journal of Applied Ichthyology, 16(1): 1-7.

Sugita, H., Miyajima, C., Fukumoto, M., Koyama, H. and Deguchi, Y. (1989) Effect of oxolinic acid on fecal microflora of goldfish *(Carassius auratus*). Aquaculture, 80: 163-174.

Sved, S., Larocque, L., Weininger, A., Schnurm, M. and Vas, D. (1990) The assay of oxolinic acid in salmon tissues by HPLC with fluorescence detection. Proceedings of Conference on residues of veterinary drugs in foods. Nordwejkerhout, Netherlands. ISBN 90-61519-011-6, pp. 356-360.

Tafalla, C., Novoa, B., Alvarez, J.M. and Figueras, A. (1999) In vivo and in vitro effect of oxytetracycline treatment on the immune response of turbot, *Scophthalmus maximus* (L.). Journal of Fish Diseases 22: 271-276.

Takahashi, Y. and Endo, T. (1987) Evaluation of the Efficacy of an Ultrafine Preparation of Oxolinic Acid in the Treatment of Pseudotuberculosis in Yellowtail. Nippon Suisan Gakkaishi, 53(12) : 2157-2162.

Takashima, N., Aoki, T. and Kitao, T. (1985) Epidemiological surveillance of drug-resistant strains of *Pausteurella piscicida.* Fish Pathology 20: 209-217.

Tassi, P., Salati, F. (1996) Microbiological study on Gram-positive bacteria isolated from diseased sea bass (*Dicentrarchus labrax Linneo* 1758) cultured in Italy. Rivista italiana di acquacoltura. Verona, 31(2): 75-80.

Tennant, D.R. (1992) Surveillance for residues in farmed fish: principles and problems. Chemotherapy in Aquaculture: from theory to reality, Michel, C.M. and Alderman, D.J. (eds). Office International des Epizooties, Paris, pp.524-535.

Theodorou, JA. (1999) Greece focuses on marketing sea bass and sea bream. SeaFood International, December 1999: pp.35-36

Theodorou, J.A., Charalambakis, G. (2001) Review of the marine fish farming development in Greece. Maraqua 2nd Workshop IMBC 20-22 March, pp. 49-51

Toften, H., Jobling, M. (1996) Development of spinal deformities in Atlantic salmon and Arctic charr fed diets supplemented with Oxytetracycline. Journal of Fish Biology, 49(4): 668-677.

Toften, H., Jobling, M. (1997) Feed intake and growth of Arctic charr, *Salvelinus alpinus* (L.), fed diets supplemented with oxytetracycline and squid extract. Aquaculture Nutrition, 3(4): 255-259.

Toranzo, A.E., Combarro, P., Lemos, M.L. and Barja, J.L. (1984) Plasmid coding for transferable drug resistance in bacteria isolated from cultured rainbow trout. Applied and Environmental Microbiology 48: 872-877.

Toranzo, A.E., Barja, J.L. (1990) A review of the taxonomy and seroepizootiology of *Vibrio anguillarum* with special reference to aquaculture in the northwest of Spain. Diseases of Aquatic Organisms, 9: 73-82.

Torkildsen, L., Samuelsen, O.B., Lunestad, B.T. and Bergh, O. (2000) Minimum inhibitory concentrations of chhlorammphenicol, florfenicol, trimethoprim/sulphadiaine and flumequinei seawater of bacteria associated with scallops (*Pecten maximus*) larvae. Aquaculture 185: 1-12.

Torsvik, V.L., Sorheim, R. and Goksoyr, J. (1988) Antibiotic resistance of bacteria from fish farm sediments. Copenhagen Denmark ICES, Copenhagen, Denmark.

Tort, L., Padros, F., Rotllant, J., Crespo, S. (1998) Winter syndrome in the gilthead sea bream *Sparus aurata*. Immunological and histopathological features. Fish & Shellfish Immunology, 8(1): 37-47.

Touraki, M., Rigas, P., Pergandas, P., Kastritsis, C. (1995) Determination of oxytetracycline in the live fish feed *Artemia* using high-performance liquid chromatography with ultraviolet detection. Journal of Chromatography B, 663: 167-171.

Touraki, M., Niopas, I., Kastritsis, C. (1999) Bioaccumulation of trimethoprim, sulfamethoxazole and N-acetyl-sulfamethoxazole in Artemia nauplii and residual kinetics in seabass larvae after repeated oral dosing of medicated nauplii. Aquaculture, 175(1-2): 15-30.

Twiddy, D.R., Reilly, P.J.A. (1995) Occurrence of antibiotic-resistant human pathogens in integrated fish farms. Research contributions presented at the ninth session of the indo-pacific fishery commission working party on fish technology and marketing. Cochin, India, 7-9 march 1994, FAO, Rome (Italy), FAO fisheries report. Rome no. 514, Suppl., pp. 23-37.

Tyrpenou, A.E. (1995) Tetracycline (OTC, TC, CTC) determination by HPLC-PDA. Quality assurance and validation procedures used at the EU-NRL of Athens-Greece. EU Workshop on quality assurance in HPLC-validation of HPLC methods for antimicrobial residues in meat. CNEVA-Laboratoire des Medicamentes Veterinaire, CRL-Community Reference Laboratory, 13-16 June, Fougeres-France.

Tzoumas, A., Alderman, D.J and Rodgers, C.J. (1989) *Aeromonas salmonicida*: development of resistance to 4-quinolone antibacterials. Journal of Fish Diseases 12: 493-507.

Ueno, R., Aoki, T. (1995) A simplified determination of oxytetracycline in serum and muscle of cultured eel by high-performance liquid chromatography. Fish Pathology, 30(3): 239-240.

Ueno, R., Okumura, M., Horiguchi, Y. and Kubota, S. (1988) Levels of oxolinic acid in cultured rainbow trout and amago salmon after oral administration. Nippon Suisan Gakkaishi 54: 485-489.

Uno, K. (1996) Pharmacokinetic study of oxytetracycline in healthy and vibriosis-infected ayu (*Plecoglossus altivelis).* Aquaculture, 143: 33-42.

Uno, K., Aoki, T., Ueno, R. and Maeda, I. (1997) Pharmacokinetics of Oxytetracycline in Rainbow Trout *Oncorhynchus mykiss* following Bolus Intravenous Administration. Fisheries Science, 63(1): 90-93.

Usher, M.L., Talbot, C. and Eddy, F.B. (1988) Drinking in Atlantic Salmon Smolts Transferred to Seawater and the Relationship Between Drinking and Feeding. Aquaculture, 73: 237-246.

Van Houweling, C.D. (1971) The Food, Drug and Cosmetic act, animal drugs and the consumer. Annales New York Academic Science 182: 411-415.

Van der Heijden, M.H.T., Helders, G.M., Booms, G.H.R., Huisman, E.A., Rombout, J.H.W.M., Boon, J.H. (1996) Influence of flumequine and oxytetracycline on the resistance of the European eel against the parasitic swimbladder *nematode Anguillicola crassus*. Veterinary Immunology and Immunopathology, 52(1-2):127-134.

Vaughan, S., Mac Gearailt, C., Gilroy, D. and Smith, P. (1993) Studies of the inhibition of the biological activity of OTC in the freshwater environment. EAFP, 6th international conference "diseases of fish and shellfish", Brest, France, 1993.

Vaughan, S., Coyne, R. and Smith, P. (1996) The critical importance of sample site in the determination of the frequency of oxytetracycline resistance in the effluent microflora of a fresh water fish farm. Aquaculture 139: 47-54.

Vera, P., Navras, J.I., Fouz, B. (1991) First isolation of Vibrio danisela from sea bream (*Sparus Aurata*). Bulletin of European Association of Fish Pathology, 11(3): 112

Vigneulle, M. (1988) Susceptibility of sea bass, *Dicentrarchus labrax*, and turbot, *Scophthalmus maximus*, to *Yersinia ruckeri*. Pathology In Marine Science. Proceedings of the Third International Colloquium on Pathology in Marine Aquaculture Held in Gloucester Point, Virginia, October 2-6, 1988., Academic Press, San Diego, CA (USA), 1990: 117-122.

Waltman, W.D. and Shotts, E.B. (1986) Antimicrobial susceptibility of *Edwardsiella tarda* from the United States and Taiwan. Veterinary Microbiology 12: 277-282.

Walton, J.R. (1992) Antibiotic resistance: An overview. Veterinary Record 122: 249-251.

Watanabe, T., Aoki, T., Ogata, Y. and Egusa, S. (1971) R-factors to fish culturing. Annales New York Academic Science, 383-410.

Weidemann, B. (1993) Monitoring of resistant organisms in man and the identification of their origin. Veterinary Microbiology 35: 275-284.

Weinstein, L. (1970) The tetracyclines. The Pharmacological Basis of Therapeutics, Goodman, L.S and Gilman, A. (Eds), 4th edn, McMillan, London, pp.1253-1268.

Whitehead. P.J.P., Bauchot, M.L., Hureau, J.C., Nielsen, J., Tortonese, E. (1986) Fishes of the North-Eastern Antlantic and the Mediterranean. UNESCO, 1: 1-510.

Wollenberger, L., Halling-Soerensen, B., Kusk, K.O. (2000) Acute and chronic toxicity of veterinary antibiotics to Daphnia magna. Chemosphere, 40(7): 723-730.

Word Health Organization Scientific Committee on Antimicrobial Resistance (1983) Antimicrobial resistance. Bull. WHO 61 pp. 383-394, 423-433.

Xu, D., Rogers, W.A. (1993) Oxytetracycline residue in hybrid striped bass muscle. Journal of the World Aquaculture Society, 24(4): 466-472.

Xu, D., Rogers, W.A. (1994a) Leaching loss from oxytetracycline medicated feeds. Journal of Applied Aquaculture 4: 29-38.

Xu, D., Rogers, W.A. (1994b) Oxytetracycline residue in striped bass muscle. J. Aquat. Animal Health, 6(4): 349-354.

Yoshida, T., Inglis, V., Misawa, N., Kruger, R., Sakai, M. (1997) In vitro adhesion of Pasteurella piscicida to cultured fish cells. Journal of Fish Diseases, 20(1): 77-80.