A STUDY INTO THE EFFECTS AND ENVIRONMENTAL RISK OF ANTIBIOTICS USED IN FRESHWATER AQUACULTURE ON ENVIRONMENTAL BACTERIA

A THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

by

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JUNE 2012

to Tiare and Juan,
for their constant love and support

A subtle thought that is in error may yet give rise to fruitful inquiry that can establish truths of great value

Isaac Asimov

Declaration

I hereby declare that this thesis has been composed entirely by myself and has not been submitted for any other degree. Except where specifically acknowledged the work described in this thesis is the result of my own investigations.

Signed

Alfredo Tello Gildemeister

Date

Acknowledgements

I am very grateful to my supervisors, Dr. Trevor Telfer and Dr. John Taggart, for their advise and constant support. Without Dr. John Taggart's advise on all things genetics, I would probably still be in the lab trying to figure out what went wrong! I am also very grateful to Professor Brian Austin, who has been constantly available to provide advice and feedback on my research, to Dr. James Bron for spending endless hours with me on the confocal microscope and for always being around for help and advise. This PhD would not have been possible without the support of Mark Gaikowski from the Upper Midwest Environmental Sciences Center (UMESC) at the USGS, who acted as an external adviser and whose lab analysed all the antibiotic samples in stream water for me. I am also grateful to Jeff Bernardy from UMESC, who actually performed the analyses of the antibiotics in stream water, and to my Chilean professors, Dr. Francisco Encina and Dr. Alfonso Mardones, who encouraged me to pursue a PhD.

I am thankful to everyone at L14 (i.e., the molecular biology lab), especially Jacquie Ireland, Dr. Oscar Monroig, Dr. Matteo Minghetti, Dr. Michael Leaver, Stephen Carmichael, Jan Heumann and Greta Carmona, for always being available to give advise and for making it a great place to work. I am also thankful to Billy Struthers from the Water Quality Lab for his valuable advise on nutrient analysis and assistance in analysing the samples, to James Dick from the Nutrition Lab for letting me use their kit for solid phase extractions and to Cathryn Dickson for always being available to help.

This PhD involved designing, building and operating a recirculating stream microcosm for the better part of 2 years, which would have been unachievable on my own. I am deeply indebted to Brian Howie for his expert help and advise in designing and building the microcosms, to my good friend Neil Handisyde for being my 'private plumbing consultant' and my 'private water collection assistant', to my good friend Ben Perry for his help during the construction and operation of the microcosms, to my good friend Dr. Juan Moreno for his help in setting up the flumes before each experiment, to Willie Thomson for setting up the lighting system and to Dr. Gabriel Singer for his advice in building the microcosms. I am also grateful to Anne Hammond and Graham McWhinnie, who also assisted me during water collection on

a few occasions and to Jane Lewis and Charlie Harrower, for dealing with my endless order of supplies through STORES.

Finally, I must express my profound gratitude to my wife and son, who literally crossed the globe with me to come to Stirling. Without their constant love, support and understanding I would have never completed this thesis. This PhD is as much their work as it is my own. I am also very grateful to my parents for their unconditional support and for encouraging me to pursue my dreams.

Abstract

Aquaculture is the fastest growing food industry in the world and it accounts for roughly half of the world's fish supply. The majority of global aquaculture production occurs in freshwater systems that are increasingly subject to multiple uses by different stakeholders. Given the overall scarcity of freshwater on a global scale, freshwater aquaculture will face increasing environmental constraints that will demand an ever better understanding of its potential impacts on the aquatic environment and human health. This thesis consists of a series of studies that, collectively, contribute to further our understanding on the effects of freshwater aquaculture effluents on aquatic ecosystems, on the effects and environmental safety of antibiotics used in freshwater aquaculture on aquatic bacterial communities and on the link between antibiotic pollution and antibiotic resistance.

Chapter 2 reviews the effects of freshwater aquaculture effluents on stream ecosystems using land-based salmonid farms as a case study. In this chapter I discuss relevant considerations related to the temporal and spatial scales of effluent discharge and ecological effects that highlight the need to characterize the patterns of stressor discharge when assessing environmental impacts and designing ecological effects studies. I also discuss the potential role of multiple stressors - with an emphasis on veterinary medicines - in disrupting ecosystem structure and function. Overall, the critical analysis presented in this chapter indicates that further research on the effects of veterinary medicines using relevant exposure scenarios would significantly contribute to our understanding of their impact in relation to other effluent stressors.

Chapter 3 is a general methods chapter that describes the stream microcosm system used to assess the effects of erythromycin thiocyanate (ERT) and florfenicol (FFC) on bacterial communities of stream biofilms. This chapter presents the results of preliminary experiments whose results provided relevant information on the overall operation of the microcosms and on the variability of major physical and biological variables. This information guided the experimental designs used to assess the effects of FFC and ERT on the bacterial community structure of stream biofilms.

Chapter 4 presents the results of the experiment conducted to assess the effects of FFC on the bacterial community structure of developing biofilms. The objective was to assess changes in bacterial community structure along a gradient of FFC

concentrations that could provide insight into the type and magnitude of effects that could be expected from episodic exposure of stream biofilms to FFC in headwater streams. At 10 and 20 days of biofilm development, bacterial community structure differentiated in a pattern consistent with the FFC concentration gradient and there was a positive relationship between bacterial richness and bacterial diversity with FFC concentration. At 15 days of biofilm development there was also a positive relationship between FFC concentration and the surface coverage of bacteria and extracellular polymeric substances. These trends declined as the biofilm developed a more complex architecture, in terms of thickness and in the surface coverage of algae. The results are consistent with an initial stimulatory effect of FFC on biofilm formation that triggered changes in bacterial community structure that were gradually compressed as the development of a complex biofilm architecture increased the relative importance of autogenic ecological processes. The results suggest that the co-occurrence of FFC with bacterial pathogens in effluents and wastewaters may favour their persistence in the environment by enhancing biofilm formation.

Chapter 5 presents the results of the experiment conducted to assess the effects of ERT on the bacterial community structure of developing biofilms. Currently, Aquamycin® 100 - a Type A medicated article (i.e., Premix) containing 100 g ERT lb^{-1} and used to produce a Type C medicated feed - is a candidate drug for approval by the US FDA to control mortality associated with bacterial kidney disease in freshwater salmonids. The objective of this experiment was to assess the effects of ERT on the bacterial community structure of stream biofilms using an exposure period consistent with the 28-day treatment regime suggested for Aquamycin® 100. The results provide no evidence to suggest that a 30-day exposure to ERT concentrations in the range of 10 $\mu g\ L^{-1}$ (i.e., 7.3 ± 3.9 $\mu g\ L^{-1}$) would lead to changes in the bacterial community structure or overall bacterial abundance of stream biofilms, while they suggest that these effects may occur at concentrations in the range of 100 $\mu g\ L^{-1}$ (i.e., 87.2 ± 31.1 $\mu g\ L^{-1}$).

Chapter 6 attempts to determine whether environmental concentrations of antibiotics and concentrations representing action limits used in environmental risk assessment may exert a selective pressure on clinically relevant bacteria in the environment. In this chapter I use bacterial inhibition as an assessment endpoint to link antibiotic selective pressures to the prevalence of resistance in bacterial populations. Species sensitivity distributions were derived for three antibiotics by fitting log-logistic

models to endpoints calculated from minimum inhibitory concentration (MIC) distributions based on worldwide data collated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Bacteria represented in these distributions were placed in a broader context by performing a brief phylogenetic analysis. The potentially affected fraction of bacterial genera at measured environmental concentrations of antibiotics and environmental risk assessment action limits was used as a proxy for antibiotic selective pressure. Measured environmental concentrations and environmental risk assessment action limits were also directly compared to wild-type cut-off values. Results suggest that measured environmental concentrations of antibiotics and concentrations representing environmental risk assessment action limits are high enough to exert a selective pressure on clinically relevant bacteria that may lead to an increase in the prevalence of resistance.

Chapter 7 presents the results of an exploratory analysis conducted to assess the abundance of class 1 integrons in stream biofilms exposed to FFC and ERT. There was no pattern in the abundance of *intI1* genes consistent with the treatment of FFC and ERT, suggesting either the absence of gene cassettes involved in dealing with selective pressures caused by these antibiotics or that the concentrations tested were below those required to give them a selective advantage.

Chapter 8 is a brief general discussion that brings together the findings of the thesis and makes suggestions for future research. Key areas identified for future research include assessing in further detail the stimulatory effect of FFC on biofilm formation in complex bacterial communities, the interactive effects of multiple aquaculture effluent stressors on aquatic bacterial communities and their potential effects on the development of antibiotic resistance, the fate of FFC and ERT in stream ecosystems, and further developing the analysis based on MIC distributions presented in chapter 6 to assess the potential effects of antibiotic pollution on the selection of multi-drug resistance in the environment.

Abbreviations and acronyms

This list contains some of the most common abbreviations used throughout the thesis. They are also defined upon first use in the text.

ANOVA: analysis of variance
Chl: chlorophyll
CLSM: confocal laser scanning microscopy
DO: dissolved oxygen
EPS: extracellular polysaccharide matrix
ERA: environmental risk assessment
ERT: erythromycin thiocyanate
FFA: florfenicol amine
FFC: florfenicol
LTP: The All-Species Living Tree Project
MEC: measured environmental concentration
NMDS: non-metric multidimensional scaling
NOEC: no observed effect concentration
OTU: operational taxonomic unit
PAF: potentially affected fraction
PCR: polymerase chain reaction
PEC: predicted environmental concentration
qPCR: real-time quantitative PCR
SSD: species sensitivity distribution
T-RF: terminal restriction fragment
tRFLP: terminal restriction fragment length polymorphism
VICH: International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products

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Chapter 1

General Introduction

1.1. Brief environmental perspective of freshwater aquaculture

Scientific and technological advances of the last 30 years have transformed aquaculture into the fastest growing food indsutry in the world, currently accounting for roughly half of the world's fish supply (Bostock et al. 2010). In a process analogous to the green revolution of the 1960s, continous diversification, intensification and expansion of the industry allowed it to rise to the challenge of meeting global demand at a time of declining natural fisheries. Although from an ecological efficiency and environmental impact perspective aquaculture has clear advantages over other sources of animal food production (Hall et al. 2011), it still poses important environmental challenges, many of which are particular to its intimate link with aquatic ecosystems.

Asia currently supplies approximately 90% of global aquaculture production, the majority of which is predominantly freshwater aquaculture (Hall et al. 2011). Global aquaculture production is estimated to reach between 79 and 110 million tonnes by 2030, a production level that may lead to environmental demands up to 2.5 times higher than those of 2008 across a range of environmental impact categories (Hall et al. 2011). Considering that only 0.26% of liquid freshwater on Earth is in lakes, reservoirs and rivers (Carpenter et al. 2011), freshwater aquaculture will increasingly interact with multiple other users of freshwater resources. This will put increasingly tighter constraints on the levels of environmental impact that are deemed acceptable for freshwater aquaculture, and demand an ever better understanding of its potential impacts on the aquatic enviornment and human health.

1.2. Overview of antibiotics used in aquaculture

Like other types of animal food production systems, aquaculture relies on the use of chemotherapeutic drugs to prevent and treat infections. Among the compounds used, antibiotics have received considerable attention due to their effects on the development of antibiotic resistance and the implications that this has for human health (e.g., Sapkota 2008; Burridge et al. 2010). Although progress in the development of vaccines has reduced the use of antibiotics in some aquaculture industries (e.g., the salmon industry), the expansion, intensification and diversification of aquaculture suggest that a reduction in their use on a global scale is unlikely in the

foreseeable future. As aquaculture diversifies to produce new species and expands the production of current species to new geographical locations, the risk of disease and need for treatment will increase. This risk is compounded by the uncertainties introduced by global climate change, which may affect the emergence and dynamics of new and existing pathogens (Tirado et al. 2010), and consequently the use of antibiotics and the prevalence AR. Importantly, while developed countries are likely to enforce guidelines on the proper use of antibiotics in aquaculture, their implementation and enforcement in developing economies may be constrained by lack of resources and a paradigm that favours the rapid growth of the industry over environmental or human health concerns.

Table 1.1. Antibiotics used in aquaculture shown as a percentage of countries within the 15 major aquaculture producers using them. Data obtained from Table 2 of Sapkota et al. 2008.

Class	Antibiotic	Percent Use
Sulfonamides	Sulfamerazine	2%
	Sulfadimidine	1%
	Sulfadimethoxine	4%
Potentiated Sulfonamides	Sulfadiazine/Trimethoprim	5%
Tetracyclines	Chlortetracycline	3%
	Oxytetracycline	12%
Penicillins	Ampicillin	4%
	Amoxycillin	3%
	Benzylpenicillin	3%
Quinolones	Ciprofloxacin	1%
	Enfrofloxacin	3%
	Norfloxacin	2%
	Oxolinic Acid	9%
	Perfloxacin	1%
	Flumequine	4%
Nitrofurans	Furazolidone	5%
Macrolides	Erythromycin	8%
	Spiramycin	1%
	Gentamicin	3%
Other Antibiotics	Chloramphenicol	9%
	Florfenicol	4%
	Thiamphenicol	1%
	Tiamulin	1%
	Nalidixic Acid	3%
	Miloxacin	1%

Currently available data indicates that, on a global scale, several of the major classes of antibiotics are being used or have been used in aquaculture. Among these are the sulfonamides, penicillins, macrolides, quinolones and tetracyclines (Sapkota et al. 2008). Table 1.1 shows the antibiotics used in aquaculture as a percentage of countries - within the 15 major aquaculture producers - using them.

In general, there is a large degree of variability in the data available on antibiotics used for veterinary medicine in different countries (Sarmah et al. 2006). While countries like the United Kingdom produce anual reports on the sales of antibitoics used in veterinary medicine (VMD 2012), similar information is lacking for many developing countries (Sarmah et al. 2006). In the United Kingdom, the use of veterinary antibiotics has remained fairly stable since 2006 (Table 1.2) (VMD 2012). While there are obvious interannual variations in the total sales of antibiotics and within specific classes of antibiotics, they are in the order of 8% of the mean (Table 1.2).

Table 1.2. Sales of antibiotics for use in veterinary medicine by chemical grouping in the United Kingdom. Data are tonnes of active ingredient. Reproduced from VMD (2012).

	2006	**2007**	**2008**	**2009**	**2010**	**2011**
Tetracyclines	192	174	174	177	200	110
Trimethroprims/Sulphonamides	71	73	70	73	75	72
β-lactams	70	72	69	76	93	86
Aminoglycosides	21	20	18	19	22	19
Macrolides	36	33	35	39	35	37
Fluoroquinolones	2	2	2	2	2	2
Other	13	14	15	16	20	20
Total	**405**	**387**	**384**	**402**	**447**	**346**

Because the majority of global aquacuture takes place in developing countries where institutional structures are not conducive to making data on sales and use of veterinary antibiotics publicly available, it is difficult to assess aquaculture's contirbution to the use of veterinary antibiotics on a global scale. In the United Kingdom in 2011, 85% of the total amount of antibiotics approved for use in food animals were sold for use in poultry and pigs, with aquaculture amounting to roughly 2% (VMD 2012). This

reflects the relative sizes of the pig, poultry and aquaculture industries in the United Kingdom and the highly technical and consolidated nature of European salmon aquaculture, whose reliance on antibiotics has decreased markedly over the last two decades.

1.3. Overview on the occurrence and effects of antibiotics in the environment

Although aquaculture can be a direct source of antibiotics to aquatic ecosystems, the occurrence of antibiotics in freshwater environments is an issue that largely trascends aquaculture. Antibiotics used in human and veterinary medicine also enter freshwater ecosystems through wastewater treatment plant effluents, hospital and processing plant effluents, surface and subsurface runoff from agricultural fields that have been ammended with manure containing antibiotic residues and leakage from waste-storage containers and landfills (Sarmah et al. 2006; Kümmerer 2009a). It is estimated that - on average - approximately 70% of all antibiotics conusmed are excreted unchanged (Kümmerer and Henninger 2003).

Sorption, leaching and degradation are considered to be the main processes determining the movement and fate of antibiotics in the environment (Sarmah et al. 2006). These processes are driven by the antibiotics' physico-chemical properties like size, shape, solubility, speciation, molecular structure and hydrophobicity (Sarmah et al. 2006). In comparison to other chemicals, pharmaceutically active compounds are considered to be complex molecules that exhibit particular properties, such as the dependance of the octanol-water partition coefficient (K_{ow}) on pH (Cunningham 2008; Kümmerer 2009b). In the range of conditions encountered in the environment, pharmaceuticals can be neutral, cationic, anionic, or zwitterionic (Kümmerer 2009b), making it difficult to predict their behaviour. For example, it was recently shown that hydrophobic interactions predicted by the K_{ow} do not control the sorption of relatively hydrophilic antibiotics to bacterial biofilms and that speciation and molecular size are important factors affecting the interactions between antibiotics and biofilms (Wunder et al. 2011). Differences in the environmental behaviour of different antibiotics is also expected to be large. The soil partition coefficient (K_d) of different antibiotics, for example, varies over several orders of magnitude, and so does their degradation half-life in different enviornments (see Sarmah et al. 2006).

Antibiotics have been detected in US streams across a range of hydrologic, climatic and land-use settings (Kolpin et al. 2002), and at different locations and relatively high concentrations in the sediments of Chinese streams (Luo et al. 2011). Several of the major classes of antibiotics have been detected in wastewater treatment plant effluents and surface waters, and some have been detected in ground water (see Kümmerer et al. 2009 for a comprehensive review on the topic). Concentrations in water compartments normally fall within the ng L^{+1} to μg L^{-1} range and may reach higher concentrations in the sediments of aquatic environments and in soil (Golet et al. 2002; Kolpin et al. 2002; Hamscher et al. 2006; Kümmerer et al. 2009; Luo et al. 2011).

Despite awareness on the ubiquity of antibiotic pollution, our knowledge on the effects of antibiotics on bacterial communities in the environment and its ecological and human health implications is still limited. Recent research, however, has provided evidence indicating that environmentally relevant concentrations of antibiotics may have effects on the structure and function of aquatic microbial communities. Verma et al. (2007) found that tetracycline inhibited bacterial production in river water at a concentration of 5 μg L^{-1}. Moreover, Quinlan et al. (2011) reported that tetracycline had significant effects on the prevalence of antibiotic resistant bacteria, bacterial abundance and productivity and algal biomass in river biofilms at a concentration of 0.5 μg L^{-1}. Similarly, Lawrence et al. (2009) showed that triclosan and triclocarban caused changes in the structure and function of river biofilms and reduced algal biomass at a concentration of 10 μg L^{-1}. In a recent metatranscriptomic analyses, Yergeau et al., (2010) showed that very low concentrations of sulfamethoxazole (i.e., 0.5 μg L^{-1}) increased the expression of a TonB-dependant receptor in river biofilms. TonB-dependant receptors are known to facilitate surface adhesion in gram-negative bacteria and there is evidence to suggest that a TonB-dependant receptor is invovled in the coaggregation of the freshwater bacteria *Blastomonas natatoria* (Rickard et al., 2003). In a complementary analysis, Yergeau et al., (2012) found that erythromycin and sulfamethoxazole - at concentrations of 1 and 0.5 μg L^{-1} - also caused the unique expression of several functional genes and variable, antibiotic and taxa-specific changes in the abundance of bacteria. The findings of these studies are consistent with current knowledge on the effects of antibiotics on bacterial isolates *in vitro* and highlight the fact that some of these effects may be largely responsible for modulating the structure of bacterial communities exposed to antibiotics in natural environments.

For example, at low concentrations, antibiotics may modulate bacterial metabolism by altering transcription patterns and induce biofilm formation, bacterial motility and bacterial cytotoxicity (Goh et al., 2002; Linares et al., 2006).

1.4. The issue of antibiotic resistance

Antibiotic resistance (AR) is widely recognised as one of the major challenges facing global public health. The increasing prevalence of AR in hospital and community acquired infections along with the scarcity of new antibiotics in the drug development pipeline paint an overall worrying picture of our current and future ability to effectively treat bacterial infections (WHO 2012). In Europe alone, antibiotic resistant hospital infections cause ~ 25,000 deaths per year and have an associated healthcare cost in excess of € 1.5 billion (WHO 2012).

The use of antibiotics in the animal farming industry has been the subject of research and debate for the past 20 years, and it remains a central environmental health and food security issue (Love et al. 2011). Although advances in husbandry practices and in the regulation of veterinary medicines have reduced the use of antibiotics and other compounds that may favour the development of AR, there are key factors indicating that a reduction in their use on a global scale is unlikely in the foreseeable future.

It has been clearly established that the wider environment is the ultimate reservoir of AR genes (D'Costa et al. 2006; D'Costa et al. 2011), and there is evidence supporting the transfer of AR genes from environmental bacteria to human pathogens. A well-documented case is the environmental origin of CTX-M extended spectrum β-lactamases and *qnrA* quinolone resistance determinants found in enteric bacteria (Olson et al. 2005; Poirel et al. 2002; Poirel et al. 2005).

Recent research has shown that selection of AR can occur at very low concentrations of antibiotics (Gullberg et al. 2011) and that pollution by veterinary antibiotics may increase the prevalence of AR in clinically relevant bacteria in the environment (Tello et al. 2012). Additionally, there is increasing evidence suggesting that environmental pollution by metals and disinfectants may co-select for AR (Berg et al. 2005; Gaze et al. 2011; Knapp et al. 2011). Metals, such as copper, are essential trace elements in animal diets and are also extensively used as biocides in animal production systems (Braithwaite et al. 2007; Frías-Espericueta 2008; Nicholson et al. 1999). Within this

context, the World Health Organization recently recognised the need to consider environmental aspects in strategies aimed at reducing AR (WHO 2012).

1.5. Environmental risk assessment of veterinary medicines

The environmental risk assessment (ERA) of veterinary medicines - including antibiotics - in most developed countries is conducted within the framework set by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products (VICH).

The objective of VICH is to harmonize the data requirements for the registration of veterinary medicines in Europe, the United States, Japan, Canada, Australia and New Zealand (de Knecht et al. 2009). The VICH guidance uses a tiered approach to ERA. Under VICH phase I guidance, the ERA of a veterinary medicine for aquatic environments - except for parasiticides - stops if the concentration in an effluent (i.e., the so called environmental introduction concentration [EIC]) is expected to be < 1 ppb. In the terrestrial environment, the ERA stops if the predicted environmental concentration is < 100 ppb. If these concentrations - referred to as 'action limits' - are exceeded, the ERA proceeds to phase II, which involves more refined analysis' and includes decision trees for aquaculture (Figure 1.1), intensively reared terrestrial animals and pasture animals (de Knecht et al. 2009).

The VICH phase II guidance for ERA is based on a risk quotient approach that determines whether the predicted environmental concentration (PEC) of a given veterinary medicine exceeds the predicted no-effect concentration (PNEC) for any of a series of standard toxicity tests (Figure 1.1). For veterinary medicines used in aquaculture, these tests include the standard algal growth inhibition test, the Daphnia immobilization test and the fish acute toxicity test as defined by the Organisation of Economic Co-operation and Developemnt (OECD) or the International Organization for Standardization (ISO) (Figure 1.1; de Knecht et al. 2009). For veterinary medicines used in aquaculture, the VICH phase II guidance does not recommend tests to assess the risks on microorganisms, while for veterinary medicines used in the terrestrial environment risks on microorganisms are assessed using nitrogen transformation tests in soil (US FDA 2006).

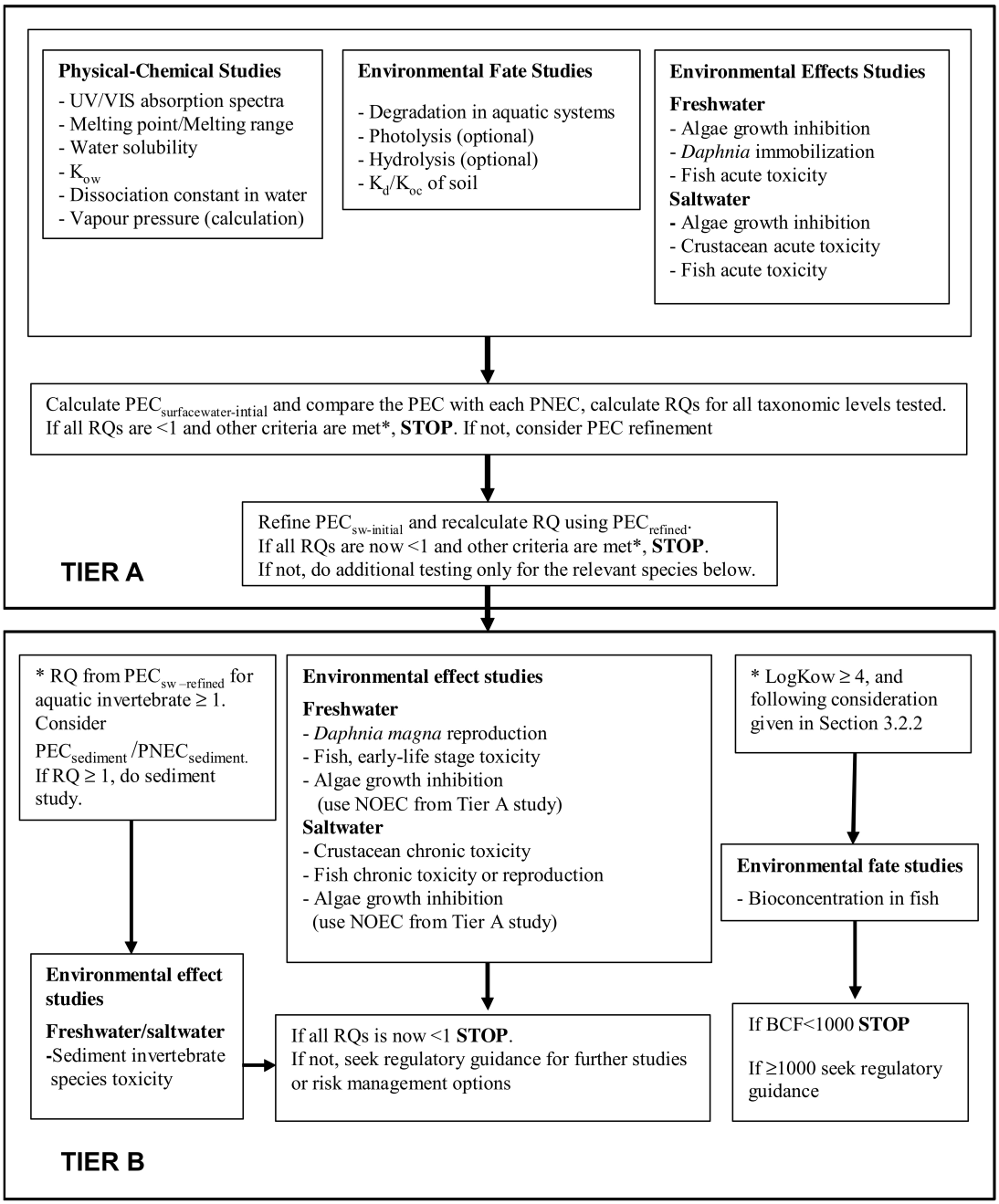

Figure 1.1. VICH phase II decision tree for the ERA of aquaculture veterinary medicines (source: US FDA 2006).

The exclusion of tests on microorganisms in the VICH phase II guidance for veterinary medicines used in aquaculture, and the recommendation of exclusively functional endpoints in the ERA of veterinary medicines for use in the terrestrial environment reflect, to a certain extent, the traditional focus of ecotoxicology on 'toxic' effects, and the view of microbial communities as a 'black box', where changes

in community structure are deemed irrelevant as long as ecological function is preserved. Although functional endpoints are undoubtly important, antibiotics may cause selective pressures leading to, for example, an increase in the prevalence of resistant phenotypes capable of driving functional processes. These tests have therefore key limitations from an environmental and public health perspective, and they have been shown to fail to assess the effects of antibiotics on environmental bacteria (Kümmerer et al., 2004). Effectively addressing the issue of antibiotic pollution in the environment requires the use of appropriate tests, appropriate assessment endpoints and a conceptual framework that acknowledges the link between antibiotic pollution, environmental bacteria and public health. Research acknowledging these considerations is critical to advance environmental management and policy of antibiotics.

1.6. Thesis layout

This thesis consists of a series of studies that, collectively, contribute to further our understanding on (a) the effects of freshwater aquaculture effluents on aquatic ecosystems (b) the effects and environmental safety of antibiotics used in freshwater aquaculture on aquatic bacterial communities and (c) the link between antibiotic pollution and antibiotic resistance. Chapter 2 presents a critical review on the effects of land-based salmonid farm effluents - with an emphasis on veterinary medicines - on stream ecosystems. Although the chapter focuses on land-based salmonid farms, insofar it addressess issues related to the temporal and spatial scale of ecological effects and the role of multiple effluent stressors in disrupting ecosystem structure and function, it provides a good reference for the enviornmental management of freshwater aquaculture effluents in general. This review made it apparent that there was a knowledge gap regarding the effects and environmental risk of veterinary medicines used in freshwater aquaculture on stream ecosystems, and therefore largely set the stage for the questions/problems addressed throughout the rest of the thesis. Chapter 3 describes the stream microcosms used to assess the effects of florfenicol (FFC) and erythromycin thiocyanate (ERT) on the bacterial community structure of stream biofilms. Several preliminary assessments and trials were performed to optimize the operation of the microcosms, and these are described in detail in this chapter. Chapter 4 and 5 present and discuss the results of the experiments conducted

to assess the effects of FFC and ERT on the bacterial community structure of stream biofilms. Chapter 6 presents and discusses the results of a study that uses minimum inhibitory concentration (MIC) distributions of antibiotics to characterize selective pressures on bacteria and link antibiotic pollution to antibiotic resistance. This chapter focuses on antibiotic pollution in general, and the analysis was not performed using an aquaculture scenario. The methods and results discussed, however, have broad implications for the ERA of antibiotics, and are therefore highly relevant to the ERA of antibiotics used in aquaculture. Chapter 7 presents and discusses the results of an exploratory analsyis conducted to assess the abundance of class 1 integrons in stream biofilms exposed to FFC and ERT. This is a small chapter that is very preliminary in nature, yet it is informative and complements well with the other chapters of the thesis. Chapter 8 is a general discussion that brings together the findings of the thesis and makes recommendations for future research.

1.7. Thesis objectives

The objectives of this thesis are:

1) to assess the current knowledge and gaps on the effects of freshwater aquaculture effluents on stream ecosystems with an emphasis on veterinary medicines

2) to further our understanding on the effects and environmental safety of antibiotics used in freshwater aquaculture on stream biofilm bacteria

3) to develop a framework to link antibiotic pollution with antibiotic resistance in the environmental risk assessment of antibiotics

Chapter 2

Effects of aquaculture effluents on stream ecosystems: land-based salmonid farms as a case study.

This chapter was published in: Tello, A., Corner, R.A. and Telfer, T.C. 2010. How do land based salmonid farms affect stream ecology? Environmental pollution 158: 1147-1158.

A. Tello conceived the study, performed the research and wrote the manuscript. R.A. Corner and T.C. Telfer had editorial input.

2.1. Introduction

Inland waters and freshwater biodiversity are valuable natural resources and their conservation and management are critical to the interests of human societies (Dudgeon et al., 2006). The ecological services that ecosystems provide are critical to the functioning of the Earth's life-support system (Constanza et al., 1997). A subset of these services, provided by running waters, are amongst the most immediately and tangibly important to human societies, particularly through water supply, nutrient re-cycling and waste treatment. Small size streams are important in regulating water chemistry in large drainages because their large surface-to-volume ratio favours rapid nitrogen uptake and processing (Alexander et al., 2000; Peterson et al., 2001; Bernot and Dodds, 2005). As a consequence, maintaining the nitrogen removal functions of stream ecosystems has been recognized as a management imperative (Mullholand et al., 2008). Headwater systems are intimately linked with downstream ecosystems through the longitudinal coupling of biogeochemical processes and their geographic isolation can support an important component of biodiversity in watersheds (Gomi et al., 2002). Upstream areas are also a critical habitat required by several resident fish (i.e., fish that complete their entire life-cycle in freshwater) and diadromous fish (i.e., fish that migrate between fresh and salt water in either direction throughout their life-cycle) to complete their life-cycle (Schlosser, 1991).

Freshwater land-based salmonid farms commonly discharge their effluents into low order headwater streams (e.g., Oberdorff and Porcher, 1994; Loch et al., 1996; Doughty and McPhail, 1995; Boaventura et al., 1997; Villanueva et al., 2000). This is in part a consequence of the fact that freshwater resource requirements for production are often met in areas with relatively low anthropogenic disturbance. Salmonids are sensitive to low levels of dissolved oxygen (DO), are intolerant to pollution and their optimum temperature range is between 5 and 15º C (Monahan, 1993) (± species-specific variability).

While several reviews have been written on freshwater salmonid farm effluents, their focus has been on the nature of the effluents themselves and/or their treatment, and not necessarily on in-stream ecological effects. With this in mind and the potential effect that freshwater land-based salmonid farm effluents can have on their structure and function, a critical review of the current knowledge and gaps, on

potential effects on stream ecosystems, is not only timely and pertinent but long overdue.

This review presents a concise overview of the effects of freshwater land-based salmonid farm effluents on stream ecosystems as revealed by an analysis of published research dating back to 1990. We discuss relevant considerations related to the temporal and spatial scales of disturbance and ecological effects and the potential role of multiple stressors, with an emphasis on aquaculture veterinary medicines, in the disruption of stream ecosystem structure and function

2.2. Freshwater production trends and geographic distribution

Between 1990 and 2006 the worldwide production of salmonid species in freshwater systems increased at an average rate of 8707 mt per year (2.76%), totalling 400 681 mt in 2006 (FAO, 2008). Freshwater production systems include land-based systems (i.e., aquaculture production systems located on land in specially designed facilities to circulate water through the production units, e.g., tanks, ponds and raceways) and artificial enclosures in freshwater lakes (e.g., cages). As of 2006, 56 countries on six continents produced freshwater salmonids, the bulk of which was concentrated in Europe (50%) and Asia (33%) (FAO, 2008). Freshwater salmonid production in Europe peaked in the 1990s, yet it showed increasing production trends in Asia, South America and North America for the period 1990-2006 ($p<0.05$) (FAO, 2008).

While the culture of anadromous species (fish that migrate to spawn in freshwater after spending a period of growth and maturation in the sea, e.g., Atlantic salmon - Salmo salar) is restricted to temperate areas in the northern and southern hemisphere where marine environmental conditions are within ranges that allow an adequate grow-out of the fish to market size, the culture of resident species (e.g., rainbow trout - Oncorhynchus mykiss) is only restricted by the adequate supply of freshwater resources. Culture of rainbow trout in countries like Brazil, Peru, Mexico, Costa Rica and Venezuela (all of which had statistically significant increasing linear production trends during the period 1990-2006; $p<0.0001$) (FAO, 2008), all of which lie within 33ºS – 33ºN Lat, highlight the fact that there is potential for the expansion of salmonid aquaculture throughout a wide latitudinal range outside the world's temperate regions in areas where geographical,

geomorphologic and environmental variables interact to produce well oxygenated, pollution free and thermally stable water resources.

These freshwater salmonid production statistics include salmonid production in freshwater lakes and thus are not exclusive of land-based production systems. Land-based production systems are, however, a major means of production in leading producing countries. In Iran and Turkey – the leading producers in Asia - trout production is performed mostly in concrete raceways (Abdolhay, 2005; Ersan, 2005), and in Chile - leading producer in South America - the expansion of freshwater salmonid production is limited to land-based farms, as licenses to culture fish in lakes are not currently being granted (León-Muñoz et al., 2007). In Europe and North America, freshwater trout production has traditionally taken place in earthen ponds and raceways (Sedgwick, 1990; Hargreaves et al., 2002), whereas 90% of Atlantic salmon smolt (fish at a stage in development to go to sea) production in Europe takes place in land-based, single-pass flow-through farms, except in Scotland where there are still a large number of smolts grown using cages in lake systems (freshwater lochs) (NCC, 1990; Bergheim et al., 2009).

2.3. Land-based salmonid farm effluents

The quantity and quality of effluents from freshwater land-based salmonid farms differs among production systems and is affected by treatment processes prior to discharge. Effluents from flow-through systems, for example, are characterized by large volumes of very dilute waste (Rosenthal, 1994), while effluents from recirculating aquaculture systems present low volume of highly concentrated waste (Chen et al., 2002). Currently, flow-through systems with varying effluent treatments are the major means of freshwater salmonid production.

The main components of land-based salmonid farm effluents that can cause adverse effects on stream ecosystems are nutrients (mainly nitrogen and phosphorus), biochemical oxygen demand (BOD), suspended solids (SS), pathogens and chemical residues. Most of the studies on effluents have focused on the first three components (e.g., Kendra, 1991; Cripps, 1995; Hennessy et al., 1996; Viadero et al., 2005; Brinker and Rosch, 2005) and few have studied the presence of pathogens (e.g., Blazer and LaPatra, 2002) and chemical residues (e.g., Smith et al., 1994; Lalumera et al., 2004; Rose and Pedersen, 2005).

This review gives particular emphasis to the presence of veterinary medicines within the effluents and their potential ecological effects. A comprehensive review on effluent characteristics from land-based fish farms which focuses primarily on nutrients, BOD and solids has been written by Hinshaw and Fornshell (2002). Comprehensive reviews on production systems and effluent treatment technology have been written by Cripps and Bergheim (2000), Chen et al. (2002), Summerfelt et al. (2001), Bergheim and Brinker (2003) and Sindilariu (2007), among others. Readers are also referred to Rosenthal et al. (1994) and Bergheim and Brinker (2003) for details on the control of fish farm effluents in Europe and to Long and Crane (2003), Montforts et al. (2004), VICH (2000) and VICH (2004) for details on the regulation of veterinary medicines in developed countries. The presence of pathogens in land-based salmonid production effluents, their fate and potential effects are beyond the scope of this review.

2.3.1. Nutrients, BOD and solids

Several environmental, production and management factors influence the output of nitrogen, BOD and solids from land-based salmonid farms and their concentration in the effluents.

In aquaculture effluents, BOD reflects the amount of particulate and dissolved organic matter derived from uneaten feed and faeces and is thus related to the production of dissolved and suspended solids. Solids production is mostly a function of feeding efficiency and is affected by management actions such as tank cleaning (Hinshaw and Fornshell, 2002).

Inorganic phosphorus (e.g., PO_4 - P) is produced by direct excretion by the fish (Clark et al., 1985) and through leaching processes from uneaten feed and faeces (Brinker and Rosch, 2005). The relative percentage of inorganic phosphorous (e.g., PO_4 - P) from total phosphorous (TP) is mainly a function of the concentration and availability of phosphorus in the feed and soluble phosphorus excretion which occurs when the available phosphorus intake is above levels sufficient for retention (Coloso et al., 2003). The ratio of soluble to particulate phosphorus is also negatively correlated with temperature in the range 4 - 13º C (Foy and Rosell, 1991)

Table 2.1. Land-based salmonid farm effluent concentrations of nutrients, BOD and solids reported in studies published between 1991 and 2005 (for an extensive list dating back to 1970 see Hinshaw and Fornshell, 2002).

	Units	TN	NH_4 - N	NO_2 - N	NO_3 - N	TP	PO_4 - P	BOD_5	TSS
[a] Bergheim et al. (1991)	(mg l^{-1}) [g kg $fish^{-1}day^{-1}$]	0.30 - 0.93 [0.17 - 0.81]	0.08 - 0.17 [0.05 - 0.09]	-	-	0.04 - 0.29 [0.03 - 0.16]	-	-	1.0 - 11.3 [0.62 - 11.7]
[b] Kendra (1991)	(mg l^{-1})	0.29	0.19	0	0	0.06	-	3.0	1.0
[c] Doughty and McPhail (1995)	(mg l^{-1})	<0.10 - 1.35	<0.01-0.72	-	-	<0.005 - 0.16	-	<1.0 - 6.0	<1.0 - 17.0
[d] Hennessy et al. (1991)	(mg l^{-1}) [g kg $fish^{-1}day^{-1}$]	0.004 - 2.8 [0.17 - 7.2]	0 - 1.6 [0.07 - 5.5]	-	-	0 - 0.90 [0.03 - 1.9]	-	0 - 181 [1.1 - 549]	0 - 201 [0.98 - 36]
[e] Boaventura et al. (1997)	(mg l^{-1})								
15 mt production		-	0.42 ± 0.17	<0.2	1.0 ± 0.3	-	0.098 ± 0.034	2.0 ± 0.6	1.8 ± 0.3
55 mt production		-	0.32 ± 0.12	<0.2	2.1 ± 0.4	-	0.065 ± 0.030	3.1 ± 1.2	2.4 ± 1.6
500 mt production		-	1.52 ± 0.25	<0.2	1.0 ± 0.2	-	0.59 ± 0.1	15.6 ± 8.4	17.8 ± 4.9
[f] True et al. (2004)	(mg l^{-1})								
raceway biomass ~ 1700 kg		-	-	-	-	0.09 ± 0.01	0.06 ± 0.01	-	1.35 ± 0.40
raceway biomass ~ 7000 kg		-	-	-	-	0.10 ± 0.01	0.06 ± 0.01	-	2.5 ± 0.21
[g] Viadero et al. (2005)	(mg l^{-1})	-	0.03 - 0.33	-	-	-	-	0 - 3.3	1.9 - 9.0

[a] Smolt farm
[b] Median efluent-influent values, except for BOD_5, which is effluent value. TN = NH_4 - N + Org. Nit (Org. Nit. not shown)
[c] Range obtained from quarterly sampling of 19 discharges during 1993
[d] Smolt farms
[e] Flow rates for the three farms in the same order as above were: 72 ± 22 l s^{-1}, 250 ± 31 l s^{-1} and 624 ± 33 l s^{-1}.
[f] Flow rates at farms sampled ranged from 5400 - 510,000 min^{-1}. Samples collected prior to treatment.
[g] Values are effluent-influent.

The NH_4 - N : TN ratio in salmonid effluents is affected by factors such as feed loss in the early stages of salmonid rearing - resulting in a larger fraction of organic nitrogen waste - and differences in the protein source of the feed, which can affect excretion of NH_4 - N (Ackefors and Enell, 1994).

The output of nutrients, BOD and solids in effluents can thus vary as a function of feed quality, feeding strategy, time (e.g., daily and annual cycles) and location (e.g., latitude). Table 2.1 shows that the range of outlet concentrations and loadings from salmonid smolt farms (i.e., fish farms in which salmonids are grown up to the smolt stage and thus stock juvenile salmonids) is generally larger than from salmonid grow-out farms (i.e., fish farms in which salmonids are grown up to market size and thus stock a mixture of juvenile and adult or exclusively adult salmonids). Smolt farm effluents are extremely variable over daily and annual cycles, with much of the variation being related to the life-stage of the stocked fish (Hennessy et al., 1996).

The discharge of nutrients, BOD and solids into stream ecosystems can have multiple and interrelated effects on structural and functional ecological processes, a good example of which is the routes through which they can affect the nitrogen cycle. The input of nitrogen - primarily as NH_4 - N -, can directly increase the nitrogen load of the stream and affect the rate of processes that determine its fate in the streambed (i.e., biotic uptake, nitrification, volatilization, adsorption - Kemp and Dodds, 2002). Nitrification rates in streams are positively related to concentrations of NH_4 - N and dissolved O_2 (Seitzinger, 1988; Kemp and Dodds, 2002) and the nitrification rate of a stream can affect the downstream export of nitrogen because NO_3 - N is more mobile than NH_4 - N (Niyogi et al., 2003; Bernot and Dodds, 2005). Organic pollutants (i.e., biochemical oxygen demand - BOD) and suspended solids can reduce dissolved O_2 in the streambed and hence limit nitrification, as NH_4

- N provides an energy source for nitrifying bacteria as long as dissolved O_2 is available (Bernot and Dodds, 2005). Increased C:N ratios (i.e., high concentrations of dissolved organic carbon) can also inhibit nitrification because heterotrophic bacteria will be subject to nitrogen limitation and outcompete nitrifying bacteria for NH_4 - N (Strauss and Lamberti, 2000). Denitrification often depends on nitrification to provide substrate (i.e., NO_3 - N) (Seitzinger, 1988; Bernot and Dodds, 2005), and hence depending on the degree of coupling between nitrification and denitrification, BOD and suspended solids can indirectly decrease denitrification rates despite generating other favourable conditions for the process (i.e., reduced dissolved O_2 and increased supply of labile carbon).

2.3.1. Veterinary medicines

Pharmaceutically active compounds are complex molecules that are developed and used because of their somewhat specific biological activity (Kümmerer, 2004). Veterinary medicines are widely used to treat disease and improve the health of animals, and they are also used as feed additives to improve the feed conversion ratio in livestock (Boxall et al., 2003).

The environmental fate and potential effects of veterinary medicines used in aquaculture have been addressed by a series of studies and technical reports (e.g., Burka et al., 1997; Jorgensen and Sorensen, 2000; Boxall et al., 2002; Thurman et al., 2002; Boxall et al., 2006). Commonly used veterinary medicines in salmonid aquaculture include anaesthetics (e.g., benzocaine, tricaine methane sulphonate), medical disinfectants (e.g., chloramine-t, formalin, bronopol), parasiticides (e.g., dichlorvos, pyrethroids) and antibiotics (e.g., oxytetracycline, amoxicillin, sulfadimethoxine) (Burka et al., 1997). In freshwater salmonid production, application of medical disinfectants is commonly used to control fungal infections of eggs during hatchery operations (e.g. saprolegniasis) and to treat external fungal and parasitic infections of skin and gills in early feeding and older fish (e.g., costiasis, white spot) (Roberts and Shepherd, 1997). Antibiotics are used to deal primarily with bacterial infections (e.g., furunculosis, bacterial septicaemias) and they are most often administered on a therapeutic basis, although prophylactic application has sometimes been used (Weston, 1996).

Most research related to the use of chemicals in land-based freshwater salmonid production and its potential environmental effects has focused on antibiotics (e.g., Smith et al., 1994; Lalumera et al., 2004; Rose and Pedersen, 2005) and studies on the fate of fungicides and parasiticides are scarce (e.g., Boxall et al., 2006). The same can be said for anaesthetics. This can be partly due to the fact that data on the amounts of veterinary medicines used in aquaculture besides antimicrobials has not been readily available (Boxall et al., 2002).

Thurman et al. (2002) found measurable concentrations of the antibiotics oxytetracycline and sulfadimethoxine in intensive and extensive fish hatcheries in the United States. Analytically detected oxytetracycline concentrations were in the range 0.17 to 10 ugl^{-1} (with 10 ugl^{-1} being measured in the settling pond of one hatchery), suggesting that concentrations in this order of magnitude could eventually reach the environment.

A recent risk assessment of compounds within a veterinary medicine priority list in the United Kingdom included a trout hatchery (egg treatment) and a land-based trout farm (fish treatment) as treatment scenarios for amoxicillin, oxytetracycline and bronopol (a fungicide) (Boxall et al. 2006). Risk characterization ratios calculated using predicted environmental concentrations (PEC) and predicted no effect concentrations (PNEC) determined from aquatic ecotoxicity data were > 1 for the three compounds and ranked bronopol highest, followed by oxytetracycline and amoxicillin. Simulated peak water column concentrations of the compounds through the farm system over a 24-hour period were 531 μgl^{-1} for amoxicillin, 236 μgl^{-1} for oxytetracycline and 9.5 μgl^{-1} for bronopol, and after 24 hours the three compounds had dissipated and were assumed to be undetectable. This emission pattern for oxytetracycline agrees with on-site sampling studies, which suggest that all of the administered drug would be released from the culture system within a 24 hour period (Smith et al., 1994).

In a different study, Rose and Pedersen (2005) indicated that 10 - 15% of the administered oxytetracycline would be released as a pulse to the receiving water during treatment and in the first 5 days thereafter. Median water column oxytetracycline concentrations modelled by Rose and Pedersen (2005) were considerably smaller in the fish farm's settling pond ($< 10^{-6}$ ngl^{-1}) and receiving stream segments (0.57 - ~ 0.8 ngl^{-1}). Their model considered association of oxytetracycline with bed sediments, settleable particles and organic and inorganic

colloids and their oxytetracycline dosing scenario was comparatively smaller than the one used by Boxall et al. (2006).

Concentrations of oxytetracycline and flumequine have been analytically measured in the sediments of stream segments receiving trout farm effluents, with peak oxytetracycline concentrations reaching 246.3 μg kg^{-1} d.w. (Lalumera et al., 2004). The half life of oxytetracycline in stream sediments has been estimated at 118 days using a modelled stream temperature of 13.1ºC (Rose and Pedersen, 2005). Sensitivity analysis of model simulations have shown that the concentration of settling pond influent biosolids, oxytetracycline biosolid depuration rate, biosolid settling velocity and hatchery discharge rate are the most relevant parameters influencing the in-stream fate of oxytetracycline and that its predominant fate in streams is sediment deposition (Rose and Pedersen, 2005).

2.4. Effects of effluents on stream ecosystems: overview of current studies

Several studies have assessed the effects of freshwater land-based salmonid farm effluents on stream ecosystems. In this section we review their results to provide a general overview of what is known about the type, extent and magnitude of their effects on stream ecosystems. The section is structured according to the biological components on which the studies reviewed focused (i.e., microbial communities, primary producers, benthic macroinvertebrates and fish communities).

2.4.1. Microbial communities

Microbial communities in lotic ecosystems play a key role in the recycling and remineralization of nutrients and can constitute a pathway of energy to higher trophic levels (Allan and Castillo, 2007).

The effects of land-based salmonid farm effluents on stream microbial communities have been studied by Carr and Goulder (1990a), Brown and Goulder (1996, 1999), and Boaventura et al. (1997) (Table 2.2). Carr and Goulder (1990a) studied the effects of land-based salmonid farm effluents on the downstream microbial abundance and heterotrophic activity of bacterial populations in three calcareous rivers in north east England (Table 2.2). During four sampling dates in the Pickering Beck and the River Hull, which received effluents from trout farms

with a production of approximately 40 and 300 tonnes respectively, they measured significant increases in acridine orange direct counts (AODCs) and colony forming units (CFUs) in the water column. In the downstream top sediment layer (1.0 cm and considered on a sediment dry weight basis) both rivers had increased levels of CFUs, AODCs and heterotrophic activity. However, heterotrophic activity in the water column only significantly increased downstream of the trout farm with the highest annual production (200 - 300 tonnes). The trout farm discharging its effluent into the third stream (Drifield Canal) had the smallest annual production (12 - 15 tonnes) and appeared to have little downstream impact. Boaventura et al. (1997) achieved similar results in a comparative study which measured the highest concentrations of mesophilic bacteria and BOD_5 downstream of the trout farm with the highest annual production. Carr and Goulder (1990a) attributed the observed increases in bacterial abundance and heterotrophic activity to the accumulation of organic matter originating as suspended solids in the fish farm effluents. As they pointed out, increased aerobic metabolism as a consequence of increased organic matter can lead to oxygen depletion and mineralization of organic nutrients (i.e., N and P), with consequent effects on the trophic state of the river itself and its receiving water bodies (e.g., lakes, estuaries).

In successive studies, Carr and Goulder (1990a) and Brown and Goulder (1996, 1999) measured the abundance of extracellular enzyme activity downstream of the trout farm in River Hull (Table 2.2). Significant increases in water column alkaline phosphatase activity (APA) were measured up to a considerable distance downstream of the effluent discharge point (Carr and Goulder, 1990a; Brown and Goulder, 1996). Carr and Goulder (1990a) did not find the increased APA to be associated to the river's microflora and attributed its presence to direct excretion by farmed rainbow trout. Similarly, Brown and Goulder (1996) found that a considerable proportion of the significantly increased extracellular activity of the enzymes they measured was free in the water column (i.e., not associated to bacteria and/or algae), suggesting that they might be released by fish-farm sediments or excretion by the farmed fish. In epilithic biofilms, Brown and Goulder (1999) observed a decline in phosphatase activity that was concurrent with an increase in the overlying water column PO_4 - P concentration and suggested that this indicated end product repression of phosphatase synthesis. Aminopeptidase activity in the

biofilms was significantly increased but not when expressed per unit bacterial cell, suggesting its incorporation into the biofilms from the overlying water column.

Besides the studies mentioned above, which directly quantified microbial parameters, Loch et al., (1996) and Doughty and McPhail (1995) observed the presence of sewage fungus in reaches receiving land-based salmonid farm effluents. Sewage fungus is composed mainly of filamentous bacteria, algal filaments and stalked protozoa, but its specific composition varies depending on the nature of the effluent (Becker and Shaw, 1955). Doughty and McPhail (1995) described the presence of sewage fungus as 'the most obvious manifestation of impact on the microbial community' in a review of 18 land-based fish farms in Scotland during the period 1989-1993. They indicated that in some situations sewage fungus extended beyond the stream reach receiving the effluent and across the whole width of the watercourse. Loch et al. (1996) reported the presence of sewage fungus in great abundance 20-50 m downstream of all trout farms studied and in considerably smaller amounts in the stream bed 1500 m downstream.

The modification of microbial communities due to the presence of aquaculture effluents may have effects on ecosystem structure and function. Although elemental process rates were not measured in these studies, higher tier effects of the effects observed on the microbial community are likely. This has been to a certain extent confirmed by observations of differences in nitrogen species concentrations between upstream and effluent receiving stream segments by Kendra (1991), Carr and Goulder (1990b) and Oberdorff and Porcher (1994), which suggest significant disruption of the nitrogen cycle.

2.4.2. Primary producers

Primary producers (e.g., stream algae) are most directly affected by physical and chemical factors (Barbour et al., 1999) and are thus very susceptible to changes in environmental conditions produced by the discharge of fish farm effluents.

Table 2.2. Effects of land-based salmonid farm effluents on stream ecosystems, indicating parameters/endpoints measured, downstream response and the maximum downstream distance sampled with observed response. Effluent treatments are indicated for those studies that reported it.

Reference	Effluent treatment system	Measured parameter	Units	Downstream response[a]		Maximum downstream distance sampled with observed response[b]	
[c] Carr and Goulder (1990a)	settling pond			water column	sediments	water column	sediments
		CFU	(x 10^5 ml^{-1}) - (x 10^7 g^{-1})	significant increase	significant increase	10 - 200 m	10 - 200 m
		AODCs	(x 10^6 ml^{-1}) - (x 10^9 g^{-1})	significant increase	significant increase	720 m	10 - 200 m
		Activity	(µg l^{-1} h^{-1}) - (µg g^{-1} h^{-1})	significant increase	significant increase	10 - 200 m	10 - 200 m
		APA	µg PO_4-4 released l^{-1} h^{-1}	significant increase	-	720 m	-
		Phytoplankton chl *a*	µg l^{-1}	no significant increase	-	-	-
				water column			
[c] Carr and Goulder (1990b)	settling pond	PO_4-4	µg l^{-1}	significant increase		720 m	
		NH_3-N	µg l^{-1}	significant increase		10 - 200 m	
		NO_2-N	µg l^{-1}	no significant response		-	
		NO_3-N	mg l^{-1}	no significant response		-	
		Algae growth potential	cells x 10^6 ml^{-1}	significant increase		0 - 270 m	
		Periphyton chl *a*	µg cm^{-2}	significant increase		0 - 1000 m	
		Extension and adventitious root growth potential of *Ranunculus sp.*	mm	significant increase		0 - 600 m	
		TN and TP concentrations in *Ranunculus sp.* tissues	% dry weight	significant increase		600 m	
Kendra (1991)	7 out of 20 farms had solids removal systems	Benthic macroinvertebrates - EPT	relative abundance	decrease		~ 500 m	
Camargo et al. (1992)	settling pond	Benthic macroinvertebrates - Species richness and diversity	total number of taxa / index (H')	decrease		1000 m	
		- Functional feeding groups	relative abundance	trophic structure modification		1000 m	
Oberdorff and Porcher (1994)		Index of Biotic Integrity (IBI)	composite index	reduction in IBI score		~ 9000 m	
[d] Doughty and McPhail (1995)		Benthic macroinvertebrates	relative abundance	increased abundance of tolerant groups			
[d] Doughty and McPhail (1995)		Benthic macroinvertebrates	relative abundance	increased abundance of tolerant groups		-	

Loch et al. (1996)	settling pond	Benthic macroinvertebrates			
		- Taxa richness (EPT)	total number of taxa	significant decrease	1500 m
		- Functional feeding groups (EPT)	relative abundance	trophic structure modification	1500 m
[e] Brown and Goulder (1996)	settling pond	Extracellular-enzyme activity			
		- Leucine aminopeptidase	nmol l^{-1} h^{-1}	increase	~ 2000 m
		- β - Glucosidase	nmol l^{-1} h^{-1}	increase	~ 2000 m
		- Phosphatase	nmol l^{-1} h^{-1}	increase	~ 2000 m
		Microbial abundance			
		- Free-living bacteria	x 10^6 ml^{-1}	increase	~ 1000 m
		- Particle-bound bacteria	x 10^6 ml^{-1}	increase	~ 400 m
		Phytoplankton chl *a*	µg l^{-1}	increase	~ 4000 m
[f] Boaventura et al. (1997)		BOD_5	mg l^{-1}	increase	12000 m
		NH_4-N	mg l^{-1}	increase	12000 m
		PO_4-4	mg l^{-1}	increase	12000 m
		Mesophillic bacteria colonies	ml^{-1}	increase	12000 m
Brown and Goulder (1999)		Epilithic extracellular-enzyme activity			
		- Leucine aminopeptidase	nmol cm^{-2} h^{-1}	significant increase	~ 200 m
		- Phosphatase	nmol cm^{-2} h^{-1}	significant decrease	~ 200 m
Villanueva et al. (2000)		Periphyton			
		- chl *a*	µg cm^{-2}	significant decrease	100 m
		- species composition	relative abundance	modification	100 m
[g] Hurd et al. (2008)		$\delta^{13}C$	permil	sediment and biota enrichment	-
Selong and Helfrich (1998)	2 out of 5 farms studied had settling ponds	NH_4-N	mg l^{-1}	significant increase	400 m
		NO_2-N	mg l^{-1}	significant increase	400 m
		TP	mg l^{-1}	no significant increase	-
		Periphyton chl *a*	mg cm^{-2}	significant increase	400 m
		Benthic macroinvertebrates			
		- RBP metrics	composite index	decrease in RBP score	400 m [h]
		- Index of Biotic Integrity (IBI)	composite index	no significant response	400 m [i]

[a] The term 'significant' is used when a statistical significance test accompanied the results.

[b] Maximum downstream distances sampled with observed response presented in ranges (e.g., 10 - 200 m) indicates that upstream and downstream samples were aggregated previous to statistical comparison. A single definite distance cannot be inferred from the studies.

[c] Carr and Goulder (1990ab) studied three streams. The results presented here correspond to those of River Hull, UK, which was more extensively studied. CFU = colony forming units; AODCs = acridine orange direct counts; Activity = glucose mineralization rate to CO_2; APA = alkaline phosphatase activity.

[d] Effects observed in 18 land-based salmonid farms sampled between 1989 - 1993 by the Clyde River Purification Board, East Killbride, Glasgow, UK.

[e] Results correspond to the River Hull, UK, where Brown and Goulder (1996) studied a 13 km reach receiving effluents from 4 trout farms. The maximum downstream distance sampled with observed response was estimated from the figures presented in the original manuscript.

[f] Boaventura et al. (1997) studied 3 trout farms with increasing annual production. The 12000 m distance with observed impaired conditions may have been affected by other activities discharging wastes into the stream below the farm studied. Only selected parameters are included in this table.

[g] Hatchery feed is enriched in ^{13}C relative to freshwater autotrophic sources and hence Hurd et al. 2008 used $\delta\ ^{13}C$ to track hatchery derived carbon through 3 stream ecosystems.

[h] Out of 5 trout farm studies, 2 generated impaired environmental conditions downstream and the other 3 produced slightly impaired or unimpaired downstream conditions.

[i] One out of 5 trout farms had a reduced downstream IBI score with respect to the upstream reference station.

The effects on downstream primary production have been studied by Carr and Goulder (1990a, 1990b), Brown and Goulder (1996), Selong and Helfrich (1998) and Villanueva et al. (2000) (Table 2.2). Carr and Goulder (1990a) found no significant increase in phytoplankton chlorophyll a downstream of a trout farm in River Hull, while Brown and Goulder (1996) found an increase in phytoplankton chlorophyll a in the same river during a later study. In the same stream segment, Carr and Goulder (1990b) found a significant increase in periphytic chlorophyll a that stretched 1000 m downstream and which they experimentally demonstrated was due to a significant increase in PO_4-P concentrations. Similarly, Selong and Helfrich (1998) found significantly increased periphyton chlorophyll a directly beneath the farm where the effluent mixed with the receiving water and which was positively correlated with feed loading rates. In their study, periphyton chlorophyll a returned to upstream reference conditions 400 m downstream in 4 of the 5 farms studied.

Contrasting with the results of Carr and Goulder (1990b) and Selong and Helfrich (1998), Villanueva et al. (2000) found a significant downstream decrease in periphyton chlorophyll a concurrent with a shift in community structure. All prostrate species (e.g., Nitzschia supralitorea, Navicula cryptocephala) decreased downstream of the effluent discharge point. They attributed the decrease in periphyton chlorophyll a to a potential effect of fish-farm derived solids deposition on benthic photosynthesis, which led to a downstream shift towards heterotrophy. These apparently conflicting results may be due to the fact that Villanueva et al. (2000) only sampled at upstream controls and 100 m downstream of the effluent, where the effects of solids deposition on the streambed could have overridden any stimulatory effects on primary production by nutrient enrichment. Further downstream effects on benthic primary production once the effects of solids deposition were attenuated could have been missed due to the short downstream sampling distance. Differences in physical variables such as stream flow and stream bed structure (i.e., size and shape of substrata) can also modulate the effects of solids by influencing their deposition rate (and hence the area over which they are dispersed) and adherence to the substrata. Factors such as these can also account for the conflicting results between studies.

Although some studies observed responses in periphytic chlorophyll a in opposing directions (e.g., Carr and Goulder 1990b and Selong and Helfrich, 1998 vs.

Villanueva et al., 2000), they all report a quantifiable effect with respect to reference conditions. Overall, these results indicate that land-based salmonid farm effluents can affect the species composition of periphytic communities and the primary production of both periphyton and phytoplankton in streams.

2.4.3. Benthic macroinvertebrates

The response of the benthic macroinvertebrate community seems to be fairly consistent across studies (Table 2.2). Kendra (1991) and Loch et al. (1996) found a decrease in the taxa richness of Ephemeroptera, Plecoptera and Trichoptera (EPT), Camargo et al. (1992) found a decrease in species richness and diversity and Selong and Helfrich (1998) reported a decrease in the Rapid Bioassessment Protocol (RBP) score, which integrates measures of diversity, similarity, indicator taxa, community structure and a family level biotic index. Doughty and McPhail (1995) concluded that the most commonly observed impact across 18 land-based salmonid farms in Scotland was an increase in numbers of individuals in tolerant groups (e.g., oligochaetes, chironomids), albeit without the loss in abundance within sensitive groups. This can be interpreted as a decrease in diversity (e.g., the Shannon-Wiener information theory index H'), as the increase in tolerant groups decreases species evenness. Camargo et al. (1992) also found a significant shift in the functional groups of the benthic macroinvertebrate community which reflected structural alterations typical of organic pollution. The increase in food supply generated by the fish farm induced the dominance of collector species (e.g., tubificids, baetids, simulids, and chironomids) and the disappearance of detritivore shredders, which did not return to upstream values 1000 m downstream of the fish farm. Loch et al. (1996) found decreased abundance of scrapers at a station located 20-50 m downstream of the effluent discharge and increased abundance of collectors at two downstream stations up to a distance of 1500 m and suggested that they were utilizing fine organic matter derived form the farms. However, contrasting with the results of Camargo et al. (1992), shredders were not significantly different between an upstream reference station and a 20-50 m downstream station, suggesting that natural allochtonous inputs remained a relevant energy source to the stream food web. Hurd et al. (2008) recently showed using ^{13}C that the contribution of hatchery

derived C to the diet of pollution-tolerant isopods was 39 - 51% in a stream receiving limestone spring water via hatchery effluent.

Specialized feeders like shredders and scrapers are expected to decrease with increasing perturbation, while generalists like collectors and filter feeders are more tolerant to pollution that may alter the availability of food resources (Barbour et al., 1999). Benthic macroinvertebrate shredders are an extremely relevant trophic pathway in headwater streams involving the transformation of coarse particulate organic matter (CPOM), and significant modifications in their abundance in a stream segment inevitably modifies the downstream linkage of energy sources. A potential route through which salmonid farm effluents could affect the distribution of shredders is by affecting the quality of their available food resources. Shredders select their food based on leaf litter characteristics such as tenderness, nutrient content, phytochemistry and microbial conditioning (Graca, 2001). Of these characteristics, microbial conditioning is the most likely to be affected by land-based salmonid farm effluent pollution. Microbial conditioning of leaf litter is associated primarily to aquatic hyphomycetes, which account for approximately 95% of the total microbial biomass associated with submerged decaying plant litter (Baldy and Gessner, 1997; Hieber and Gessner, 2002; Gulis and Suberkropp, 2003a). Pascoal and Cássio (2004) found maximum fungal biomass in leaf litter to be positively correlated with DO and observed a decrease in microbial leaf litter decomposition in a high nutrient/low DO sampling site. Hence by increasing downstream nutrient concentrations and decreasing DO, effluents could potentially affect the conditioning of leaf litter by aquatic hyphomycetes and consequently the distribution and abundance of shredders.

2.4.4. Fish communities

The distribution of fish species within river networks is determined by a combination of factors set at large scales (i.e., climate, geology and topography) - which influence smaller scale processes that influence the availability of different habitat types within the network - and by the presence of unique habitats and the occurrence of disturbance events at specific locations that influence properties of the system in either direction (Fausch et al., 2002). The terrestrial-aquatic interface in upstream reaches of fluvial networks provides environmental conditions that are

considered critical for fish reproduction (Schlosser, 1991). Physico-chemical degradation of these areas as a result of anthropogenic pollution can restrict the availability of suitable habitats for certain species and constrain them from completing important life history processes.

Oberdorff and Porcher (1994) used a modified version of the Index of Biotic Integrity (IBI) for fish assemblages (Karr, 1986), which integrates species richness and composition, trophic composition and fish abundance and condition to assess the impact of 9 trout farms in 6 streams in Brittany. They found lower IBI scores downstream of the 9 fish farms studied and found the impact on fish assemblages to be positively correlated with fish farm production. Selong and Helfrich (1998), on the other hand, used the standard IBI in conjunction with other ecological endpoints (i.e., measurable parameters that provide information on the quantitative and/or qualitative state of an ecological process or biotic community within an ecosystem) to assess the effects of 5 trout farms. Their IBI scores did not indicate downstream impairment, which contrasted to the results they obtained using the RBP metrics for benthic macroinvertebrates and attributed to the fact that the IBI may have not accurately reflected the environmental conditions in headwater systems with low fish richness and abundance.

Oberdorff and Porcher (1994) attributed the observed effects on fish assemblages to organic and inorganic enrichment and siltation and concluded that 'fish farming can cause both structural and functional changes in wild fish assemblages'. The fact that they observed the impacts on fish assemblages to be negatively correlated with catchment area suggests that larger catchments could help buffer the effects of effluent pollution through scale-dependent processes of species distribution.

2.5. Temporal and spatial scales in effluent discharge and ecological effects

The studies reviewed in section 4 show that land-based salmonid farm effluents affect a series of in-stream structural and functional ecological endpoints, and can therefore be considered a disturbance (USEPA, 1997). To provide meaningful information on their potential ecological effects, disturbances should be defined by the nature of their damaging properties, along with parameters describing their spatial extent and temporal characteristics (frequency, predictability and temporal duration) (Lake, 2000). In addition, defining appropriate measures to assess

anthropogenic effects on ecosystems is essential to identify ecologically relevant impacts, their link to larger scale ecological processes and to define adequate assessment endpoints to be used in environmental management (see USEPA, 1997 for a definition of assessment endpoint).

2.5.1. Temporal scales

Lake (2000) defined three types of disturbance and ecological responses to them in stream ecosystems to characterize flow generated disturbances and the response of the biota to them; presses, pulses and ramps. These types of disturbance and ecological response can be applied to anthropogenic pollution and provide an explicit framework to characterize their subsequent temporal patterns. Presses are disturbances that arise sharply and then reach a constant and maintained level, while pulses are short-term and clearly delineated disturbances (Lake, 2000). Ramps are disturbances whose strength increases steadily over time that may or may not reach an asymptote after an extended period of time (Lake, 2000).

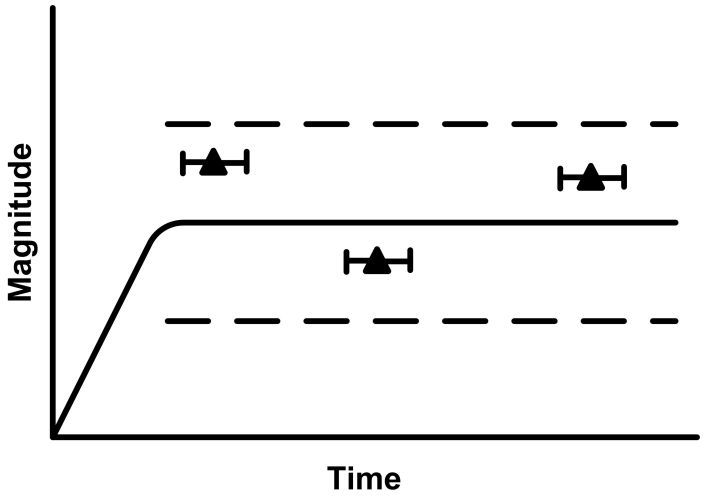

Figure 2.1. Temporal pattern of effluent discharge on a hypothetical yearly timescale. The solid line represents the press disturbance generated by nutrients, BOD and solids and the dashed lines account temporal variation in their magnitude. Solid triangles represent pulses of veterinary medicines released within the effluent as discrete events in time that vary in their duration (horizontal bars) and magnitude (displacement along the ordinate) (adpated with permission from Lake, 2000).

The temporal pattern of effluent discharge from land-based salmonid farms can be best defined as a press disturbance with nested pulse disturbances (Figure 2.1). Although the concentrations of BOD, nutrients and solids in the effluents vary with time (e.g., daily pulses and annual cycles) due to factors such as feeding and excretion, the rearing stage of the fish, temperature and unpredictable events (e.g., system failure), their release is continuous on a yearly timescale and can be considered to fluctuate within a flexible range of values (e.g., Table 2.1). Alternatively, the use of veterinary medicines on a therapeutic basis is discrete and its frequency of occurrence (i.e., number of events from t_1 - t_n) is related to epidemiological factors of the disease being treated. Their prophylactic use should follow a similar discrete pattern, as preventive treatments are likely to be associated to specific rearing stages and/or times of the year. The occurrence of other chemical compounds, such as disinfectants, will also be discrete as they are associated to non-continuous management actions (e.g., tank cleaning).

The solid line in Figure 2.1 represents the effluent discharge of BOD, nutrients and solids and the dashed lines account for temporal variability in their concentrations that are not monotonically increasing or decreasing. The pulse disturbances generated by the discharge of veterinary medicines nested within the press disturbance generated by BOD, nutrients and solids are represented by black triangles in Figure 2.1. Pulses can vary in their duration and concentration from time to time, represented by the triangle's horizontal bars and the displacement of the triangles along the ordinate in Figure 2.1, respectively. However, the fact that certain veterinary medicines might persist in stream sediments (e.g., OTC - Rose and Pedersen, 2005) implies that their frequency of occurrence as a disturbance in the environment may not be synchronous with their frequency of occurrence in the effluent. If a compound remains bioactive or regains bioactivity through e.g., sediment resuspension, its frequency of occurrence may result in pulses that exceed its frequency of discharge in the effluent or effectively in a press type disturbance. In continuous, multiple cohort production programmes where specific life stages (e.g., alevins) are successively and continuously produced, specific compounds may be continuously used and discharged within the effluents (e.g., to treat Saprolegnia sp.).

The response of ecological endpoints to disturbance (i.e., press, pulse or ramp) (Figure 2.2) is dependent on the resolution of the ecological metric used to assess

the response. A ramp response, as shown in Figure 2.2, can eventually lead to the extinction of an ecological endpoint and is most likely to be observed when considering structural endpoints of low biological organization. A press response in community level parameters of benthic invertebrates, for example, can imply a ramp response (i.e., local extinction) from one or several species. The disappearance of shredders from the stream segment affected by trout farm effluents in the study of Camargo et al. (1992) (see section 5.3) is an example of such a case. The studies reviewed in section 5 suggest that several structural and functional endpoints at the community and ecosystem level of organization in stream reaches receiving salmonid farm effluents respond as a press (i.e., they have shifted to new perturbed states). This is particularly apparent in the case of the benthic macroinvertebrate community, whose consistent response across studies indicates a shift towards taxonomic and functional groups tolerant of altered physicochemical variables and able to efficiently exploit the niches created by disturbance. The degree to which pulse responses of particular ecological endpoints might be nested within press responses and the role of multiple stressors in eliciting these nested responses, however, is not clearly understood.

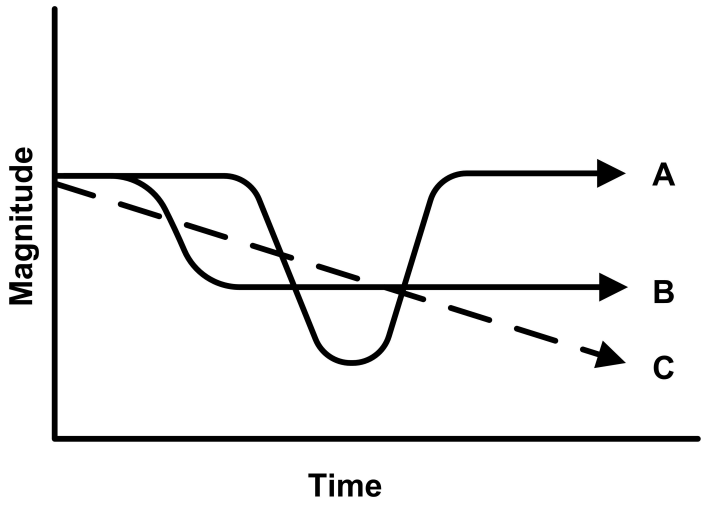

Figure 2.2. Temporal response pattern of ecological endpoints to disturbance. (A) pulse, (B) press and (C) ramp. The x-axis is on a generic timescale (adapted with permission from Lake, 2000).

The long term effects of a continuous pollution source can be assessed by considering the ecosystem's resilience once the polluting events finish (e.g., once the salmonid farm stops operating). Streams have the ability to displace the effects of stressors by diluting them and exporting them downstream (Pratt and Cairns, 1996), giving them a remarkable natural ability to recuperate from stress. The time-scales involved in stream recovery from land-based salmonid farm effluent disturbances once they have ceased to operate are relatively unknown. Hurd et al. (2008) recently showed using ^{13}C that the sediments of a spring creek remained enriched with hatchery derived C for several years after hatchery closure. To our knowledge, this is the only study that has made a partial assessment of the recovery of stream food webs after the cessation of effluent discharge.

2.5.2. Ecological costs

Despite the complete recovery of both ecosystem structure and function after the cessation of disturbance, there can be ecological 'costs' associated to the biota and to aggregate functional processes. Genetically inherited tolerance due to stress (i.e., microevolution due to pollution - Medina et al., 2007) can lead to trade-offs in a population's ability to efficiently exploit its niche (e.g., functional role) and cope with future stress, thus modifying ecosystem stability in relation to pre-disturbance conditions. Vinebrooke et al. (2004), for example, noted that functional groups that had been previously exposed to agricultural stressors may exhibit stress-induced community sensitivity and be especially sensitive to the impacts of future urban development. Costs associated to relevant stream ecosystem functions can be estimated in terms of e.g., increment of nutrient export and removal using standard methodologies in stream ecology (e.g., see Hauer and Lamberti, 2007) and more sophisticated approaches recently proposed (e.g., Mullholand et al., 2004; Payn et al., 2005; Runkel et al., 2007). The relevance of quantifying such functional costs in stream ecosystems has become evident from recent research in which the importance of in-stream process rates to maintaining whole watershed water quality has been highlighted, particularly in small streams (e.g., Alexander et al., 2000; Peterson et al., 2001; Mullholland et al., 2008).

2.5.3. Spatial scales

The spatial extent of the disturbance generated by land-based salmonid farm effluents can be defined as the furthest downstream distance at which physical, chemical and biological parameters in the stream remain significantly different from background/reference values due exclusively to the effluent discharge. This in turn will also depend on their intrinsic properties. Dissolved elements can be expected to travel further downstream than suspended elements, as the latter will tend to settle to the streambed faster. The dynamics of conservative solutes will be driven mainly by advection and dispersion, but can be affected by biological processes that affect transient storage (Battin et al., 2003). Non-conservative solutes (e.g., nutrients) are also subject to processes such as adsorption, desorption, precipitation and dissolution as well as to biotic uptake, storage, removal and release (Webster and Valett, 2006), which will influence the spatial extent to which downstream communities are exposed.

Due to the multiple ecological interactions and the downstream linkage of stream ecosystems, the spatial extent of ecological effects can extend beyond the spatial scales that define the environmental fate of individual stressors. Headwaters are source areas of aquatic and terrestrial invertebrates and detritus that link upland ecosystems with lower habitats in the catchment and support growth and density of fisheries (Wipfli and Gregovich, 2002). Hence disruptions in local species distribution and material processing (i.e., stream segment in direct contact with the effluent) can have downstream ecological consequences. From the published literature (i.e., Table 2.2) it is not possible to give specific predictive estimates of the downstream distances impaired due to effluent discharges, as studies usually focus on a subset of ecological parameters that are often not sampled sufficiently downstream to determine recovery. Conversely, in studies in which downstream conditions have been observed to fully recover, the spatial extent of effects only applies to measured ecological endpoints and extrapolation to other ecological parameters is not straightforward. Each type of study will therefore only give a partial view of full in-stream effects.

Recent studies within the context of 'wider' ecosystem-level effects of aquaculture in marine ecosystems have pushed the scales of analysis beyond cage footprint areas either by directly increasing the spatial extent of analysis (e.g., Ford et al.,

2007) or by studying aquaculture-environment interactions that have direct implications for the up-scaling of relevant ecological processes (e.g., Kutti et al., 2008). In the context of stream ecosystems, the natural scale at which aggregate impacts from aquaculture effluents can be assessed is the catchment. The catchment scale is considered appropriate for conservation (Dudgeon et al., 2006) and downstream impacts due to headwater functional disturbance (e.g., Alexander et al., 2000; Peterson et al., 2001) are likely to be seen in full perspective when considered on a catchment-scale basis. Aggregate impacts at the catchment scale can be expressed as the length of stream impaired, defined by specified ecological metrics, within that particular catchment. Additionally, cumulative catchment scale effects can be expressed in terms of ecosystem services not provided within their normal range by e.g., quantifying biogeochemical process rates relevant to management targets. At increasing spatial scales of analysis, the relative contribution of land-based salmonid farm effluents in relation to other sources of anthropogenic stress will vary, requiring an integrative approach to distinguish the contribution of these different sources. In doing so, explicit consideration of the natural processes operating at different locations within the catchment is important. Hydrogeomorphic and biological processes between headwaters and network systems have fundamental differences, and the spatio-temporal variations of processes in headwater systems are critical to the dynamics of stream ecosystems and the heterogeneity of riparian and riverine landscapes in channel networks (Gomi et al., 2002). The use of weighted approaches to consider different anthropogenic sources of stress might be necessary to account for the differential ecological relevance of the processes being disrupted at different spatial locations within a catchment.

2.6. Distinguishing the effects of multiple stressors

Distinguishing types of disturbance and response (i.e., perturbation - Glasby and Underwood, 1996) is important because it enables the design of studies to determine the exact cause(s) of measured effects and because it allows the implementation of specific management actions to reduce or eliminate responsible stressors (Glasby and Underwood, 1996). This can at times be a difficult task, as multiple stressors can have multiple and interacting effects on determined ecological processes. In a

recent study using short-term bioassays, for example, antibiotic mixtures used in aquaculture were shown to have synergistic effects on Pseudokirchneriella subcapitata and activated sludge microorganisms (Christensen et al., 2006). In aquaculture facilities culturing several batches of fish, more than one compound can be used at the same time and they could thus reach the effluent simultaneously. This can occur not only with mixtures of antibiotics, but with mixtures consisting of several different groups of veterinary medicines (e.g., antibiotics and fungicides, fungicides and disinfectants, antibiotics and disinfectants, fungicides and anaesthetics, etc.).

The relative contribution of pulses of veterinary medicines to the overall effect produced by land-based salmonid farm effluents in stream ecosystems has not received considerable attention by researchers and it is currently a knowledge gap for environmental impact assessments and risk characterization procedures. To implement effective management actions, it is desirable that the probability of a specific compound - and mixtures of compounds - in the effluent reaching or exceeding a certain value (i.e., $P(x \geq X)$) can be causally linked to predicted effects in the receiving ecosystem. This allows the implementation of more targeted management actions and can avoid potential under and over environmental regulation. While decades of research in stream ecology (e.g., Allan & Castillo, 2007) provide a general mechanistic understanding on how nutrients, BOD and solids discharged from effluents can potentially affect stream ecosystems, the effects of veterinary medicines and other pharmaceuticals on ecological structure and function is still a matter of ongoing research. Potentially, there may be as many patterns of ecological effects as groups of compounds being discharged into the environment.

Studies assessing the ecological effects of aquaculture veterinary medicines have mainly focused on biogeochemical processes in marine sediments (e.g., Hansen et al., 1992; Capone et al., 1994). Capone et al. (1994) found no measurable effects of oxytetracycline on microbial density, NH_4-N flux, sulphate reduction rates or O_2 consumption. Conversely, Hansen et al. (1992) reported a significant reduction in microbial density and a sharp decrease in sulphate reduction upon exposure to high doses of oxytetracycline, oxolinic acid and flumequine. In a freshwater microcosm, Klaver and Mathews (1994) found that oxytetracycline had a dose-dependant inhibitory effect on nitrification which spanned a range of oxytetracycline

concentrations. The concentrations of antibiotics tested in theses studies, however, are orders of magnitude above modelled and measured concentrations in stream segments receiving salmonid farm effluents (see section 3.2).

The minimum inhibitory concentration and minimum bactericidal concentration of oxytetracycline hydrochloride have been shown to decrease significantly in the presence of river sediment (Vaughan and Smith, 1996a), suggesting that the bioavailability of oxytetracycline and consequently its ability to disturb biogeochemical processes in stream sediments might be strongly reduced. However, these results must be interpreted with caution, as sediment bound oxytetracycline might undergo processes under natural environmental conditions that would allow it to regain its biological activity (e.g., sediment resuspension) (Vaughan and Smith, 1996a). Test organisms (e.g., Y. ruckeri and S. aureus) and experimental conditions (e.g., culture temperature) might also not accurately represent the range of sensitivities encountered in natural microbial populations and adequately reflect in-stream environmental conditions, respectively.

Guardabassi et al. (2000) observed a decrease in genetic diversity concurrent with an increase in resistance to oxolinic acid in Acinetobacter spp. isolated downstream of a trout farm 21 days after the fish had been treated with the antibiotic. It is important to highlight that Guardabassi et al.'s study showed that the increase in antibiotic resistance was caused by oxolinic acid and not by non-antibiotic effluent constituents as observed for oxytetracycline by Vaughan et al. (1996b). These results suggest that antibiotic-induced selective pressures led to a decrease in diversity and an increase in the abundance of resistant mutants in the stream's microbial community.

Recent laboratory studies have shown that environmental concentrations of pharmaceuticals can have measurable subtle effects on ecologically relevant microbial parameters (Schreiber and Szewzyk, 2008), and testing of these results in experimental systems which represent natural ecosystems adequately awaits further research. Schreiber and Szewzyk (2008) showed that environmentally relevant concentrations of pharamaceuticals influenced the inital adhesion of bacteira, and Maul et al. (2006) found a shift in function of leaf-associated microbial communities in samples exposed to ciprofloxacin and attributed the result to reduced carbohydrate substrates in exposed leaves. Using food selection experiments with Gammarus pulex, Hahn and Shulz (2007) found that the

amphipod clearly preferred leaves conditioned in the absence of oxytetracycline and sulfadiazine. Bundschuh et al. (2009), on the other hand, found Gammarus fossarum to prefer leaf discs conditioned in the presence of an antibiotic mixture of sulfamethoxazole, trimethoprim, erythromycin-H_2O, roxithromycin, and clarithromycin which was related to a shift in fungal communities. Experimental studies on the interactions between aquatic hyphomycetes and bacteria associated with decomposing leaf litter have shown that gains in fungal biomass and sporulation rates are delayed in the presence of bacteria (Gulis and Suberkropp, 2003b), suggesting that the effects of antibiotics on leaf litter associated bacterial communities can reduce and/or eliminate antagonistic and competitive interactions that control fungal abundance.

To understand the relative contribution of veterinary medicines to the overall ecological effect of effluents on stream ecosystems, future studies must consider exposure scenarios that are representative of their use in aquaculture production. Careful consideration of the timescales involved in exposure and measurement of ecological effects is critical to generate meaningful results for ecological risk assessments. Furthermore, studies will eventually need to incorporate drug metabolites and other effluent constituents (e.g., nutrients) to provide decision makers with information that is increasingly representative of the disturbance to which the ecosystem is subject.

2.7. Conclusion

Freshwater salmonid aquaculture can be a relevant source of anthropogenic pollution to otherwise undisturbed stream ecosystems, particularly when aquaculture operations are located in the headwaters of river networks. The discharge of effluents from land-based salmonid farms into stream ecosystems can affect a series of in-stream ecological endpoints which are important to sustain the ecosystem services they provide. Effects have been documented on all biological components of stream ecosystems, from microbial communities to vertebrates and up to variable and at times considerable downstream distances from the effluent discharge point.

Stream ecology provides a sound theoretical framework to understand the ways in which nutrients, BOD and solids can affect stream ecosystems. Our current

knowledge on the effects of veterinary medicines on relevant in-stream ecological endpoints, however, is insufficient to thoroughly understand their potential medium and long-term effects under realistic exposure scenarios. With increasing evidence suggesting that trace amounts of certain pharmaceutical compounds can have subtle effects on important ecological processes (e.g., Maul et al. 2006; Hahn and Shulz, 2007; Schreiber and Szewzyk, 2008), studies aimed at assessing the effects of aquaculture veterinary medicines on the structure and function of stream ecosystems is crucial. At the moment, we cannot effectively and causally link the presence of veterinary medicines in aquaculture effluents with potential in-stream ecological effects. Thus their relative contribution to the overall effect produced by the discharge of effluents remains, to a great extent, unknown. This is currently a knowledge gap that prevents the implementation of effective benchmarks in the ecological risk assessment of aquaculture production processes and thus requires further research. Future research should also explicitly consider the temporal patterns of stressors occurring in the effluents and assess the effects of co-occurring stressors and of stressors occurring in successive temporal intervals.

As aquaculture expands to meet the demands of an increasing human population, knowledge on the impacts of pollution associated with the production of particular species should guide efforts to efficiently and effectively prevent and/or mitigate adverse effects on ecosystems.

Chapter 3

General Methods

3.1. Introduction

Assessing the effects of antibiotics on bacterial communities in the field is challenging due to the presence of confounding factors that may be difficult to account for. In stream ecosystems, the occurrence of physicochemical gradients at multiple spatial and temporal scales makes it particularly difficult to link changes in bacterial communities to chemical pollution. Patchy hydrodynamics, for example, can influence bacterial community structure and biodiversity at very small spatial scales (Besemer et al. 2009).

To gain insights into the potential effects of antibiotic pollution on stream bacterial communities it is necessary to use test systems that can effectively isolate experimental factors while maintaining the representation of key ecosystem variables. The use of model streams to this effect have had a long standing role in stream ecology and ecotoxicology (Belanger 1997), and new experimental systems with varying features continue to be used by different researchers (e.g., Lawrence et al. 2000; Battin et al. 2003; Singer et al. 2006).

The main objective of this chapter is to describe the stream microcosm system used to assess the effects of ERT and FFC on bacterial communities of stream biofilms (i.e., Chapters 4, 5 and 7) and the preliminary analysis conducted to assess the reproducibility of important biological and physical variables in the system. The analytical methods used to quantify these variables are described in this chapter and later referred to throughout the thesis by their corresponding section (e.g., 'DNA was extracted as described in section 3.3.4.1'). Details of other methods and the experimental designs used in the research discussed in chapters 4, 5, 6 and 7 are described in each chapter individually and in appendices at the end of the thesis to which the reader is referred accordingly.

Two pilot experiments were conducted between 2009 and 2010 using a subset of the recirculation units comprising the entire microcosm system. An initial 45-day pilot experiment was conducted on three header tank - sink recirculation units to assess the stability of flow throughout biofilm development, total chlorophyll (Chl) concentrations between recirculation units and the stability of FFC and ERT in the system. Biofilm DNA was also extracted from two random samples during this experiment to assess the technical variability in the molecular method used for DNA fingerprinting of bacterial communities. A second, 15-day pilot experiment also

using three header tank - sink recirculation units was carried out to assess the reproducibility of bacterial community structure between recirculation units. In a way, several of the subsections of this chapter constitute 'mini experiments' whose results were used to guide the design and implementation of the full scale experiments whose results are discussed in chapters 4, 5 and 7.

3.2. Microcosm design and operation

Microcosms were built following the microcosm design described by Singer et al. (2006) to study stream microbial biofilms. Stream microcosms consisted of 24 Plexiglas flumes distributed into six header tank - sink recirculation units (i.e., 6 independent water bodies with 4 replicate flumes each) that could be allocated to experimental treatments (e.g., different antibiotic concentrations) (Figure 3.1).

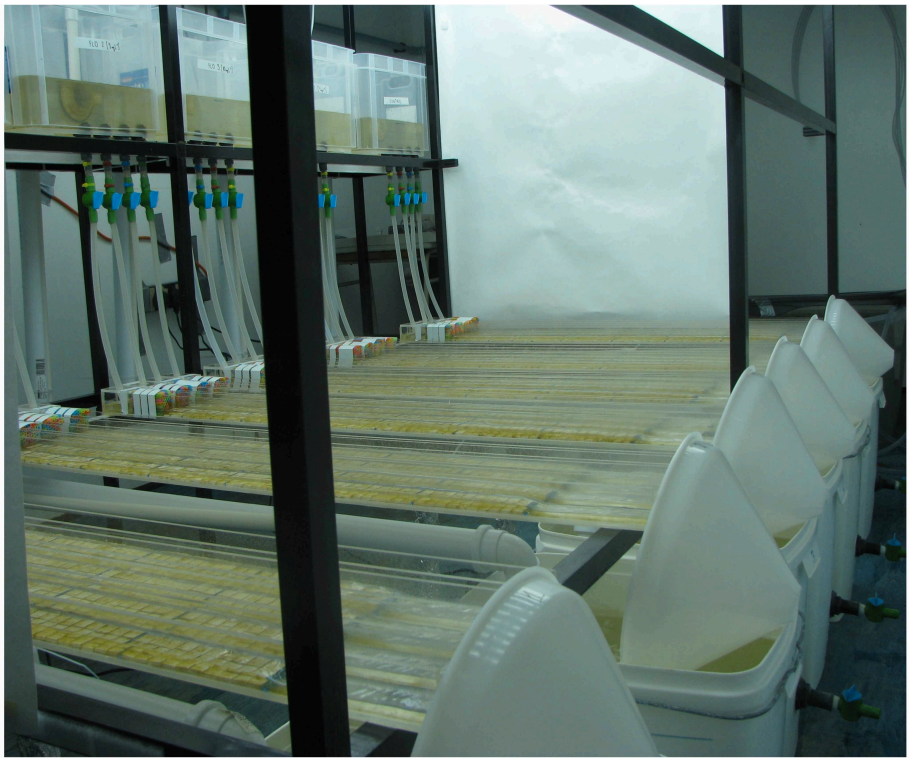

Figure 3.1. Photograph of the microcosm system in operation. Four of the six sets of replicate flumes recirculating between a sink and a header tank can be seen clearly. Both ends/sides of the system had a white sheet that acted as a diffuser to aid in the even spread of light (seen in this picture at the far end, right after the last header tank).

Plexiglas flumes were 120 cm long, 3 cm wide (internal width) and 3.5 cm high (internal height). Header tanks (18.5cm x 31cm x 33 cm) were made of transparent polypropylene (Curver Ltd. UK) and they were covered with aluminum foil to minimize growth of algae during operation. Sinks (32cm x 22.5cm x 33.5cm) were made of white polypropylene (Mailbox Stanford Product Ltd. UK). White polypropylene (PVC-U) 40mm and 20mm pipes were used as overflow and recirculation pipes, respectively. A 26 cm diameter polypropylene funnel (Fisherbrand®) was placed on each sink to prevent splashing (i.e., water flowing through the flumes hit the inner wall of the funnel first and then dropped into the sink without splashing). Baffles consisted of 8cm long polypropylene tubes (5mm diameter) and served the purpose of stabilizing flow (Singer et al., 2006). One Eheim® Pump 5500+ (Deizisau, Germany) was used for each header tank - sink recirculation unit (i.e., 4 flumes). They were operated as dry pumps (i.e., not submerged) and set at the same speed, yielding homogeneous head (i.e., water level) across all treatments. Lighting was provided by 14 Phillips TL-D 58W daylight fluorescent tubes (Figure 3.2).

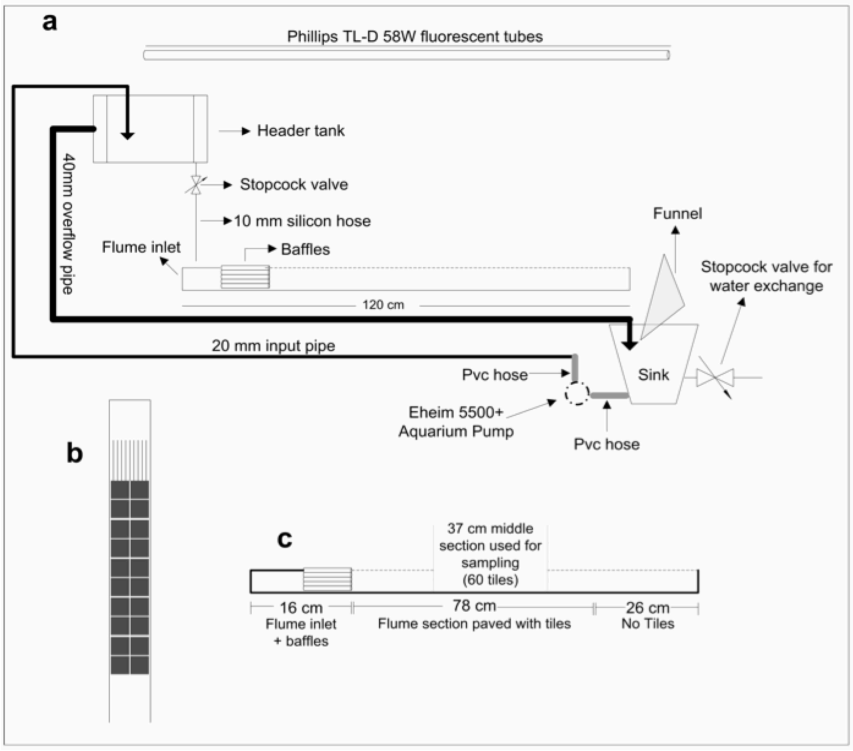

Figure 3.2. Diagram depicting the features of the recirculating microcosms built after the design proposed by Singer et al., (2006). a) lateral view of a recirculation unit . b)top view of a flume showing the side by side layout of the tiles within each flume. C) lateral view of

a single flume showing the portions of the flume allocated to inlet, baffles and tiles. A 37 cm long middle section of the flume was adopted as the sampling section in the experiments described in Chapters 3, 4 and 5.

Microcosms were operated with stream water collected from an unnamed first order stream draining an agricultural catchment at the foot of the Ochil Hills in upper Bridge of Allan (Stirling, UK) (Figure 3.3). Water was filtered through a 40 µm mesh upon collection and once again before it was added to the microcosms to exclude macroinvertebrate larvae and meiofauna (Palmer et al., 2006). A 75% water exchange every 72 hours was performed by doing two 50% water exchanges in a period of 90 minutes. Source water was collected approx. 24 hours before each water exchange to allow the temperature of the water to stabilize to room temperature before exchange. To ensure uniform water chemistry and inoculum across treatments, stream water was thoroughly mixed prior to every water exchange in a source tank kept exclusively for this purpose. Each header tank - sink recirculation unit operated at a volume of 29 litres. Standardized hydrodynamic conditions across all 24 flumes were achieved by individually adjusting each flume's flow using miniature stopcock valves (Aquatic Eco-Systems Inc. USA).

Figure 3.3. Photograph of the stream from which water was collected to operate the microcosms and of the water collection equipment.

Each Plexiglas flume was paved with 126 unglazed, square ceramic tiles (length x width 154 ± 6.0 mm^2, height 3.0 ± 0.3 mm) (Aldershaw Handmade Tiles Ltd., UK)

that were used to grow and sample the biofilms. Clay tiles were acid washed and combusted as described in Singer et al., (2006) and then autoclaved (121^{o}C/15 min) in sterilizer bags (SPS Laboratories, UK), where they were stored until used. Before the start of each experiment, the entire system was flushed with 2% (v/v) Decon 90® (Decon Laboratories, Sussex, UK) and 2% (w/v) sodium hypochlorite, after which it was thoroughly rinsed and left to air-dry. All experiments were run in an indoor aquarium facility in an independent room at ambient temperature with a 12:12 light-dark cycle.

3.3. Microcosm evaluation

3.3.1. Light distribution

Light distribution in the microcosms was assessed using a Skye Instruments Ltd. (UK) light meter that measured photon flux density in the range 400 – 740 nm. Readings were adjusted for flume water depth using an empirical correction factor. Light was measured along the length of each flume by threading the light sensor trough a 20mm polypropylene pipe as shown in Figure 3.4.

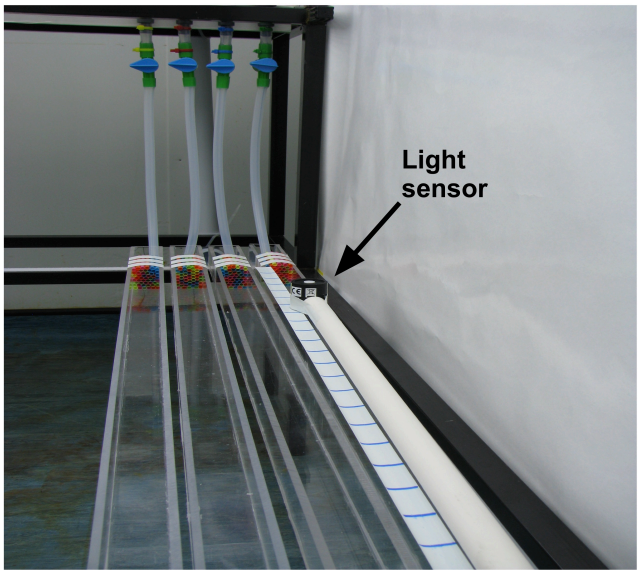

Figure 3.4. Photograph showing how light intensity distribution was measured in the microcosms.

Each flume was divided into 26, 4 cm sections from the baffle outlet onwards that matched the dimensions of the light sensor. Photon flux was measured at these sections in each of the 24 flumes. Light intensity increased from the inlet towards the middle section of each flume and then declined towards the outlet (Figure 3.5). Mean light intensity across all flumes was 102 ± 7 µmol m^{-2} s^{-1} (coefficient of variation = 7%). There was a clear threshold distance at which point the variance in photon flux between flumes increased. This threshold occurred at approximately 80 cm from the baffle outlets (i.e., Flume section 20 in Figure 3.5).

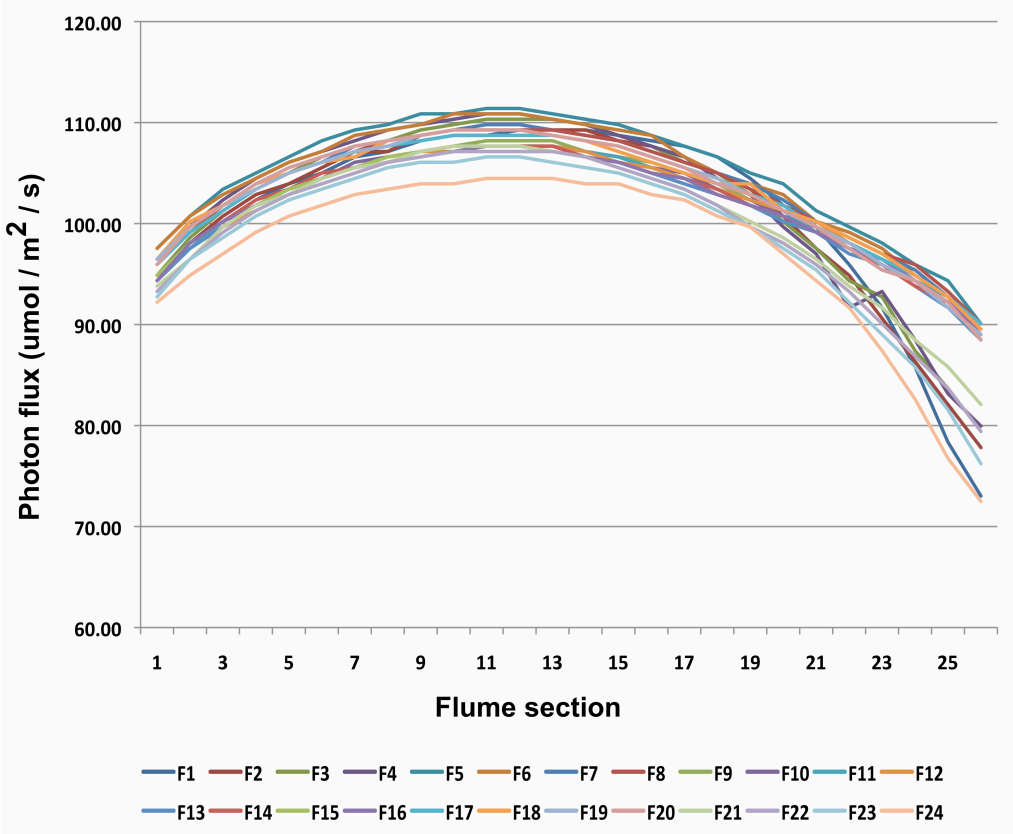

Figure 3.5. Light distribution profiles throughout the length of each of the 24 flumes in the microcosms. Each flume is represented by a different color as described in the legend.

A profile of mean photon flux between section 7 and 20 was plotted for each flume in order to assess the distribution of light across flumes (i.e., from flume 1 to 24) (Figure 3.6). This middle section was assessed because it was considered to be a suitable section for sampling based on the light distribution profile shown in Figure 3.4 and sufficiently 'downstream' of the flume inlet. There was a slight trend of decreasing photon flux from flume 1 towards flume 24. Mean photon flux density

across this flume cross-section was 106 ± 1.4 µmol m^{-2} s^{-1}, with a range of 6.31 µmol m^{-2} s^{-1}. Within flume variation in this longitudinal section increased slightly in the edges of the microcosm and remained fairly constant between flumes 5 and 20.

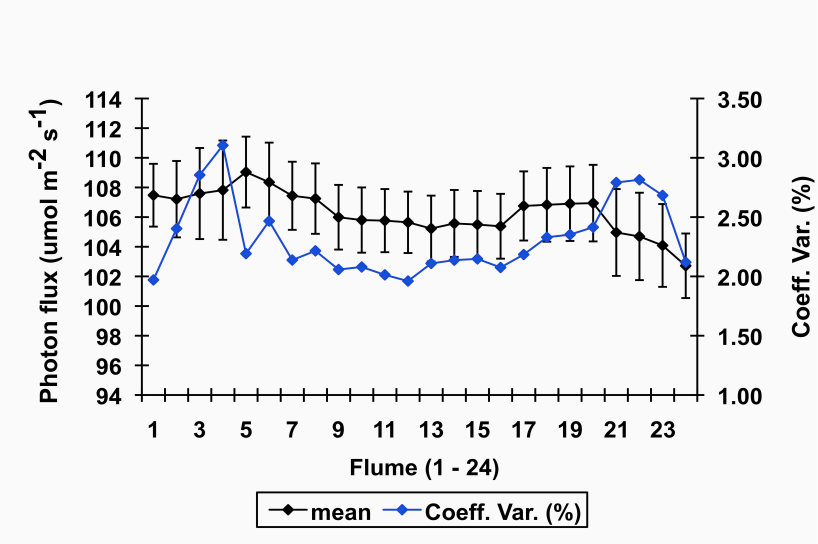

Figure 3.6. Profile of mean photon flux density between section 7 and 20 of each flume. Flumes 1-4 belong to header - tank sink recirculation unit 1, flumes 5 -8 to header tank - sink recirculation unit 2 and so forth until flumes 21 -24, which belong to header tank - sink recirculation unit 6.

3.3.2. Hydrodynamics

The stability of flume hydrodynamic conditions was assessed during a 45 - day pilot experiment using 3 header tank - sink recirculation units (i.e., 12 flumes). Flow was set in all flumes to 52.7 ± 0.84 ml s^{-1} at the start of the experiment and measured every 5 days thereafter by volumetric analysis. Initial flow was set to yield a turbulent hydrodynamic regime with a Reynolds number of approximately 1200, estimated by the equation:

$$R = v\left(\frac{Lr}{kv}\right)$$

where v is water velocity, Lr is the hydraulic radius of the flume and kv is the kinematic viscosity of water at 20 °C (Singer et al. 2006). Valves of individual

flumes were not adjusted throughout this pilot run in order to assess the change in flow as the biofilms developed and get an estimate of the periodicity with which valves would have to be adjusted.

There was a clear trend of decreasing flow with time in all flumes throughout the experiment (Figure 3.7). The mean decrease in flow over time across all 12 flumes was well described by a linear dependence of flow on the number of days since the start of the experiment ($y = 52.5 - 0.04x$; $R^2 = 0.67$; $F_{1,31} = 64.06$; $p<0.0001$). Variability in flow between flumes throughout the 45 - day trial was low, with a mean standard deviation of 1 ml s^{-1} (range 0.86 - 1.1 ml s^{-1}).

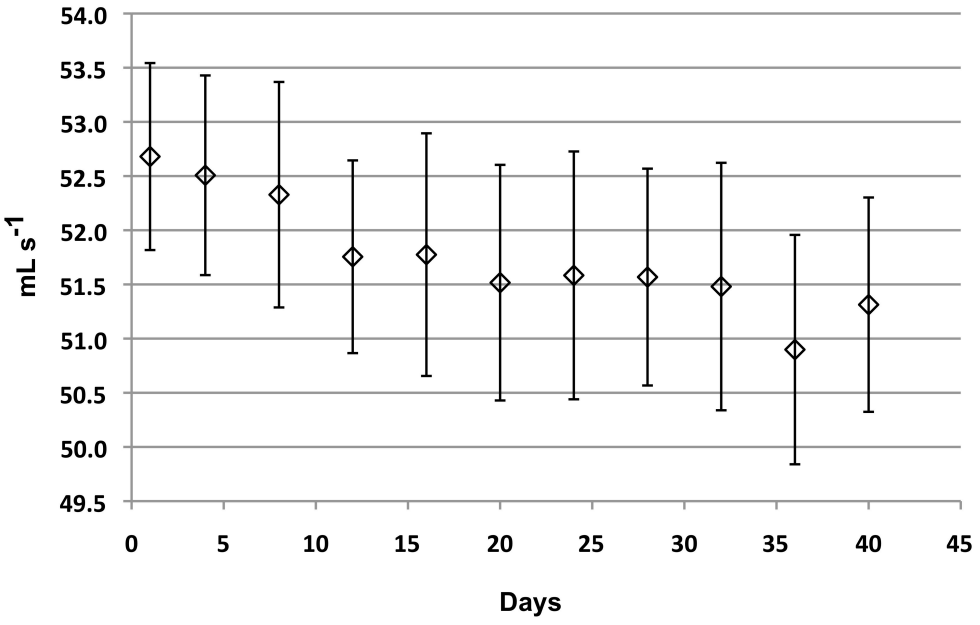

Figure 3.7. Temporal series of flow for each flume. Error bars represent the standard deviation from the mean.

Based on the decreasing trend of flow with time, an arbitrary decision was made to adjust valves manually once a week in subsequent experiments. Based on the empirical relationship between flow and days of experiment determined above, this would be expected to prevent flow from decreasing beyond 0.5 % of the initially set flow rate (i.e., decrease in flow of 0.04 ml s^{-1} per day * 7 days = 0.28 ml s^{-1} ; 0.28 ml s^{-1}/52.5 ml s^{-1} = 0.5 %).

3.3.3. Total chlorophyll

The variability in total chlorophyll (Chl) concentration between header tank - sink recirculation units, flumes and within flumes was assessed during the same 45 - day pilot experiment used to assess the stability of flume hydrodynamics described in the previous section. The header tank - sink recirculation units used in the pilot experiment spanned the entire gradient of photon flux densities shown in Figure 3.6. Samples for total Chl were taken at 16 and 26 days of biofilm development. Each sample consisted of a single tile. For each time point, 3 tiles were collected from each flume in a longitudinal gradient from flume inlet to flume outlet to assess potential differences in total Chl concentration along the length of the flumes (i.e., 3 tiles per flume x 12 flumes x 2 time points = 72 samples). Samples were kept in the dark on ice and processed for total Chl determination within 2 hours of collection. Samples were not taken beyond day 26 because at day 29 a trial was started to assess the stability of FFC and ERT in the microcosms (section 3.4.4), which would have introduced a confounding factor in the assessment. Average nutrient concentrations in the stream water used to run the microcosms were 7.7 ± 7 μg L^{-1} NH_4, 203 ± 46 μg L^{-1} NO_3 and 14 ± 6 μg L^{-1} PO_4. Total ammonia and nitrate were analysed on a Bran Luebe AutoAnalyser 3 following the protocols provided by the manufacturer (Bran+Luebe 2003a; Bran+Luebe 2003b). Phosphate was analysed by the ascorbic acid method with absorbance reading at 690 nm (APHA 1998).

Total Chl analysis was performed following the method described by Thompson et al. (1999) for the determination of total Chl concentrations in epilithic microbial biofilms. Briefly, 15 mL of 100% methanol were added to the sample collection vials (i.e., containing one tile each). Vials were briefly vortexed and then incubated in the dark at room temperature (~20°C) overnight. Total Chl was determined spectrophotometrically by measuring sample absorbance at 663 ($Å_{663}$) and 750 ($Å_{750}$) nanometers. Total Chl in μg cm^{-2} was then calculated by:

$$TotalChl(ugcm^{-2}) = \frac{13 \times A_{net} \times v}{d \times s}$$

where 13 is a constant for methanol, A_{net} = $Å_{663}$ - $Å_{750}$ (corrected for blank readings), v is the methanol extraction volume, d is the cell path length and s is the

surface area of each tile. To assess differences in total Chl concentration between header tank - sink recirculation units, flumes and along longitudinal gradients within flumes, total Chl values were z-standardised within sampling times using the equation:

$$z - score = \frac{x - m}{s}$$

where x is the total Chl value for each sample and m and s are the mean and standard deviation for each sampling time, respectively. Z-standardization removes the temporal variation from the data and therefore reduces the number of factors while concurrently increasing the number of cases (Singer et al. 2006). This is desirable when the main focus of the statistical analysis is not on temporal patterns of the variable of interest and which in this case was the reproducibility of total Chl concentrations at different experimental levels. A mixed - model analysis of variance (ANOVA) was used to assess the effects of the factor 'Header Tank' (fixed), 'Flume' (random) and 'Tile Position' (random) on total Chl concentrations. Untransformed total Chl concentrations were also analysed to check for differences between header tank - sink recirculation units at 16 and 26 days of biofilm development separately. Linear regressions between total Chl concentrations and photon flux density measured for each flume and sampling section were also performed to assess the effect of light intensity on algal biomass.

Figure 3.8a shows boxplots of total Chl concentration (untransformed) at 16 and 26 days of biofilm development across all 12 flumes. Total Chl increased from 2.9 ± 0.96 μg cm^{-2} to 4.1 ± 1.1 μg cm^{-2} (i.e., 41%) between 16 and 26 days of biofilm development. This increase in total Chl agreed with the visible increase in algal biomass in the microcosms during this time period. The boxplots in Figure 3.8b and 8c show slight differences in total Chl concentrations between header tank - sink recirculation units and along the length of flumes that seemed somewhat consistent with the longitudinal and cross-sectional patterns of photon flux density depicted in Figures 3.5 and 3.6 (section 3.3.1). However, although photon flux density had a statistically significant effect on z-standardised total Chl concentrations, it only explained a small percent of the observed variance (linear regression: $F_{1,69} = 5.35$, $R^2 = 0.072$, $p = 0.02$, $\alpha = 0.05$). Linear regressions performed for each time point separately using untransformed total Chl concentrations gave similar results ($F_{1,33} =$

1.54, $R^2 = 0.016$, $p = 0.22$, $\alpha = 0.05$ at 16 days of biofilm development; $F_{1,34} = 3.98$, $R^2 = 0.10$, $p = 0.054$, $\alpha = 0.05$ at 26 days of biofilm development).

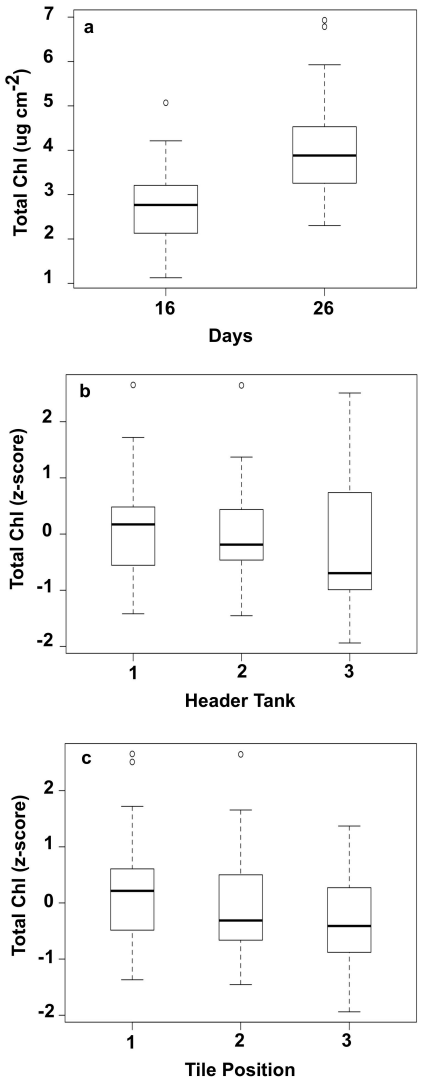

Figure 3.8. Boxplots of (a) total Chl concentration at 16 and 26 days of biofilm development across all flumes (b) z-standardised total Chl concentration in flumes belonging to each header tank - sink recirculation unit and (c) z-standardised total Chl concentrations at different positions along the flume length (i.e., 1: closest to inlet; 2: middle section; 3: closest to outlet).

The mixed - model ANOVA (Table 3.1) showed there were no significant differences in total Chl concentrations between header tank - sink recirculation units, flumes and within flume sections. Total Chl concentrations between header

tank - sink recirculation units were also not significantly different when analysed for each time point separately (ANOVA: $F_{2,32} = 1.98$; $p = 0.15$; $\alpha = 0.05$ at 16 days of biofilm development and $F_{2,33} = 0.07$; $p = 0.94$; $\alpha = 0.05$ at 26 days of biofilm development).

Table 3.1. Mixed-model ANOVA for the effects of the factors 'Header Tank', 'Flume' and 'Tile Position' on total Chl concentrations. 'Flume' and 'Tile Position' are random factors nested within the fixed factor 'Header 'Tank'. Df, degrees of freedom; SS, sums of squares; MS, mean squares; F, F-test statistc; P, p-value.

	Df	SS	MS	F	P
Header Tank	2	2.1	1.0	1.3	0.29
Flume (within Header Tank)	9	14.2	1.6	1.9	0.08
Tile Position (within Flume)	24	24.1	1.0	1.2	0.29
Residuals	35	28.7	0.8		

3.3.4. Bacterial community structure

3.3.4.1. Biofilm DNA extraction

All samples for biofilm DNA extraction consisted of three tiles randomly sampled from the middle section of each flume (see Figure 3.2). Tiles were sampled using flame - sterilised forceps and immediately placed in 50 ml centrifuge tubes containing 35 ml of filter sterilized (0.2 μm) source stream water. Biofilms were detached from the tiles by sonicating them with a Branson® Sonifier 150 at 45 W for 90 s and the biofilm pelleted by centrifugation at 4°C and 5000 x g for 30 minutes. Sonication was used to detach biofilms over other methods (e.g., scraping) because it has been shown to efficiently and reliably dislodge biofilm bacteria (Bjerkan et al. 2009). DNA was extracted from the biofilm pellet using the PowerFood™ Microbial DNA Isolation Kit (MoBio Laboratories Inc.) following the manufacturers instructions. DNA was quantified and quality checked spectrophotometrically and by agarose gel electrophoresis before storage at -20°C.

3.3.4.2. DNA fingerprinting of bacterial communities

Several molecular techniques have been developed and used to assess changes in bacterial communities without the need of culturing individual bacteria. Almost invariably these techniques exploit the conserved and hypervariable regions of the 16S rRNA gene, which is widely used as a phylogenetic marker for bacteria (Woese and Fox 1977). The general feature of these methodologies is that they allow the assessment of changes in the dominant bacteria taxa within a community. Due to their coarse resolution, they are unable to adequately characterize the tail of the species-abundance distribution. Although this may be viewed as a drawback, it is normally accepted that the most abundant taxa within a community play a predominant role in shaping its structure and function, and therefore characterizing them provides important ecological insights. Perhaps the most widely used DNA fingerprinting techniques are denaturant gradient gel electrophoresis (i.e., DGGE) (Muyzer 1999) and terminal restriction fragment length polymorphism (i.e., tRFLP) (Liu et al. 1997). Although they both have strengths and weakneases, the reproducibility and higher resoultion of tRFLP have favoured its use by many research groups (Osborn et al. 2000; Abdo et al. 2006). Currently, developments in next generation sequencing technologies are enabling the 'deep' sequencing of 16S rDNA amplicons amplified from environmental samples (e.g., Caporaso et al. 2010). The use 16S rDNA amplicon sequencing provides a significant increase in resolution and it is likely to gradually displace DNA fingerprinting techniques in the future.

Throughout this thesis, bacterial communities were characterized by tRFLP of 16S rRNA genes, which allows changes in dominant bacterial taxa within a community to be determined (Liu et al., 1997). In tRFLP, one or both 16S primers are labelled with a fluorescent dye at their 5' ends, which are incorporated into the PCR product. After restriction digestion of the PCR products, terminal restriction fragments are fluorescently labelled and detectable by an automated fluorescence based DNA sequencer. Each terminal restriction fragment putatively corresponds to a different bacterial Operational Taxonomic Unit (OTU), and the full data set generated by tRFLP can be analysed with the statistical techniques normally used in numerical ecology.

Different sets of 16S primers and restriction enzymes were used to assess changes in bacterial communities in the different experiments performed throughout this PhD project. Initial tests on a small subset of samples were performed in each experiment to assess which primer pair/enzyme combination revealed the highest number of terminal restriction fragments (T-RFs) upon visual inspection of the profiles. The primer pair/enzyme combination that gave the highest number of T-RFs was always selected for analysis. Restriction fragment, capillary electrophoresis and data pre-processing and analysis details are given throughout the thesis.

3.3.4.3. Methodological variability in tRFLP profiles

tRFLP is a highly reproducible DNA fingerprinting technique that is adequate to assess broad changes in microbial communities. Systematic evaluations of tRFLP have shown that the greatest source of variation in tRFLP is between replicate profiles generated during capillary electrophoresis, while variation between replicate DNA extractions, PCR products and restriction digests is low (Osborn et al. 2000). Because the level of technical variability may vary between researchers, it is good practice to assess these sources of variation during the initial optimization stages of the method. The reproducibility between replicate PCRs and replicate restriction digests was assessed using two biofilm DNA samples collected randomly from a flume at 20 and 45 days of biofilm development during the 45 - day pilot experiment mentioned in sections 3.3.2 and 3.3.3. Biofilm DNA was extracted following the method described in section 3.3.4.1. Per sample, duplicate PCR and triplicate restriction digest reactions were performed and each restriction digest was analysed in triplicate (i.e., 2 samples x 2 PCR reactions x 3 restriction digests x 3 runs = 36 tRFLP profiles). Variability between replicate DNA extractions was not assessed because it was considered unfeasible to perform replicate DNA extractions per flume during full scale experiments.

An approximately 1300 base pair (bp) fragment of the 16S rRNA gene was amplified using primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') (Marchesi et al., 1998) and 1389R (5'-ACGGGCGGTGTGTACAAG-3') (Marchesi et al., 1998; Osborn et al., 2000). Forward and reverse primers were labelled at their 5' ends with CY5 (blue) and C55 (green), respectively. Each PCR reaction (50 µl)

contained 5 µl of 10X PCR buffer, 0.2 mM dNTPs, 0.4 µM of each primer, 1.25 U Taq DNA polymerase, 1.5 mM $MgCl_2$ and approximately 50 ng of template DNA. PCR amplification consisted of an initial hot start step of 95 °C for 15 min; 10 cycles of touchdown PCR consisting of 94 °C for 1 min, 60 – 55.5 °C for 1 min and 72 °C for 2 min; 15 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min; final extension step at 72 °C for 10 min. PCR products were cleaned with the Invitek MSB® Spin PCRapace cleanup kit to remove PCR reagents and approximately 80 ng were digested at 37 °C for 4 hours in 15 µl reactions containing 10 U of HhaI restriction enzyme and 1.5 µl of 10X restriction enzyme buffer.

Terminal restriction fragments (T-RFs) were analyzed on a Beckman Coulter CEQ 8800 sequencer. Fragments were size called in GeneMarker v1.85 using the local southern algorithm and only fragments between 60 and 550 bp were retained for analysis. Noise filtering and peak alignment were performed in TREX (Culman et al., 2009) following the procedure described by Abdo et al., (2006) with a standard deviation multiplier of 3 and the procedure described by Smith et al., (2005) with a clustering threshold of 0.5 bp, respectively. This resulted in a data matrix with the relative peak height of terminal restriction fragments or OTUs for each sample.

The Bray-Curtis dissimilarity index was used to characterize community dissimilarities. The dissimilarity between the *i* th and *j* th sample is expressed as:

$$d_{ij} = \sum_{k=1}^{S} \frac{| x_{ij} - x_{ik} |}{(x_{ij} + x_{ik})}$$

where x_{ij} is the relative abundance of taxon k in sample i. The variation in community dissimilarities in response to the factors 'PCR' and 'Restriction Digest' was partitioned using a non-parametric permutational multivariate analysis of variance (Anderson, 2001; McArdle and Anderson, 2001; implemented in the function *adonis* in the R package VEGAN v.1.17-6 and referred to as *adonis* for simplicity) (Okansen et al. 2011). *adonis* lacks formal assumptions about the distribution of variables (Anderson, 2001) and accepts categorical and/or continuous variables as predictors. A nested model (i.e., 'Restriction digest' nested within 'PCR') was specified using both factors as

categorical variables. *adonis* was run using the Bray-Curtis dissimilarity index calculated on untransformed relative peak heights, hellinger transformed peak heights (i.e., square root of relative peak heights) and presence-absence data (i.e., Sorensen index). Untransformed relative peak heights emphasize changes in abundance of the most common OTUs, while a reduction to presence-absence data firmly shifts the emphasis towards patterns in the intermediate and rarer OTUs (Clarke and Warwick, 2001). Hellinger transformed peak heights constitute a moderate transformation that allows intermediate abundance OTUs to contribute in the analysis (Clarke and Warwick, 2001). The use of this sequence of transformations in *adonis* allowed us to assess whether data transformations likely to be used in the analysis of future experimental data would differentially and significantly reflect technical variability.

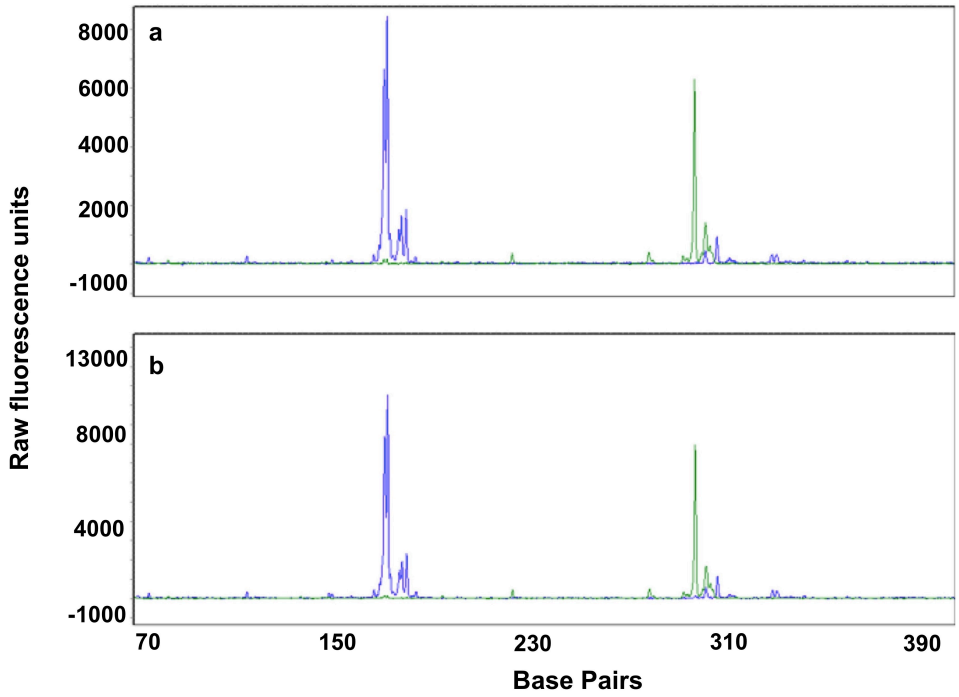

Figure 3.9. tRFLP profiles from replicate PCRs (a and b) from the biofilm DNA sample taken at 20 days of biofilm development. There were no peaks beyond 390 base pairs.

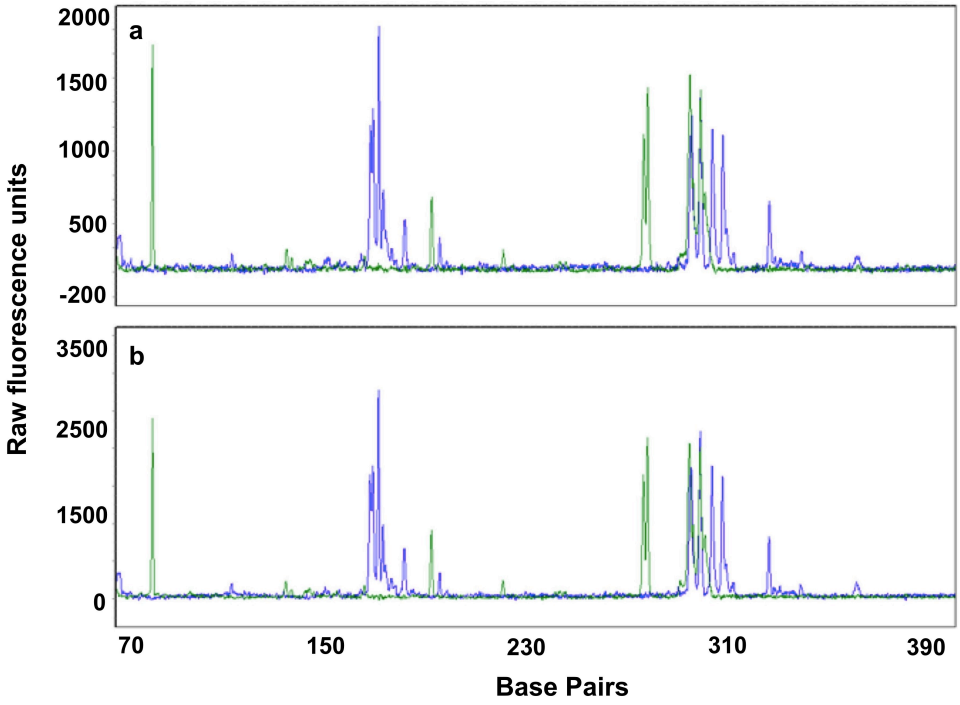

Figure 3.10. tRFLP profiles from replicate PCRs (a and b) from the biofilm DNA sample taken at 45 days of biofilm development. There were no peaks beyond 390 base pairs.

Figures 3.9 and 3.10 show tRFLP profiles from replicate PCRs for the two samples analysed (i.e., samples taken at 20 and 45 days of biofilm development, respectively). These figures highlight the reproducibility between tRFLP profiles from replicate PCR reactions, restriction digestions and capillary electrophoresis runs in samples that have a clear difference in the complexity of their bacterial communities. Variance partitioning of community dissimilarities with *adonis* showed that the factors 'PCR' and 'Restriction Digest (within PCR)' did not have a significant effect on community dissimilarities in either sample (Table 3.2). Shifting the emphasis in community dissimilarities from composition (i.e., presence/absence) to the relative abundance of the commonest OTUs (i.e., relative peak height) did not significantly affect the contribution of these factors to the observed variation in community dissimilarities either. These results indicate that variability in tRFLP profiles from replicate PCR and restriction digestion reactions is low and that technical replication at these stages is not essential.

Table 3.2. Variance partitioning of community dissimilarities with *adonis* for tRFLP profiles from 20 day (Sample 1) and 45 day old biofilms (Sample 2). $\alpha = 0.05$. Significance is based on 5000 permutations.

	Experimental factor	
Sample 1	**PCR**	**Restriction Digest (within PCR)**
Presence/absence	$F_{1,16} = 0.20$; $R^2 = 0.012$; $P = 0.94$	$F_{5,12} = 1.1$; $R^2 = 0.32$; $P = 0.36$
Hellinger	$F_{1,16} = 0.25$; $R^2 = 0.015$; $P = 0.97$	$F_{5,12} = 0.94$; $R^2 = 0.28$; $P = 0.55$
Relative peak height	$F_{1,16} = 0.21$; $R^2 = 0.012$; $P = 0.99$	$F_{5,12} = 0.7$; $R^2 = 0.24$; $P = 0.86$
Sample 2		
Presence/absence	$F_{1,15} = 0.13$; $R^2 = 0.008$; $P = 0.91$	$F_{5,11} = 1.0$; $R^2 = 0.32$; $P = 0.46$
Hellinger	$F_{1,15} = 0.30$; $R^2 = 0.019$; $P = 0.88$	$F_{5,11} = 1.1$; $R^2 = 0.34$; $P = 0.36$
Relative peak height	$F_{1,15} = 0.64$; $R^2 = 0.04$; $P = 0.64$	$F_{5,11} = 1.2$; $R^2 = 0.35$; $P = 0.29$

3.3.4.4. Microcosm variability in tRFLP profiles

The reproducibility in bacterial community structure - as determined by tRFLP - between header tank - sink recirculation units was assessed during a 15-day pilot experiment. Biofilms were grown from raw stream water as described in section 3.2 using twelve flumes distributed in three header tank - sink recirculation units that spanned the entire gradient of photon flux densities shown in Figure 3.6. During the first pilot experiment it was observed that turbulent hydrodynamic conditions caused the clay tiles to be occasionally dislodged from the flume bed, and therefore the decision was made to adjust individual valves to yield a transitional flow with an estimated Reynolds number of approximately 880. Mean flow across all twelve flumes throughout the 15-day pilot experiment was 40 mL s^{-1}, with a coefficient of variation of 0.6%.

At 15 days of biofilm development, DNA was extracted following the method described in section 3.3.4.1. An approximately 500 base pair (bp) fragment of the 16S rRNA gene was amplified using primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') (Marchesi et al., 1998) and 519R (5'-

GTATTACCGCGGCTGCTG-3') (Lane 1991). The forward primer was labelled at its 5' end with CY5 (blue). PCR was performed in 25 µL reactions using 0.4 µM of each primer and 12.5 µL of 2X Promega® PCR master mix. Thermocycling was performed as described in section 3.3.4.3. PCR products were cleaned with the QIAGEN® QIAquick PCR purification kit and approximately 70 ng were digested at 37 °C for 4 hours in 20 µL reactions containing 12 U of AluI restriction enzyme and 2 µL of restriction enzyme buffer. PCR and restriction digests were run as single reactions and tRFLP profiles were analysed in duplicate, yielding a total of 24 tRFLP profiles (3 header tank - sink recirculation units x 4 flumes x duplicate tRFLP profiles = 24). Duplicate profiles were averaged within flumes in TREX (Culman et al. 2009) and data processing and analysis were performed as described in section 3.3.4.3.

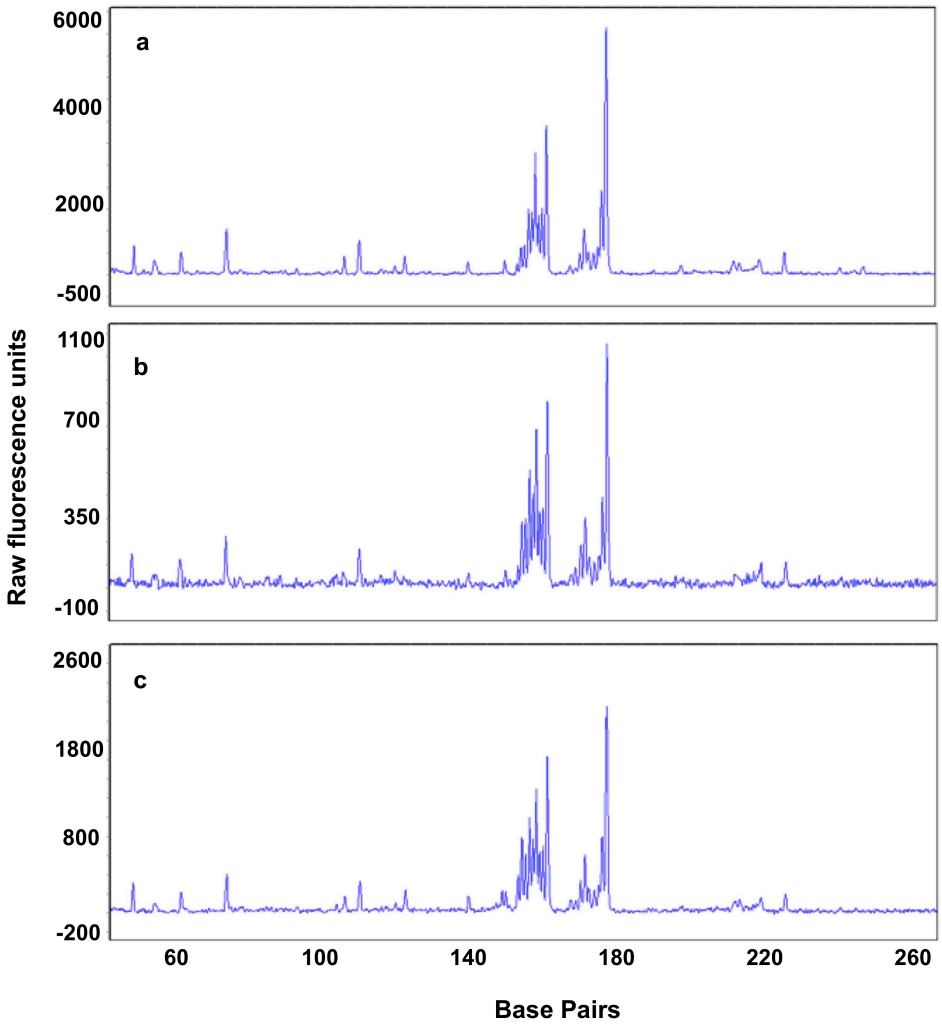

Figure 3.11. tRFLP profiles from different header tank - sink recirculation units (a, b and c) after 15 days of biofilm development. There were no peaks beyond 260 base pairs.

Additionally, a non-metric multidimensional scaling (NMDS) was performed on the Bray-Curtis dissimilarity matrix based on presence-absence data using the metaMDS function in the R package VEGAN to visualize dissimilarities between samples from different header tank - sink recirculation units. Given the cross-sectional pattern of photon flux density in the microcosms (Figure 3.5), *adonis* was also run using photon flux density as a quantitative explanatory variable to assess its potential effect on biofilm bacterial community structure.

Figure 3.11 shows the tRFLP profiles from three flumes corresponding to different header tank - sink recirculation units. These profiles show overall good reproducibility, although there are visible differences in the presence/absence of minor peaks and also in the relative abundance of some of the major peaks. Variance partitioning of the entire dataset with *adonis* showed that the variation in dissimilarities between header tank - sink recirculation units (i.e., categorical variable 'header tank') was not statistically significant, indicating that biofilm bacterial communities - as assessed by tRFLP - were not significantly different between flumes belonging to different header tank - sink recirculation units after 15 days of biofilm development (Table 3.3). Photon flux density, on the other hand, did not have a significant effect on bacterial community structure with any of the data transformations used (p-values between 0.10 and 0.34). The fact that no significant differences were found with any of the data transformations shown in Table 3.3 indicate that differences in the presence/absence of minor OTUs and in the relative abundance of major OTUs was not significantly different between flumes from the three different header tank - sink recirculation units.

Table 3.3. Variance partition of community dissimilarities with *adonis* for tRFLP data from three different header tank - sink recirculation units after 15 days of biofilm development. α = 0.05. Significance is based on 5000 permutations.

Presence/absence	$F_{2,11} = 1.59$; $R^2 = 0.26$; P = 0.18
Hellinger	$F_{2,11} = 2.08$; $R^2 = 0.32$; P = 0.09
Relative peak height	$F_{2,11} = 2.37$; $R^2 = 0.35$; P = 0.09

In line with these results, the NMDS ordination plot shows that there is no clear distinction in the composition of bacterial communities from the three different header tank - sink recirculation units, with most of the samples from the different groups overlapping to some extent (Figure 3.12). In all, these results show good reproducibility between flumes belonging to different header tank - sink recirculation units and suggest that their assignment to different treatments (e.g., antibiotic concentrations) is adequate.

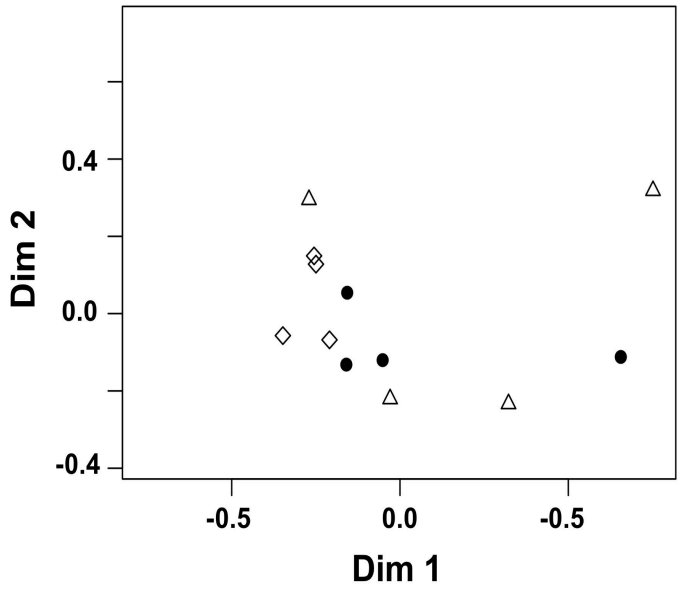

Figure 3.12. Non-metric multidimensional scaling of the Bray-Curtis dissimilarity matrix calculated from presence absence data for the entire tRFLP dataset. Dim 1: first NMDS dimension. Dim 2: second NMDS dimension. Dimensions in final solution: 2. Stress: 0.015. Symbols correspond to replicate flumes from the three different header tank - sink recirculation units.

3.4. Analysis of antibiotics in stream water

Concentrations of FFC and ERT in stream microcosm water samples were determined at the United States Geological Survey (USGS) Upper Midwest Environmental Sciences Center (UMESC). For FFC analysis, samples were collected, filtered and stored frozen at -70 °C by A. Tello. For ERT analysis, samples were collected, processed by solid phase extraction (SPE) and stored at 4 °C by A. Tello. The analytical determination of FFC and ERT was performed by Jeffry A. Bernardy, who contributed the specific method descriptions detailed in sections 3.4.1 and 3.4.2 below.

3.4.1. FFC analysis

Water samples for FFC analysis were filtered using MILLEX® HV 33 mm, 0.45 µm PVDF syringe filters and stored at -70°C in 700 µL propylene HPLC vials until analysis. The samples and a minimum of seven external standards (FFC and florfenicol amine [FFA]) were injected (100 µL) onto a 40°C Kinetex, 2.6 µm, 50 x 3.0 mm, PFP analytical column (Phenomenex, Torrance, CA, USA) with an 1100 liquid chromatograph, detected on an G1946D single quadrupole mass selective detector and quantified using Rev. B.03.02-SR2 Chemstation software (all Agilent Technologies, Santa Clara, CA, USA). Briefly, the LC mobile phase (A = water : methanol : acetic acid (898:100:2) and B = methanol; A:B ratio = 88:12) was pumped isocratically at 0.5 mL min^{-1} for 7.5 min per run. The gas temperature (350 °C), drying gas flow (12.0 L min^{-1}), nebulizer pressure (35 psig) and both positive and negative capillary voltages (3000 V) of the electro-spray interface were not varied. The FFA and one qualifier were detected using positive polarity, SIM ions 248.0 and 230.0 respectively, with the fragmentor set at 125 V for both ions. The FFC and one qualifier were detected using negative polarity, SIM ions 355.9 and 335.9, with the fragmentor set at 110 and 155 respectively. The standards bracketed the expected sample concentrations and were injected at the beginning, end, and after every ten samples during the sequential runs. The FFC and FFA peaks were identified by retention times and qualifier ion ratio (± 10%) comparisons with the standards. Peak areas of the FFC and FFA standard ions were used to construct the

standard curves and the concentration of the FFC and FFA sample ions were determined by comparison with the standard curves.

3.4.2. ERT analysis

Oasis® HLB 3 mL, 60 mg extraction cartridges were conditioned with 6 mL of methanol and 2 mL of acetonitrile and then equilibrated with 2 mL of HPLC grade water. 12 mL of aqueous sample were loaded into the cartridges and allowed to drip without vacuum, after which cartridges were vacuum dried at approximately 30 kPa for 5 minutes. Cartridges were then eluted with 650 µL methanol and 1300 µL acetonitrile : methanol (50:50). After the last eluent had stopped dripping, all of the eluent remaining in the packing was drawn through the cartridge with vacuum. The eluent was brought to a final volume of 2 mL with 5 µL acetontrile and then filtered through 13 mm, 0.2 µm PTFE membrane syringe filters into amber glass, rubber septa HPLC vials. Vials were stored at 4 °C until analysis.

The samples and a minimum of six external erythromycin (ERT) standards were diluted (7 µL sample mixed with 28 µL water) and injected using a programmable autosampler onto a 40 °C Kinetex, 2.6 µm, 50 x 3.0 mm, C_{18} analytical column (Phenomenex, Torrance, CA, USA) with an 1100 liquid chromatograph, detected on a G1946D single quadrupole mass selective detector, and quantified using Rev. B.03.02-SR2 Chemstation software (all Agilent Technologies, Santa Clara, CA, USA). Briefly, the LC mobile phase (A = water : acetonitrile : acetic acid (798:200:2) and B = acetonitrile; A:B ratio = 95:5) was pumped isocraticly at 0.5 mL/min for 5 min per run. The gas temperature (350 °C), drying gas flow (12.0 L/min), nebulizer pressure (35 psig) and positive capillary voltage (3000 V) of the electro-spray interface were not varied. The ERT and two qualifier ions were detected using positive polarity, SIM ions 734.4, 576.4 and 158.1, with the fragmentor set at 120, 260, and 300 V respectively. The standards bracketed the expected sample concentrations and were injected at the beginning, end, and after every ten samples during the sequential runs. The ERT peaks were identified by retention times and qualifier ion ratio (± 10 %) comparisons with the standards. Peak areas of the ERT standard ions were used to construct the standard curves and the concentration of the ERT sample ions were determined by comparison of the sample peak areas with the standard curves.

3.4.3. Preliminary mixing experiment with a conservative solute

A preliminary mixing experiment was conducted to calculate the time required for a conservative solute to mix in the microcosms. The objective of this mixing experiment was to get a rough estimate of the time it would take for FFC and ERT to mix in each header tank - sink recirculation unit and therefore of the minimum time after injection of the antibiotics at which samples could be taken for analysis.

A standard curve covering the range 1 - 100 mM of NaCl was constructed by serial dilution of a stock NaCl solution and measuring conductivity using a portable Hach® conductivity meter (Figure 3.13).

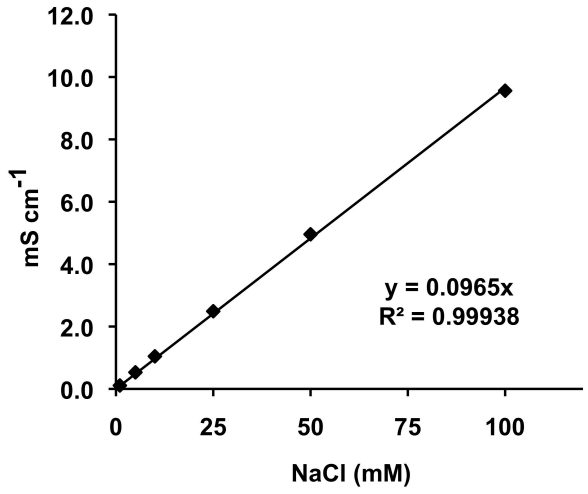

Figure 3.13. Standard curve to determine NaCl concentration from conductivity readings.

Based on the equation from this standard curve, a final concentration of 43 mM was targeted in one header tank - sink recirculation unit (total volume = 29 lt) by injecting 500 mL of a 2.5 M solution. Conductivity was monitored continuously until it stabilised at a conductivity reading close to that predicted by the standard curve (i.e., Cond (mS cm^{-1}) = 0.0965 x 43 mM = 4.1 mS cm^{-1}).

Figure 3.14 shows a graph of conductivity over time after the injection of the 500 mL 2.5 M NaCl solution. Conductivity increased sharply during the first 5 minutes after injection and stabilised at 4.08 mS cm^{-1} after 12 minutes of injection, indicating the complete mixing of the solute in the system.

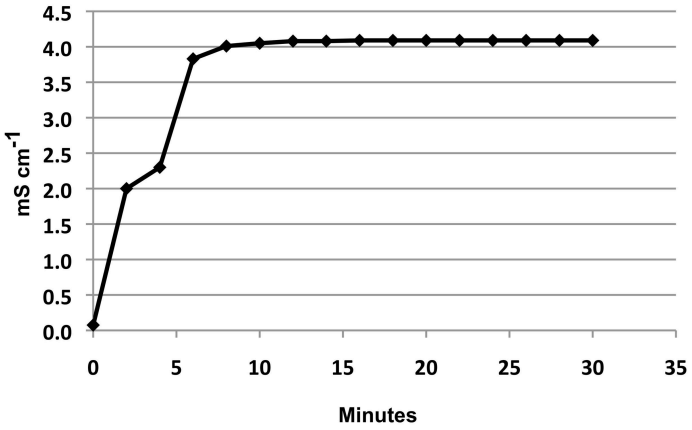

Figure 3.14. Graph showing the increase in conductivity over time after injection of a 2.5 M solution of NaCl into a 29 lt header tank - sink recirculation unit.

The results of this reference mixing experiment indicated that injection solutions of FFC and ERT should dissolve fairly quickly in the microcosms. Because it was not feasible to conduct a mixing experiment with the antibiotics themselves, a 'time safety factor' was applied to the mixing time after injection at which samples for antibiotic analysis were taken. Water samples for antibiotic analysis were taken between 45 to 60 minutes after injection.

3.4.4. Stability of FFC and ERT in microcosms

The stability of FFC and ERT was assessed during a 15-day period between days 29 and 43 of the 45 - day pilot experiment previously mentioned. One header tank - sink recirculation unit operating at a total volume of 29 L was allocated to each antibiotic. For both antibiotics, nominal concentrations of 20 μg L^{-1} were targeted in the microcosms. Fresh 25 μg mL^{-1} stock solutions of FFC and ERT were made fresh each day of antibiotic injection by dissolving 10 mg of analytical standard FFC (CAS N° 73231-34-2**;** molecular weight, 358.21 g mol^{-1}; Sigma-Aldrich F1427-500MG) in 400 mL of Milli-Q® water and 10 mg of ERT (CAS number, 7704-67-

8; molecular weight, 793.02; potency = 761 μg mg^{-1} as is; Abbott Laboratories IL) in 400 mL of 30 mM triethanolamine buffer. 200 mL injection solutions at a concentration of 2.9 μg mL^{-1} were made by mixing 23.2 mL of each antibiotic stock solution with 176.8 mL of Milli-Q® water for FFC and 176.8 mL of 30 mM triethanolamine buffer for ERT. Each 200 mL antibiotic injection solution therefore contained 2.9 μg mL^{-1} x 200 mL = 580 μg of antibiotic, which dissolved in the 29 L microcosms yielded a nominal concentration of 580 μg / 29 L = 20 μg L^{-1}. Injection solutions were delivered as a single injection into the header tank of each microcosm after 75% water exchanges on four occasions during the 15-day trial period. Water samples for the determination of both antibiotics were taken on different occasions as follows: 1 hour after antibiotic injection (4 samples each); 24 hours after antibiotic injection (2 samples each); 72 hours after antibiotic injection (3 samples each). In addition, microcosm water samples spiked to the targeted nominal antibiotic concentrations were analysed for ERT to determine sample loss and degradation during pre-processing, storage and transport.

Table 3.4 shows the concentrations of FFC determined in the microcosm throughout the 15-day trial period. After the initial injection, FFC concentration decreased by 17 % (i.e., from 20.5 μg L^{-1} to 17.1 μg L^{-1}) during the 72 hr period between water exchanges. Thereafter, FFC concentrations accumulated with antibiotic injections in the microcosm as a result of incomplete (i.e., 75 %) water exchanges, and all mean measured concentrations were above the nominal 20 μg L^{-1}.

Table 3.4. Measured concentrations of FFC in the microcosm at 1, 24 and 72 hours after injection of the antibiotic and at 4 successive time points throughout the 15-day trial period. Antibiotic injections 1, 2, 3 and 4 were delivered after 75% water exchanges at days 29, 36, 40 and 43 of the 45-day pilot experiment.

	FFC injection number				
Hours after injection	1	2	3	4	**mean ± sd**
1	20.5	23.5	23.2	25.1	23.2 ± 1.9
24	19.1	-	24.3	-	21.7 ± 3.7
72	17.1	29.6	-	19.8	22.2 ± 6.6
mean ± sd	18.9 ± 1.7	26.6 ± 4.3	23.7 ± 0.8	22.5 ± 3.8	

Based on this data, it was estimated that there was a build-up of FFC in the microcosm in the range of 12.5 to 33 % (mean = 21 %), calculated as the percent deviance from the nominal 20 μg L^{-1} concentration. This build-up of FFC was consistent with the residual FFC that would be expected to accumulate in the system due to incomplete water exchanges. In subsequent experiments, a correction factor of 25 % due to incomplete water exchanges and 17 % due to FFC loss in the microcosms as estimated after the first injection were used to adjust the targeted FFC concentrations.

Table 3.5. Measured concentrations of ERT in the microcosm at 1, 24 and 72 hours after injection of the antibiotic and at 4 successive time points throughout the 15-day trial period. Antibiotic injections 1, 2, 3 and 4 were delivered after 75% water exchanges at days 29, 36, 40 and 43 of the 45-day pilot experiment. Concentrations were corrected by empirical factors derived from spiked samples.

	ERT injection number				
Hours after injection	1	2	3	4	**mean ± sd**
1	21.7	14.7	12.9	16.9	16.5 ± 3.8
24	20.0	-	9.2	-	14.6 ± 7.6
72	5.4	2.7	-	1.5	3.2 ± 2.0
mean ± sd	15.7 ± 8.9	8.7 ± 8.5	11.1 ± 2.6	9.2 ± 10.1	

Mean concentrations of ERT in the microcosm deviated considerably more than FFC concentrations and they dropped drastically after 72 hrs in the system. (Table 3.5). The concurrently analysed batches of spiked samples showed that there was a loss of ERT between 19 and 35 % during pre-processing, storage and transport. In previous analysis, the recovery of ERT has been observed to vary from day to day (i.e., from 69 to 84 %), and this seems to be a function of the SPE procedure (Jeffry A. Bernardy, personal communication). Variation in the accuracy of SPEs can thus account in part for the differences observed in ERT concentrations 1 hr after injection between successive injections (Table 3.5). Nonetheless, the drop in ERT

concentrations after 72 hrs suggest that ERT underwent degradation in the microcosms.

3.5. Discussion

This chapter has described the evaluation of major physical (i.e., water flow and light intensity) and biological (i.e., total Chl as a proxy for algal biomass and bacterial community structure) variables in the stream microcosm system built to assess the effects of antibiotics on the bacterial community structure of stream biofilms. Light and hydrodynamics are critical variables influencing the biological structure and process rates of benthic microbial communities. Light directly influences photosynthetic rates and primary production in benthic algae, which in turn affect the levels of nutrient uptake and the temporal patterns of dissolved oxygen in stream water (Hauer and Hill 2006). Stream hydrodynamics affects mass transfer between stream water and benthic biofilms and is a determinant of algal and bacterial community structure, biofilm thickness and the uptake and transport of solutes (Battin et al. 2003; Besemer et al. 2007).

The two pilot experiments conducted between 2009 and 2010 showed that it was easy to maintain stable hydrodynamic conditions across flumes by adjusting the individual valves that regulate their flow. The coefficient of variation in flow across flumes during the second 15-day pilot experiment, in which valves were adjusted weekly, was less than 1%. Photon flux density, on the other hand, exhibited gradients within and across flumes (Figures 3.5 and 3.6), yet it only explained 7% of the variation observed in total Chl concentrations (section 3.3.3). The fact that total Chl concentrations were not significantly different within longitudinal sections in flumes, between flumes and between header tank - sink recirculation units (Table 3.1) suggests that differences in photon flux density were not of a sufficient magnitude to significantly affect primary production and algal biomass at these experimental levels during the 26 day period throughout which samples were collected. Photon flux density did not have a significant effect on bacterial community structure either, and bacterial communities were not significantly different between flumes belonging to different header tank - sink recirculation units (section 3.3.4.4).

The reproducibility in bacterial community structure and total Chl concentrations observed between header tank - sink recirculation units is consistent with the evaluation of a very similar microcosm system performed by Singer et al. (2006), who observed little variation between different header tank - sink recirculation units and suggested that sets of flumes from different recirculation units could be allocated to different treatments. Likewise, the assessment of variability in tRFLP profiles between replicate PCR and restriction digest reactions (3.3.4.3) agreed with previous studies indicating low variability at these experimental levels and that most of the variation in tRFLP profiles occurs between replicate profiles on a capillary electrophoresis run (Osborn et al. 2000). This reinforced the need to run replicate profiles to confirm the reproducibility of peaks in the electropherograms.

The stability of FFC and ERT in the microcosms was markedly different. While variation in FFC concentrations between water exchanges in the microcosms was reasonably low, concentrations of ERT varied considerably and suggested a significant loss of ERT in the system. These results indicate that while for FFC the use of a gradient of nominal concentrations for further experiments would be possible, further experiments with ERT would require the use of a few nominal concentrations sufficiently apart to be considered distinct treatments after analytical measurement. Although the differing stability between FFC and ERT in the microcosms undoubtly reflects their distinct physico-chemical properties, it also reflects variation in sample pre-processing, particularly in the SPE of ERT. In particular, FFC is highly soluble (1.32 g L^{-1} at pH 7.0) and has a Log K_{ow} (0.37), which indicates that it will tend to remain in the water column (Vincent 1992). Erythromycin, on the other hand, sorbs more readily to soils and sediments by cation exchange and hydrophobic interactions (Kim et al. 2004c).

Quantifying the variability - and assessing the reproducibility - of key variables in experimental systems such as model streams is important to optimize their operation and the design of experiments. The series of assessments/'mini experiments' described in this chapter provided relevant information on the overall operation of the microcosms and the variability observed in major physical and biological variables. This information guided the experimental designs used to assess the effects of FFC and ERT on the bacterial community structure of stream biofilms described in chapters 4 and 5.

Chapter 4

Exposure of developing biofilms to a florfenicol concentration gradient in model streams

4.1. Introduction

Surveys conducted over the past decade have detected different classes of antibiotics in streams at concentrations in the range of nanograms to micrograms per litre (see Kümmerer, 2009a) raising concerns about their potential ecological effects. Among freshwater ecosystems, streams are increasingly recognised as critically important due to the biogeochemical and ecological processes they support (e.g., Peterson et al., 2001). All antibiotics - including some synthetic compounds – have been shown to have a hormetic effect on bacteria (Davies et al., 2006), suggesting that they are likely to influence the dynamics and evolution of bacterial communities via stimulatory and inhibitory effects.

Biofilms are multispecies bacterial communities that grow attached to surfaces enclosed in an extracellular polysacharide matrix (Stoodley et al. 2002). In streams, biofilms are also composed of algae and fungi, and their relative abundance at any given time point depends on the substratum in which the biofilm develops (e.g., epilithic vs. epyxilic biofilms) (Allan and Castillo 2007). Bacteria are primarily responsible for biofilm formation via cell-surface attachment and modulate biofilm development through complex cell-cell signaling interactions (Stoodley et al., 2002). Biofilm formation begins with the attachemnt of primary colonizers onto a substratum conditioned with polysacharides and proteins (Rickard et al. 2003). Cell growth, cell division, the production of extracellular polysaccharides and colonization by secondary colonizers gradually leads to the development of a mature, differentiated and complex multi-species community (Stoodley et al. 2002; Rickard et al. 2003). The emergent properties of biofilms, such as thickness, density and porosity - sometimes referred to collectively as biofilm architechture - are fundamental in determining its functional properties (Stoodley et al. 2002; Battin et al. 2002; Besemer et al. 2007; Bottacin-Busolin et al. 2009). Biofilm thickness, for example, influences mass transfer, the difussion of solutes and consequently the biogeochemical processing of elements (Battin et al. 2002; Battin et al. 2003; Battin et al. 2007). In streams, biofilms are central to the biogeochemical processing of elements and link dissolved substrates to higher trophic levels (Battin et al., 2003; Allan and Castillo, 2007; Bottacin-Busolin et al. 2009). Bacterial community composition is also a determinant of the level of ecosystem functioning (Bell et al., 2005), underscoring the need to assess how it is affected by ubiquitous pollutants.

In this study we used model streams to assess the effects of the broad spectrum antibiotic FFC ([R-(R*,S*)-2,2-dichloro-N-[1-fluoromethyl-2 hydroxy-2-(4-methylsulfonylphenyl)] ethyl acetamide]) on the bacterial community structure of stream biofilms. FFC is a major veterinary antibiotic used to treat bacterial infections in cattle, swine, poultry and fish (CVMP 1999a, 1999b, 2000) whose bacteriostatic activity is based on a reversible binding to the 50S subunit of 70S ribosomes that prevents peptide elongation (Schwarz *et al.*, 2004). Its use in aquaculture - where it is licensed for use to control certain bacterial diseases in several countries including the USA, UK, Norway and Chile - represents a particularly relevant exposure scenario given the extent of freshwater aquaculture and the fact that many freshwater aquaculture facilities discharge their effluents directly into headwater streams (Bostock et al. 2010; Tello et al., 2010).

We grew biofilms under a gradient of FFC concentrations for a time period consistent with its use in land-based freshwater aquauculture and monitored changes in bacterial community structure and biofilm composition using terminal restriction fragment length polymorphism (tRFLP) (Liu et al. 1997) and confocal laser scanning microscopy (CLSM). The objective of this study was to assess patterns of change along the gradient of FFC concentration that could provide insight into the type and magnitude of effects that could be expected from episodic exposure of stream biofilms to FFC in headwater streams.

4.2. Methods

4.2.1. Microcosm operation and experimental design

Microcosms were operated as described in section 3.2. Biofilms were grown under a gradient of FFC concentrations for 33 days. A FFC nominal concentration gradient of 0, 1, 3, 10, 31 and 100 μg L^{-1} was set by randomly assigning each concentration to one of the six header tank - sink recirculation units. Results from the preliminary experiments described in chapter 3 showed that physicochemical and biological variables were reproducible accross header tank - sink recirculation units and that, therefore, flumes belonging to the same header tank - sink recirculation unit could be allocated to different treatments. In Appendix 1 we provide further support for the experimental design and validity of the results

discussed in this chapter. Samples for bacterial community structure were collected on days 10, 20 and 33 of biofilm development. Samples for confocal laser scanning microscopy (CLSM) were taken at 15 and 29 days of biofilm development. Water samples for nutrient analysis of the source stream water were taken prior to every water exchange. Total ammonia and nitrate were analysed on a Bran Luebe AutoAnalyser 3 following the protocols provided by the manufacturer (Bran+Luebbe 2003a; 2003b). Phosphate was analyzed by the ascorbic acid method with absorbance reading at 690 nm (APHA, 1998). Flow and water depth were monitored weekly. Temperature, pH and DO were monitored in the microcosms every other day. The experiment was run in an indoor aquarium facility from May 14th to June 15th 2010 (33 days) in an independent room at ambient temperature with a 12:12 light-dark cycle. Table 4.1 shows the main physicochemical parameters in the microcosms throughout the study.

4.2.2. FFC injection, sampling and analysis

FFC was injected into the microcosms at the start of the experiment and after every water exchange thereafter. For each injection, a fresh stock solution of FFC (25 μg ml^{-1}) was made by dissolving 10 mg of analytical standard FFC (Sigma-Aldrich F1427-500MG) in 400 mL of sterile (121^{o}C 15 min^{-1}) distilled water. This stock solution was serially diluted to make up 200 mL injection solutions targeting each of the set nominal FFC concentrations in the microcosms. The control treatment received 200 mL of the sterile distilled water used to make up the injection solutions. Solutions were delivered as a slug to their corresponding header tanks.

Table 4.1. Main physicochemical parameters of microcosms and source stream water throughout the study. Flow and water depth were monitored weekly. Temperature, pH and DO were monitored every other day. Samples for nutrient analysis were collected from the source tank prior to every water exchange.

Variable	mean ± sd
Across microcosms/flumes	
Flow (ml $^{s-1}$) [a]	39 ± 0.5
Water Depth (cm) [a]	0.7
Temperature (°C)	20. 78 ± 0.75
pH	8.13 – 8.18
DO (mg L^{-1})	9.5 ± 1.2
Photon Flux (umol m^{-2} s^{-1})	106 ± 1.4
Source stream water	
NH_4 + NH_3 (ug L^{-1})	55.20 ± 7.54
NO_3 (ug L^{-1})	1030.12 ± 25.63
PO_4 (ug L^{-1})	32.64 ± 2.81

[a] Flow and water depth settings yielded a mean estimated Reynolds Number of 886 (i.e., transitional flow) using the formula described in Singer et al., 2006 and the kinematic viscosity of water at 20 °C.

The mass of FFC injection solutions was corrected as described in section 3.4.3 so that, theoretically, FFC concentrations would always match the targeted nominal concentration after injection (e.g., 100 μg L^{-1} – (100*0.17*0.25) = 79.2 μg L^{-1} adjusted targeted concentration). Water samples for FFC analysis to determine whether measured concentrations were in agreement with the targeted nominal concentrations, were taken at days 4, 5, 7, 12, 19, 26, 28 and 30. On two occasions, samples were taken 1, 24 and 48 hours after antibiotic injection into the microcosms, and 3 times 72 hours after injection. This resulted in a total of 45 samples analysed over the 33-day experimental period (i.e., 9 samples per nominal FFC concentration). Samples for FFC and FFA analysis were processed and analysed as described in section 3.4.1.

4.2.3. DNA fingerprinting of bacterial communities

Bacterial communities were characterized by tRFLP of the 16S rRNA gene using labelled forward and reverse primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') (Marchesi et al., 1998) and 1389R (5'-ACGGGCGGTGTGTACAAG-3')

(Marchesi et al., 1998; Osborn et al., 2000). Each sample for bacterial community structure analysis consisted of three tiles randomly sampled from the middle section of each flume and pooled together, which resulted in 4 replicate samples per treatment for every time point sampled (i.e., 1 sample consisting of 3 pooled tiles per flume x 4 flumes per treatment). DNA was extracted from biofilms as described in section 3.3.4.1 and PCR, restriction digestion and tRFLP were carried out as described in section 3.3.4.3. All PCR reactions included no template controls and restriction digest reactions included no-restriction enzyme and no-PCR product controls. PCRs and restriction digests were run as single reactions.

4.2.4. CLSM of stream biofilms and image analysis

Each sample for CLSM analysis consisted of a single tile randomly sampled from the middle section of each flume. Individual tiles were sampled with sterile forceps and placed upright in fix pots containing 35 µL of 0.2 µm filter sterilized stream water. Bacteria and extracellular polymeric substances (EPS) were stained with Syto 13® (Invitrogen) and tetrameythylrhodamine wheat-germ agglutinin (TRITC WGA), respectively, following the method described by Neu and Lawrence (2005). Tiles were mounted upside down on a custom-made coverslip chamber with the biofilm layer immersed in 0.2 µm filter sterilized stream water. The weight of each clay tile was insufficient to compress the biofilm against the coverslip and thus spacers were not required to separate the biofilm from the coverslip. Biofilms were scanned on an inverted Leica TCS SP2 AOBS CLSM using a 488 nm Argon laser at 50% deflection intensity and a HC PL Fluotar 10X 0.3 numerical aperture dry lens that provided a good compromise between a broad field of observation and axial and lateral resolution. Biofilm thickness was systematically measured in each tile by focusing through the biofilm using the *z*-position dial (i.e., 5 depth measurements per tile). At the centre of each tile, 5 equidistant *xy* optical sections were collected from the middle section of the biofilm to estimate the coverage of bacteria, EPS and algae. (i.e., the thickness of the biofilm at the centre of each tile was divided by 7 and images were collected at the 5, equidistance depth steps between the surface and bottom of the biofilm). Excitation/emmission wavelengths for Syto 13® stained bacterial cells, TRITC-WGA stained EPS, and algae autofluorescence were 488/496-535 nm, 543/550-600 nm, and 663/677-795 nm, respectively.

Measurements and image collection were performed using a 4X optical zoom and a field of observation of 375 x 375 μm (2.4 μm *z*-step size). All CLSM settings were kept constant throughout the experiment.

A total of 720 *xy* sections were analysed in ImageJ v.1.45s (Abramoff et al. 2004) to estimate the surface coverage of bacteria, EPS and algae. All images showed an overall good signal and background noise was removed using a 3 x 3 pixel median filter and manual background substraction in images which exhibited higher background noise. Images were then converted to binary using automatic threshold detection based on the histogram of each image. The surface area of bacteria, EPS and algae was quantified from these binary images.

4.2.5. Statistical analysis

4.2.5.1. Bacterial community structure

Based on initial observations of scatterplots, a linear and log-linear model were fit by least squares regression to the total number of OTUs (i.e., species richness) and to the Gini coefficient calculated on relative peak heights to assess the presence of trends consistent with the FFC concentration gradient. The Gini coefficient - which can be used as a measure of diversity - is the probability that two randomly selected individuals from a community are different species (Lande, 1996), and it is calculated as 1-λ, where λ is Simpson's concentration index (Simpson, 1949) described by:

$$\lambda = \sum_{i=1}^{S} p_i^2$$

where S is the number of species and p_i the frequency of species i in a community. The best model for each variable and sampling time was selected based on Akaike's 'an information criterion' (AIC). The Bray-Curtis dissimilarity index was used to characterize community dissimilarities (see section 3.3.4.3). A non-metric multidimensional scaling (NMDS) was performed using the *metaMDS* function in the R package VEGAN (v. 1.17-6) (Okansen et al., 2011) to visualize dissimilarities between treatments throughout the experiment. *metaMDS* uses several random starts

to find a stable solution and standardizes the scaling in the result for ease of interpretation. The non-parametric permutational multivariate analysis of variance implemented in the function *adonis* in the R package VEGAN was used to partition variation in community dissimilarities (Anderson, 2001; McArdle and Anderson, 2001). To partition the variation in the entire dataset (i.e., data from 3 sampling times) in response to FFC concentration, days of biofilm development and their interaction, a model using FFC concentration as a continuous variable and days of biofilm development as a categorical covariate was specified. *adonis* was then used to determine the percent of variation in community dissimilarities attributable to FFC in each of the 3 time points separately using FFC concentration as a continuous variable. *adonis* was run using the Bray-Curtis dissimilarity index calculated on untransformed relative peak heights, hellinger transformed peak heights (i.e., square root of relative peak heights) and presence-absence data (i.e., Sorensen index). The use of this sequence of transformations in *adonis* allowed assessment of the relative contribution of changes in abundance vs. changes in composition to the overall change due to FFC along the 3 sampling times. Unless otherwise stated, tables and figures present the results of analysis performed using hellinger transformed peak heights, and relevant differences with respect to results obtained using untransformed relative peak heights and presence-absence data are discussed within the text. There was only a minimal quantitative difference in the results if performed using measured vs. nominal FFC concentrations and it did not alter our conclusions. In the interest of clarity, therefore, the results presented correspond to analysis performed using nominal FFC concentrations. Qualitative conclusions were also unchanged when using Euclidean distance instead of the Bray-Curtis index as a measure of community dissimilarity. All significance tests were based on 10,000 permutations.

4.2.5.2. Biofilm thickness and composition

Linear regressions were used to assess the percent of variance in biofilm thickness and surface coverage of bacteria, EPS and algae explained by FFC concentrations. Biofilm thickness and the complete set of surface coverage measures for baceria, EPS and algae were regressed against FFC concentrations for each time point

separately. Assumptions of linearity, homocedasticity and normality were checked by visual inspection of q-q and residual plots.

4.2. Results

4.2.1. Stability of FFC in microcosms

Table 4.2 shows that measured FFC concentrations in the microcosms throughout the study were within 15% of the targeted nominal concentrations. This is to some extent as expected from FFCs chemical characteristics. FFC is highly soluble in water (1.32 g L^{-1} at pH 7.0) and has a low Log K_{ow} (0.37), indicating that it will not partition to organic material and will remain in the water column (Vincent, 1992). FFC has, however, been reported to undergo some degradation in water under biotic conditions (Vincent 1992; Lai et al. 2009). Conservatively correcting FFC concentrations presented in Table 4.2 to account for an analytical recovery of 85% (i.e., the lower recovery limit of the analytical method used in this study) and the 17% adjustment factor described in section 3.3, gives a lower limit for FFC degradation in the microcosms in the range of 7.6 – 19.5% (e.g., 31 – [29.48 μg L^{-1} * 1.15] + [31*0.17] = 7.6%; 100 - [84.77 μg L^{-1} * 1.15] + [100*0.17] = 19.5%). As can be seen in Table 4.2, this was not accounted for by its metabolite, florcenicol amine (FFA). FFA was detected at very low levels in all of the samples taken from the 30 and 100 μg L^{-1} treatments and only twice in the 10 μg L^{-1} treatment. It was not detected in the 3 and 1 μg L^{-1} treatments.

Table 4.2. Measured concentrations of FFC and FFC-amine in microcosms throughout the experiment. Concentrations are ug L^{-1} (mean ± standard deviation).

Nominal FFC concentration	Measured FFC[a]	Measured FFC-amine[b]
1	0.90 ± 0.12	0
3	2.79 ± 0.31	0
10	9.14 ± 0.85	0.11 ± 0.03
31	29.48 ± 3.85	0.073 ± 0.05
100	84.77 ± 9.67	0.18 ± 0.08

[a] n = 9; Data not corrected for analytical recoveries (85 – 100%)
[b] FFC-amine was not detected in the two lower FFC treatments

4.2.2. Bacterial community structure

The total number of OTUs detected throughout the experiment increased from 28 to 47 to 52 at 10, 20 and 33 days of biofilm development, respectively. There was a saturating trend of increasing OTUs with increasing FFC concentration at 10 and 20 days of biofilm development that was best described by a linear dependence of the number of OTUs on log -transformed FFC (Table 4.3). This relationship was marginally not significant at 10 days of biofilm development and highly significant at 20 days of biofilm development. Similarly, the Simpson evenness index showed a positive and significant relationship with FFC concentration at 10 and 20 days of biofilm development that was best described by linear and log-linear relationship, respectively (Table 4.3). At 33 days of biofilm development there was no significant relationship between these variables and FFC concentration (Table 4.3).

Table 4.3. Linear models estimating the effect of FFC on the number of OTUs ($\hat{S}$) and Simpson evenness ($\hat{E}$). $\alpha = 0.05$.

	Models	$F_{1,22}$	R^2	p-value
T_1	$\hat{S}$ = 2*log(FFC)+19	3.9	0.15	0.06
	$\hat{E}$ = 0.0002*FFC+0.9	6.1	0.22	0.02
T_2	$\hat{S}$ = 5*log(FFC)+26	9.8	0.31	0.005
	$\hat{E}$ =0.02*log(FFC)+0.8	12.4	0.36	0.002
T_3	$\hat{S}$ = -0.2*log(FFC)+38	0.02	0.001	0.9
	$\hat{E}$ = 0.002*log(FFC)+0.9	0.4	0.02	0.5

The NMDS ordination of the entire dataset showed two major patterns (Figure 4.1). The first is a strong temporal trend described by the grouping of samples along the first dimension of the NMDS and the second is a trend related to FFC treatment effects seen as a scatter of the samples from each sampling time along the first and mainly second NMDS dimensions. Despite the variability observed between replicates, Figure 4.1 shows that bacterial communities differentiated in a pattern consistent with the imposed FFC concentration gradient, most noticeably at 10 and 20 days of biofilm development.

The partitioning of variation in community dissimilarities was largely consistent with the NMDS ordination patterns seen in Figure 4.1. Table 4.4 shows that time accounted for the bulk of the total variation observed in bacterial community

structure and that FFC concentration accounted for a minor, yet significant percent of this variation. The percent of variation explained by the interaction term increased when we ran *adonis* using untransformed relative peak heights ($F_{2,66}$=4.82, p=0.003) and it was not significant when using presence-absence data ($F_{2,66}$=2.14, p=0.075), suggesting that the differential effect of FFC in time was mostly related to changes in the relative abundance of the most common OTUs.

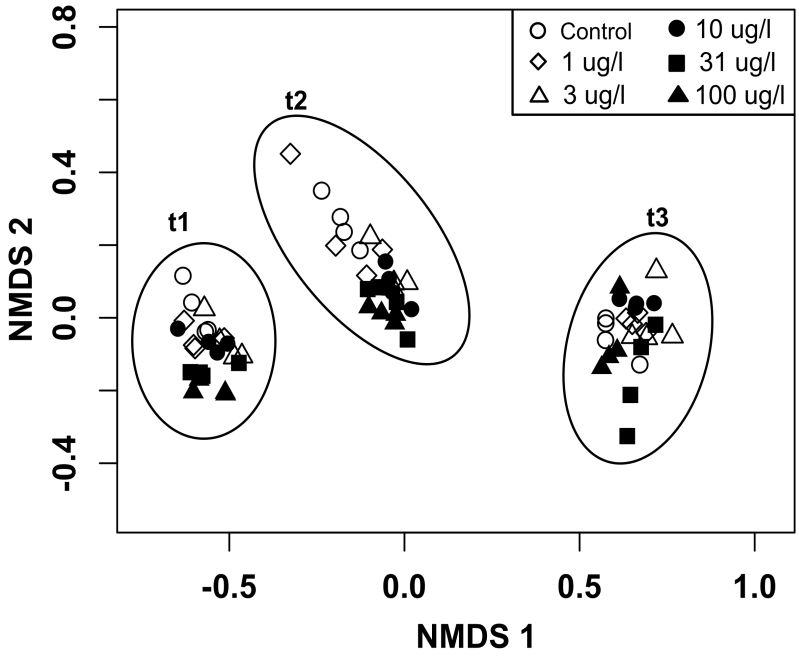

Figure 4.1. Nonmetric multidimensional scaling ordination of the entire dataset. Dimensions in final solution:2. Stress: 0.05. NMDS 1: first NMDS dimension. NMDS 2: second NMDS dimension. t1, t2 and t3 represent samples taken at 10, 20 and 33 days of biofilm development, respectively. Ellipses were hand drawn to highlihght sampling times.

Table 4.4. Variance partitioning of the entire dataset using *adonis*. Df, degrees of freedom; SS, sums of squares; MS, mean squares; F, F-test statistc; P, p-value.

	Df	**SS**	**MS**	**F**	**P**
Dose	1	0.12	0.12	8.90	<0.001
Time	2	5.34	2.67	196.39	<0.001
Dose x Time	2	0.09	0.05	3.57	0.013
Residuals	66	0.89	0.01		
Total	71	6.45			

Indeed, a comparison of the results obtained with *adonis* for each independent time point using untransformed relative peak heights, hellinger transformed peak heights and presence-absence data indicates that the variation in bacterial community structure due to FFC effects shifted from predominantly changes in the relative abundance of the most common OTUs towards differences in the presence of intermediate and rarer OTUs (Table 4.5; Figure 4.2). This is inferred from the decreasing trend in the percent of explained variance in the analysis conducted with untransformed relative peak heights vs. the slight increase in the percent of explained variance when analysing presence-absence data seen in Table 4.5. While differences in the relative abundance of common OTUs along the FFC gradient decreased steadily over time, differences due to the presence of intermediate and minor OTUs increased slightly and accounted for the bulk of the variation attributable to FFC at 33 days of biofilm development.

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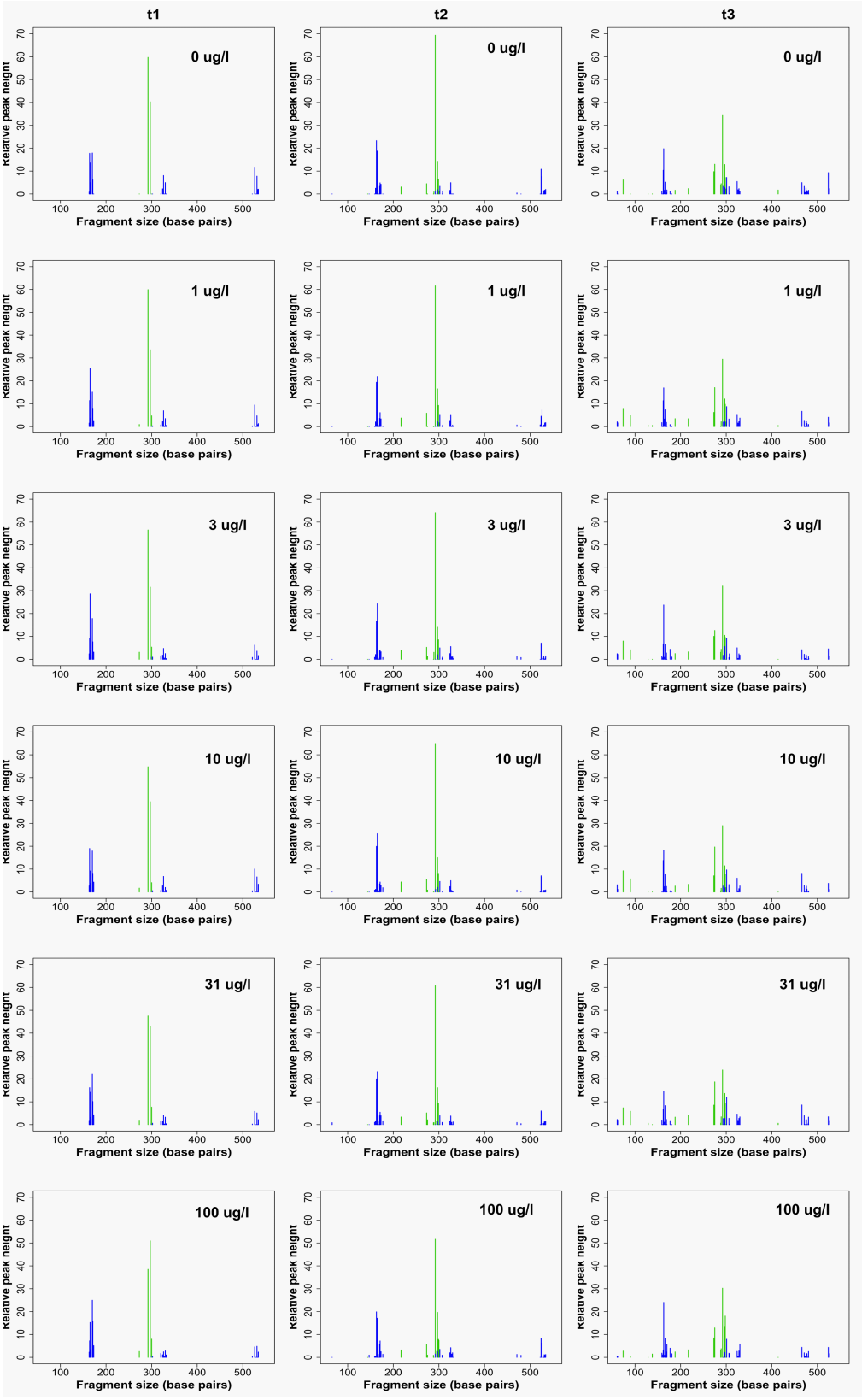

Figure 4.2. Representative synthetic tRFLP electropherograms of samples from each concentration in the gradient at 10 (t1), 20 (t2) and 33 (t3) days of biofilm development. Note how changes between treatments are very subtle compared to temporal changes. Blue: forward T-RFs. Grenn: reverse T-RFs.

Table 4.5. Variance partitioning of each independent time point using *adonis*. RPH, untransformed relative peak height; HELL, hellinger transformed relative peak heights; PA, presence-absence. P-values are conservatively corrected for multiple comparisons using the Bonferroni correction. t1, t2 and t3 represent samples taken at 10, 20 and 33 days of biofilm development, respectively.

	RPH	**HELL**	**PA**
T_1	$F_{1,22}$=11.24, R^2=0.34, P<0.0003	$F_{1,22}$=7.04, R^2=0.24, P<0.003	$F_{1,22}$=3.25, R^2=0.13, P=0.126
T_2	$F_{1,22}$=8.39, R^2=0.28, P<0.0003	$F_{1,22}$=5.79, R^2=0.21, P<0.003	$F_{1,22}$=3.91, R^2=0.15, P=0.048
T_3	$F_{1,22}$=4.51, R^2=0.17, P<0.003	$F_{1,22}$=4.89 R^2=0.18, P<0.0003	$F_{1,22}$=4.13, R^2=0.16, P=0.009

4.2.3. Biofilm thickness and composition

Biofilm thickness and the surface coverage of bacteria, EPS and algae was highly variable at all FFC concentrations (Figure 4.3). However, at 15 days of biofilm development there was a significant positive relationship between the surface coverage of bacteria and EPS and FFC concentration (Figure 4.3b and 4.3c). Although these trends had little explanatory power, they were apparent from visual inspections of composite *xy* sections, which suggested an increase in bacterial and EPS coverage with FFC concentration at 15 days of biofilm development (Figure 4.4). There were no significant relationships between biofilm thickness or the surface coverage of algae and FFC concentration at 15 and 29 days of biofilm development (Figure 4.3d and 4.3h).

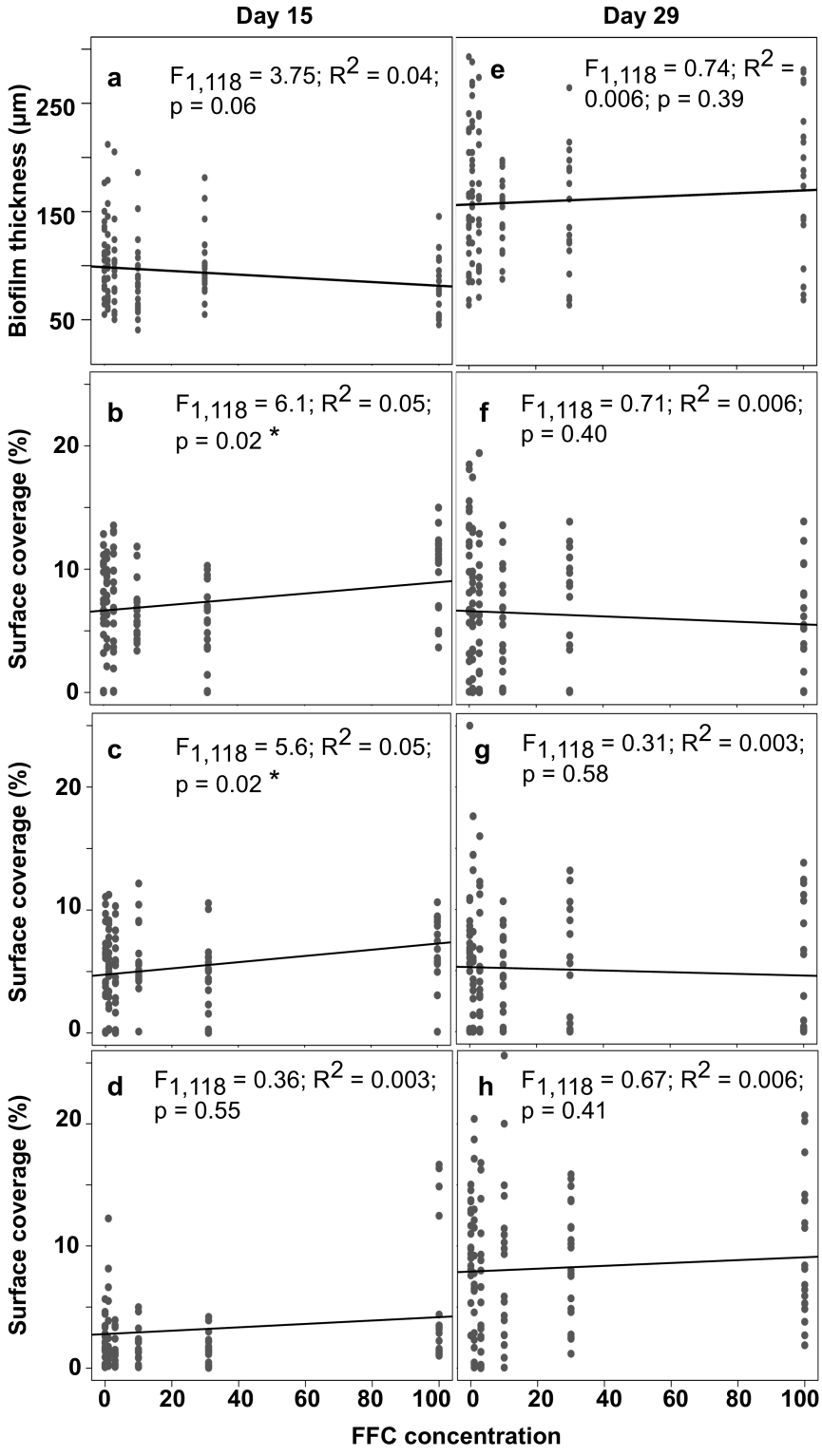

Figure 4.3. Linear regressions between FFC concentration and biofilm thickness (a e) and the surface coverage of bacteria (b f), EPS (c g) and algae (d h) at 15 and 29 days of biofilm development. $\alpha = 0.05$.

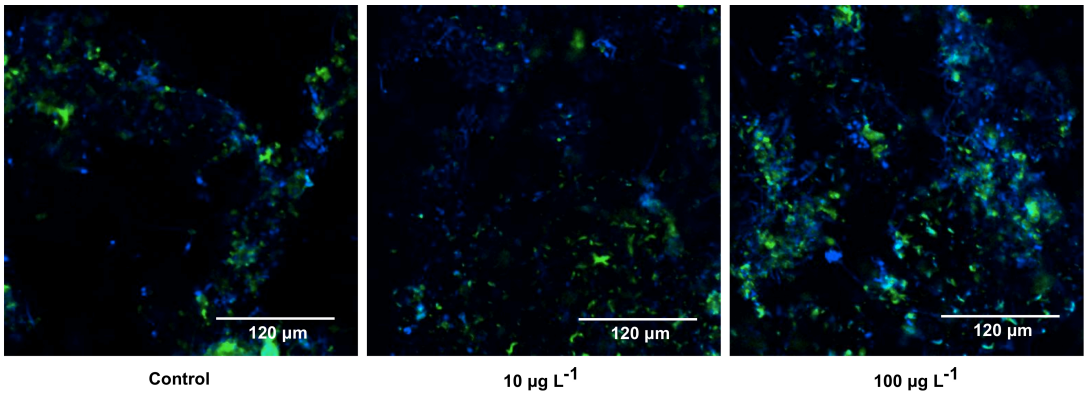

Figure 4.4. Representative composite *xy* sections of biofilm samples from the control, 10 µg L^{-1} and 100 µg L^{-1} treatments at 15 days of biofilm development. Blue: Syto 13 stained bacterial cells; Green: TRITC WGA stained EPS.

4.3. Discussion

Our results show that FFC concentration accounted for a significant percent of the variation in community dissimilarities throughout biofilm development and that differences between treatments shifted from predominantly changes in the abundance of common OTUs to the presence of intermediate and rarer OTUs. Interestingly, we observed positive trends between the number of OTUs and the Gini coefficient with FFC concentration at 10 and 20 days of biofilm development, and between the surface coverage of bacteria and EPS with FFC concentration at 15 days of biofilm development.

The positive relationship between the number of OTUs, the Gini coefficient index and the surface coverage of bacteria and EPS with FFC concentration during the first 20 days of biofilm development suggest a stimulatory effect of FFC on biofilm formation. In *S. aureus* isolates, subinhibitory concentrations of FFC have been shown to enhance biofilm formation by stabilizing transcripts coding for several adhesins (Blickwede et al., 2005). Adhesins mediate bacterial adhesion to host cells as well as intra and intergeneric bacterial coaggregation, a common phenomenon in freshwater bacteria that promotes biofilm formation (Min and Rickard, 2009; Rickard et al., 2003; Rickard et al., 2002). The positive trend between the surface coverage of EPS and FFC concentration at 15 days of biofilm development is

largely consistent with these studies. The lectin we used as an EPS probe binds to N-acetyl-glucosamine, an extracellular polysaccharide involved in biofilm formation in a number of bacteria (Jefferson and Cerca, 2006). FFC could have therefore facilitated biofilm adhesion to less successful colonisers or OTUs more likely to colonise at later stages of biofilm development, as well as increased the abundance of certain OTUs beyond the molecular detection threshold. Both of these effects may explain the positive trends seen between the number of OTUs and the Gini coefficient with FFC concentrations.

Theory suggests that as biofilms mature, the development of a complex architecture and formation of microhabitats increases the importance of autogenic factors such as resource competition and niche partitioning in determining the structure of bacterial communities (Jackson et al., 2001; Lyautey et al., 2005). Algae, on the other hand, exert a significant control on the composition of stream biofilm bacterial communities by reducing biofilm heterogeneity and creating a structural template for bacterial development (Besemer et al., 2007). Between 15 and 29 days of biofilm development, there was a clear increase in biofilm thickness and in the surface coverage of algae across all treatments (Figure 3), indicating a significant, overall change in biofilm architecture. In this context, our results agree with a scenario in which FFC enhanced biofilm formation and caused initial differences in bacterial community structure that were gradually compressed as the development of a complex architecture increased the relative importance of autogenic ecological processes.

Our study has limitations that must be considered in the interpretation of our results. Although tRFLP is a highly reproducible and powerful technique that is adequate to describe changes in bacterial communities along environmental gradients, it only describes the most abundant taxa within a community, and it can suffer from PCR sampling bias and artefacts (Abdo et al., 2006; Blackwood et al., 2007; Egert and Friedrich, 2003; Osborn et al., 2000). These limitations were partially overcome by using peak alignment and noise filtering methods that significantly remove PCR artefacts and provide an objective and un-biased method to identify true T-RFs (Abdo et al., 2006; Culman et al., 2009; Smith et al., 2005). Similarly, although TRITC WGA has been suggested as a useful general EPS probe for stream biofilms (Lawrence et al., 1998), different lectins have different binding specifities and our results may not provide a complete picture of the EPS matrix.

Therefore, the patterns we describe might not hold for the less abundant bacterial taxa and other EPS components. They do, however, reflect true differences in bacterial community structure and biofilm composition. Moreover, they are consistent with the imposed FFC concentration gradient, with previous studies showing a stimulatory effect of FFC on biofilm formation and with biofilm theory. Finally, although we cannot completely rule out indirect, top-down effects of FFC due to the disruption of grazing by protozoa, we consider them to be highly unlikely. 80S eukaryotic ribosomes are not the target of FFC, and in-vitro tests have shown it to be ineffective against protozoa at concentrations 1000 times higher than our highest nominal concentration (Iglesias et al., 2002). Additionally, at 29 days of biofilm development there was no relationship between FFC concentration and the surface coverage of bacteria and EPS, whereas adverse effects on protozoan grazing would have likely resulted in a positive relationship between these variables and FFC concentration at this stage of biofilm development (Lawrence et al. 2008).

Although our experiment was not deliberately designed to assess the dissipation and fate of FFC, it is interesting to note that the conservative estimates based on our data suggests that FFC underwent a loss in the range of that reported by Lai et al. (2009), which is higher than previously reported under abiotic and biotic conditions (Pouliquen et al., 2007; Vincent, 1992). Our model streams had a relatively large surface area covered by a multispecies biofilm through which water was continuously recirculated. Stream biofilms are known to increase the retention of solutes, influence biogeochemical process rates and have been reported to enhance contaminant removal with respect to suspended reactions (Battin et al., 2003; Bottacin-Busolin et al., 2009; Ganzter et al., 1986). We hypothesize that these factors may enhance biotic and abiotic removal processes of FFC, though this will be investigated further in the future.

5. Conclusions

Our study adds to the weight of evidence showing that antibiotics may influence the structure of aquatic bacterial communities. Importantly, it provides evidence for a stimulatory effect of FFC on biofilm formation that has direct implications for the potential effects of antibiotic pollution in aquatic ecosystems. Although the stimulatory effect of certain antibiotics on biofilm formation has been shown *in-*

vitro and in simple experimental systems (e.g., Hoffman et al., 2005; Schreiber and Szewzyk, 2008), there is only limited and indirect evidence for such an effect in experimental systems that adequately represent the complexity of aquatic ecosystems (Yergeau et al., 2010). In stream ecosystems, biofilm formation is a continuous and dynamic process that influences the persistence, dispersal and transmission of pathogens (Hall-Stoodley and Stoodley, 2005). Research aimed at further understanding how antibiotic pollution may affect these processes and its potential implications is critical to the development and implementation of benchmarks that are protective of the environment and human health. In this context, studies designed to assess the effects of FFC on the expression of specific bacterial adhesins may provide important insight into its effects on biofilm formation in bacterial communities.

Chapter 5

Effects of erythromycin thiocyanate on the bacterial community structure of stream biofilms

5.1. Introduction

Many freshwater aquaculture farms present a possible trace source of antibiotics to aquatic ecosystems. Thurman et al. (2002), for example, found antibiotics in water samples from intensive and extensive fish hatcheries in the United States and Lalumera et al. (2004) detected antibiotics in the sediments of stream segments receiving effluents from a salmonid farm. The occurrence of antibiotics in aquatic ecosystems is a matter of concern due to their potential effects on sensitive organisms and on the development of antibiotic resistance (Kümmerer 2009; Martinez 2009). Because bacteria are the natural targets of antibiotics, assessing their effects on bacterial communities in the environment is essential to determine their environmental safety prior to marketing authorization.

Currently, Aquamycin® 100 - a Type A medicated article (i.e., Premix) containing 100 g ERT lb^{-1} and used to produce a Type C medicated feed - is a candidate drug for approval by the US FDA to control mortality associated with bacterial kidney disease in freshwater salmonids (Meinertz et al. 2011). Bacterial kidney disease is a chronic systemic disease of salmonids caused by the gram-positive diplobacillus *Renibacterium salmoninarum* that causes up to 80% mortality in Pacific salmon and 40% in Atlantic salmon (Toranzo et al. 2005; Fish Health Inspectorate http://www.defra.gov.uk/aahm/files/leaflet-notifiable-diseases.pdf). Erythromycin inhibits protein synthesis by stimulating the dissociation of pepidyl-tRNA from ribosomes during elongation, resulting in chain termination and a reversible stoppage of protein synthesis (Roberts et al. 1999). Erythromycin is active mainly against gram-positive bacteria, *Chlamydia* and *Rickettsia* (Treves-Brown 2000). Surprisingly, despite being among the most frequently detected pharmaceuticals in a survey of 139 US streams (Kolpin et al. 2002), few studies have assessed the effects of erythromycin on the bacterial communities of freshwater ecosystems (Kim et al. 2005).

A major part of freshwater salmonid aquaculture is performed in land-based flow-through systems that discharge their effluents into headwater streams (Tello et al. 2010) and therefore the use of ERT to treat bacterial kidney disease in salmonids represents a potentially direct source of ERT to stream ecosystems. In this study we used model streams to assess the effects of ERT on the bacterial community structure of stream biofilms. We exposed biofilms to ERT for 30 days, a period

consistent with the 28-day treatment regime suggested for Aquamycin® 100, and monitored changes in bacterial community structure using tRFLP (Liu et al. 1997). This study complements previous studies that assessed the mineralization of erythromycin A and its influence on bacterial populations in aquaculture sediments (Kim et al. 2004a; 2005), and provides valuable additional data on the environmental safety of ERT to bacterial communities in stream ecosystems.

5.2. Methods

5.2.1. Microcosm operation and experimental design

Microcosms were operated as described in section 2.2. Biofilms were grown free of ERT for 15 days. Thereafter, a 30 - day nominal ERT treatment of 10 and 100 μg L^{-1} was set by assigning eight flumes (i.e., two header tank - sink recirculation units) to each concentration. Two header tank - sink recirculation units were used as controls, one of which served as a carrier control for the 10 mM triethanolamine buffer used to prepare the ERT injection solutions. Samples for bacterial community structure were taken at 15, 24 and 45 days of biofilm development (i.e., before ERT treatment and after 9 and 30 days of ERT exposure, respectively). Water samples for nutrient analysis of the source stream water were taken prior to every water exchange. Total ammonia and nitrate were analysed on a Bran Luebe AutoAnalyser 3 following the protocols provided by the manufacturer (Bran+Luebbe 2003a; 2003b). Phosphate was analyzed by the ascorbic acid method with absorbance reading at 690 nm (APHA, 1998). Flow and water depth were monitored weekly. Temperature, pH and DO were monitored in the microcosms every other day. The experiment was run in an indoor aquarium facility from August 24th to October 7th 2010 (45 days) in an independent room at ambient temperature with a 12:12 light-dark cycle. Table 5.1 shows the main physicochemical parameters in the microcosms throughout the study.

5.2.2. ERT injection, sampling and analysis

ERT was injected into the microcosms after every water exchange starting at day 16 of biofilm development. Stock ERT solutions were prepared and delivered as

described in section 3.4.4, with the exception that the carrier solution for ERT was 10 mM instead of 30 mM triethanolamine buffer. Water samples for ERT analysis to determine whether measured concentrations were in agreement with the targeted nominal concentrations were taken at days 16, 18, 22, 30, 37, 38 and 43. On three occasions samples were taken 1 hour after injection into the microcosms, twice 48 hours after injection and once 24 and 72 hours after injection. This resulted in a total of 28 samples analysed over the 30-day ERT exposure period (i.e., 14 samples per nominal ERT concentration). Samples for ERT analysis were processed and analysed as described in section 3.4.2. Triplicate samples spiked to each nominal concentration were processed and analysed with each batch of ERT samples to correct for loss during processing, storage and transport.

Table 5.1. Main physicochemical parameters of microcosms and source stream water throughout the study. Flow and water depth were monitored weekly. Temperature, pH and DO were monitored every other day. Samples for nutrient analysis were collected from the source tank prior to every water exchange.

Variable	**mean ± sd**
Across microcosms/flumes	
Flow (ml $^{s-1}$) [a]	39 ± 0.6
Water Depth (cm) [a]	0.7
Temperature (°C)	20.7 ± 0.5
pH	8.1 – 8.3
DO (mg L^{-1})	9.4 ± 0.3
Photon Flux (umol m^{-2} s^{-1})	106 ± 1.4
Source stream water	
NH_4 + NH_3 (ug L^{-1})	6.3± 6.0
NO_3 (ug L^{-1})	466.8 ± 218.6
PO_4 (ug L^{-1})	29.9 ± 23.7

[a] Flow and water depth settings yielded a mean estimated Reynolds Number of 886 (i.e., transitional flow) using the formula described in Singer et al., 2006 and the kinematic viscosity of water at 20 °C.

5.2.3. DNA fingerprinting of bacterial communities

Bacterial communities were characterized by tRFLP of the 16S rRNA gene using labeled forward and reverse primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') (Marchesi et al., 1998) and 518R (5'-CGTATTACCGCGGCTGCTGG-3')

(Felske and Osborn 2005). Sampling and DNA extraction were performed as described in section 3.3.4.1 and 4.2.3. PCR was performed in 25 μL reactions containing 2X Promega PCR MasterMix® and 0.4 μM of each primer, and thermocycling conditions were identical to those described in section 3.3.4.1. PCR products were cleaned with the Qiagen® QIAquick PCR purification kit and 70 ng of PCR product from each sample were digested with AluI (Promega® USA) and HhaI (New England BioLabs® UK) restriction enzymes. Samples for tRFLP were analysed in duplicate as described in section 3.3.4.3. PCRs and restriction digests were run as single reactions.

5.2.4. Abundance of 16S rRNA genes

The abundance of 16S rRNA genes was used as a proxy for bacterial abundance and determined in samples taken from two flumes from each header - tank sink recirculation unit (i.e., 2 flumes per header tank - sink recirculation unit = 4 flumes per treatment) at each sampling time. A 142 base-pair fragment of the 16S rRNA gene was targeted using primers BACT1369F (5'-CGGTGAATACGTTCYCGG-3') and PROK1492R (5'-GGWTACCTTGTTACGACTT-3') (Smith 2005). Standard curves for absolute quantification using quantitative real time PCR (qPCR) covering the range 5 x 10^2 to 5 x 10^8 copies of the target were prepared from serial dilutions of linear plasmid DNA. qPCR was performed in 20 μL reactions containing 10 μL of 2X Abgene® SYBR Green mastermix, 0.4 μM of each primer and 0.75 ng of template DNA on an Eppendorf Realplex Mastercycler® using a 4-step PCR cycling program. The linear dynamic range of the standard curve was 5 x 10^4 to 5 x 10^8 copies ($y = -4.3x + 47$; $R^2 = 0.99$; PCR efficiency = 0.71). The threshold cycle (C_t) value of the no template control (NTC) was 7 cycles higher than the lowest standard quantified and the C_t value of all samples fell within the linear range of the standard curve. All samples were analysed in duplicate and standard curves and NTCs were run in triplicate. Product specificity was checked by melting curve analysis and 2% agarose gel electrophoresis. Full details of qPCR assay validation are given in Appendix 2.

5.2.5. Statistical analysis

The Bray-Curtis dissimilarity index calculated on presence-absence data (i.e., Sorensen index) was used to characterize community dissimilarities. The data matrices generated from the AluI and HhaI tRFLP profiles were analysed separately, as they were presumed to provide complementary perspectives of the dominant bacterial taxa in each sample. The *adonis* function in the R package VEGAN (v.1.17-6) (Okansen et al., 2011) was used to partition variation in community dissimilarities. Time and ERT concentration were treated as categorical variables and samples belonging to the same nominal ERT concentration and different header tank - sink recirculation units were pooled together for analysis. Community dissimilarities from the control and carrier control header tank - sink recirculation units at 24 and 45 days of biofilm development were compared separately before pooling them together for analysis to ensure that their within group variability did not obscure treatment effects. First, a model was fit using "header tank" as a categorical variable to samples taken at 15 days of biofilm development (i.e., before the start of the ERT treatment) to assess whether bacterial communities from different header tank - sink recirculation units were reproducible before the start of ERT treatment. The effects of time and ERT concentration on bacterial community structure were then assessed by fitting a model to the combined data from samples taken at 24 and 45 days of biofilm development (i.e., after 9 and 30 days of ERT exposure, respectively). Independent models where then fit to samples from each of these time points separately.

Differences in the abundance of 16S rRNA genes between treatments were assessed for each sampling time separately by ANOVA on log-transformed abundances.

5.3. Results

5.3.1. Stability of ERT in microcosms

Mean measured ERT concentrations throughout the experiment in the 10 and 100 μg L^{-1} nominal treatments were 7.3 ± 3.9 and 87.2 ± 31.1 μg L^{-1}, respectively.

Table 5.2 shows mean measured ERT concentrations for each nominal treatment at different hours after injection into the microcosms.

Table 5.2. Measured ERT concentrations at different times after injection into the microcosms in the 10 and 100 μg L^{-1} nominal ERT concentration treatments.

Hours after injection	Nominal ERT Concentration	
	10 μg L^{-1}	100 μg L^{-1}
1 [a]	6.5 ± 1.6	81.8 ± 28.9
24 [b]	8.3 ± 0.1	101.8 ± 8.4
48 [c]	9.5 ± 7.0	96.2 ± 45.2
72 [d]	4.4 ± 0.1	70.5 ± 26.6

[a] n = 12; [b] n = 4; [c] n = 8; [d] n = 4.

The lowest ERT concentrations in each nominal treatment were measured 72 hours after injection. In samples taken 1 hour after antibiotic injection at 3 different points after the start of ERT treatment, we observed an average increase in measured concentrations in the 10 and 100 μg L^{-1} nominal treatments with successive ERT injections of 24 and 46%, respectively (i.e., after 1, 3 and 8 ERT injections). This indicates a gradual build-up of ERT in the system due to incomplete (i.e., 75%) water exchanges. Assuming no loss of ERT in the system, however, measured concentrations would have been expected to peak at 13.33 and 133.33 μg L^{-1} after 5 and 7 ERT injections due to the 25% residual water volume not changed between water exchanges (e.g., after three 10 μg L^{-1} ERT injections and two 75% water exchanges measured concentrations would equal 10*0.25+10 = 12.5 μg L^{-1} [after second injection]; 12.5*0.25+10 = 13.13 μg L^{-1} [after third injection]; and so on). Mean concentrations after 8 ERT injections in the 10 and 100 μg L^{-1} nominal treatments were 39 and 12% lower than these theoretical estimates, indicating that there was a loss of ERT in the system. There were no significant differences in measured ERT concentrations between the duplicate header tank - sink recirculation units used for each nominal ERT treatment, indicating that they could be regarded as single treatments in subsequent analysis ($F_{1,12}$ = 0.43, p = 0.52 [10 μg L^{-1} ERT nominal treatments] and $F_{1,12}$ = 0.08, p = 0.78 [100 μg L^{-1} ERT nominal treatments]; α = 0.05).

5.3.2. Effects of ERT on the abundance of 16S rRNA genes and OTU richness

At 15 days of biofilm development, before the start of ERT treatment, there was a significant difference in the abundance of 16S rRNA genes between two header tank - sink recirculation units (Tukey's HSD adjusted p-value = 0.002) (Figure 5.1a). There was also a marginally significant difference between the control and carrier control at 24 days of biofilm development ($F_{1,6} = 5.75$, $p = 0.05$); at 45 days of biofilm development there were no differences in the abundance of 16S rRNA genes between the control and carrier control ($F_{1,6} = 1.87$, $p = 0.22$). Figure 5.1 shows boxplots of 16S rRNA gene abundances for each treatment before (Figure 5.1a) and after (Figure 5.1b and 5.1c) the start of ERT treatment. At 15 days of biofilm development (Figure 5.1a) - before the start of ERT treatment - samples were grouped into their treatment groups to ease comparisons with the other time points. There were no significant differences in the abundance of 16S rRNA genes between treatments at 24 ($F_{2,21} = 2.9$, $p = 0.08$) or 45 days of biofilm development ($F_{2,21} = 0.5$, $p = 0.6$).

The total number of OTUs detected was higher in the AluI than in the HhaI tRFLP datasets. At 15, 24 and 45 days of biofilm development the number of OTUs observed in the AluI digests was 79, 66 and 60. In the HhaI dataset it was 34, 36 and 35, respectively. At 15 days of biofilm development there were no significant differences between the number of OTUs in different header tank - sink recirculation units (one-way ANOVA; $F_{5,18} = 0.68$, $p = 0.64$ and $F_{5,18} = 1.7$, $p = 0.18$ for the AluI and HhaI digests, respectively; $\alpha = 0.05$), and there were no significant differences in the number of OTUs between the control and carrier control at 24 and 45 days of biofilm development either (p-values between 0.27 and 0.92; $\alpha = 0.05$). At 24 and 45 days of biofilm development there were significant differences in the number of OTUs between treatments generated by the HhaI and AluI digests, respectively (one-way ANOVA; $F_{2,21} = 4.8$, $p < 0.05$ and $F_{2,19} = 7.2$, $p < 0.01$; $\alpha = 0.05$).

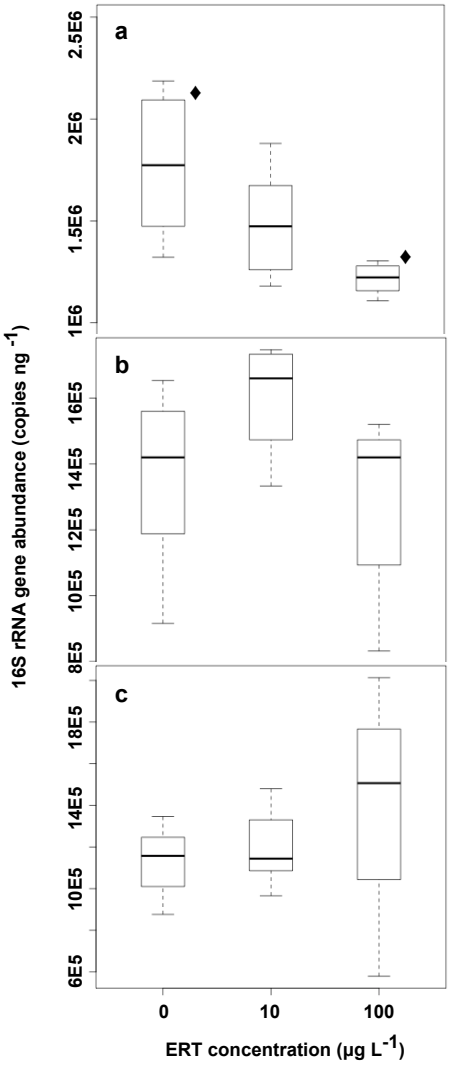

Figure 5.1. Boxplots of 16S rRNA gene abundances in each treatment at 15 (a), 24 (b) and 45 (c) days of biofilm development. Samples from Figure 5.1a are before the start of ERT treatment and they were pooled into treatment groups for ease of comparisons. Boxplots sharing the same symbol are significantly different at the $\alpha = 0.05$ significance level. Differences between treatments in samples taken at 45 days of biofilm development (Figure 5.1c) were assessed using the Mann-Whitney-Wilcoxon test.

Tukey's a-posteriori honest significant difference (HSD) test showed that these overall differences were due to differences between the control and the 100 µg L^{-1} treatment for the HhaI digests at 24 days of biofilm development and between the 10 and 100 µg L^{-1} treatment for the AluI digests at 45 days of biofilm development (adjusted p-values of 0.01 and 0.003, respectively; $\alpha = 0.05$). In both cases the 100

μg L^{-1} treatment had lower numbers of OTUs. There were no significant differences in the number of OTUs between the control and 10 μg L^{-1} treatment.

5.3.3. Effects of ERT on bacterial community structure

Variance partitioning of community dissimilarities from samples taken at 24 and 45 days of biofilm development showed a significant effect of time and ERT treatment on bacterial community structure (Tables 5.3 and 5.4).

Table 5.3. Variance partitioning of the entire (t2&t3) AluI dataset using *adonis*. Df, degrees of freedom; SS, sums of squares; MS, mean squares; F, F-test statistc; P, p-value.

	Df	SS	MS	F	R^2	P
Time	1	0.13	0.13	4.97	0.08	<0.01
Treatment	2	0.23	0.11	4.36	0.15	<0.001
Time x Treatment	2	0.16	0.08	3.06	0.10	<0.01
Residuals	39	1.03	0.03		0.66	
Total	44	1.55				

Table 5.4. Variance partitioning of the entire (t2&t3) HhaI dataset using *adonis*. Df, degrees of freedom; SS, sums of squares; MS, mean squares; F, F-test statistc; P, p-value.

	Df	SS	MS	F	R^2	P
Time	1	1.92	1.92	58.7	0.50	<0.001
Treatment	2	0.50	0.25	7.65	0.13	<0.001
Time x Treatment	2	0.11	0.06	1.74	0.03	0.11
Residuals	39	1.28	0.03		0.33	
Total	44	3.81				

The interaction term was significant in the tRFLP AluI dataset and it was not significant in the HhaI dataset. It is interesting to note that while the percent of variation in community dissimilarities explained by time in both datasets was markedly different (i.e., 8 vs. 50% in the AluI and Hha I datasets, respectively), the percent of variation explained by ERT was very similar (i.e., 15 vs. 13% in the AluI and Hha I datasets, respectively).

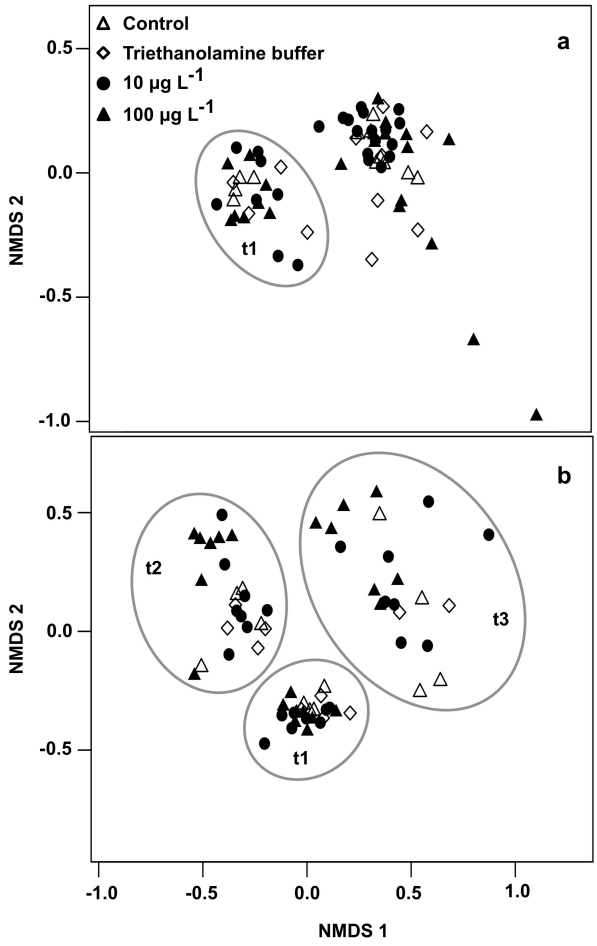

Figure 5.2. NMDS ordination of the AluI (a) and HhaI (b) tRFLP datasets. t1: 15 days of biofilm development, t2: 24 days of biofilm development, t3: 45 days of biofilm development. The ellipses were hand-drawn to identify samples from each sampling point. In Figure 5.1a, samples taken at 24 and 45 days of biofilm development are clustered together and they were not encircled in an ellipse. Note that at t1 samples had not yet been treated with ERT; the legend was kept for this time-point for comparative purposes. Figure 5.1a: stress = 0.09, dimensions in final solution = 2. Figure 5.1b: stress = 0.15, dimensions in final solution = 2.

The NMDS ordination of the AluI tRFLP dataset shows that samples taken at 24 and 45 days biofilm development (i.e., after 9 and 30 days of ERT treatment) clustered together, except for samples from the 100 µg L^{-1} treatment at 45 days of biofilm development, which form the 'tail' of samples extending towards the negative values of the second NMDS dimension (Figure 5.2a). Samples from the HhaI tRFLP dataset at 24 and 45 days of biofilm development had a pattern

somewhat consistent with ERT concentrations, although samples from different treatments overlapped to varying extents (Figure 5.2b). At 15 days of biofilm development, before the start of ERT treatment, there were no significant differences in bacterial community structure between header tank - sink recirculation units (adonis: $F_{5,18} = 1.36$, $p = 0.18$ for the AluI dataset and $F_{5,18} = 1.44$, $p = 0.14$ for the HhaI dataset; $\alpha = 0.05$), and this is reflected in the rather tight clustering of these samples in Figure 5.1.

Table 5.5. Pairwise comparisons of community dissimilarities between treatments at 24 and 45 days of biofilm development for the tRFLP datasets generated using Alu I and Hha I restriction enzymes. Significance levels: ^{NS} not significant; * <0.05; ** <0.01; *** <0.001. $\alpha = 0.05$. P-values are conservatively corrected for multiple comparisons using the Bonferroni correction.1

	Alu I		Hha I	
	24 days	45 days	24 days	45 days
Ctrl vs 10 µg L^{-1}	$F_{1,15} = 1.5$; $R^2 = 0.10$ ^{NS}	$F_{1,14} = 3.1$; $R^2 = 0.19$ ^{NS}	$F_{1,15} = 3.9$; $R^2 = 0.22$ *	$F_{1,13} = 1.7$; $R^2 = 0.12$ ^{NS}
Ctrl vs 100 µg L^{-1}	$F_{1,14} = 2.5$; $R^2 = 0.16$ ^{NS}	$F_{1,13} = 2.9$; $R^2 = 0.20$ ^{NS}	$F_{1,15} = 10.6$; $R^2 = 0.43$ ***	$F_{1,12} = 6.7$; $R^2 = 0.38$ **
10 vs 100 µg L^{-1}	$F_{1,14} = 3.4$; $R^2 = 0.21$ ^{NS}	$F_{1,14} = 6.9$; $R^2 = 0.35$ **	$F_{1,15} = 3.7$; $R^2 = 0.21$ ^{NS}	$F_{1,14} = 4.8$; $R^2 = 0.27$ **

Bacterial community structure between the control and carrier control were only significantly different at 24 days of biofilm development in the HhaI tRFLP dataset (adonis: $F_{1,7} = 5.55$, $p < 0.05$), yet pooling these samples together did not obscure comparisons between treatments (i.e., treatment effects for this dataset and time point were significant despite this variation at the control replicate level). Independent adonis models for samples taken at 24 and 45 days of biofilm development and digested with both restriction enzymes showed that ERT explained between 19 and 37% of the variation observed in community dissimilarities at 24 days of biofilm development (i.e., AluI and HhaI datasets, respectively) and between 31 and 32% at 45 days of biofilm development (i.e., HhaI and AluI datasets, respectively). To further explore these differences, we performed

pairwise comparisons of community dissimilarities between treatments (Table 5.5). Table 5.5 shows that the AluI and HhaI datasets provided a somewhat different assessment of the effects of ERT on bacterial community structure, likely reflecting effects on different taxonomic groups. A relevant consistency between both datasets is the absence of significant differences between the control and 10 μg L^{-1} treatment after 30 days of ERT treatment.

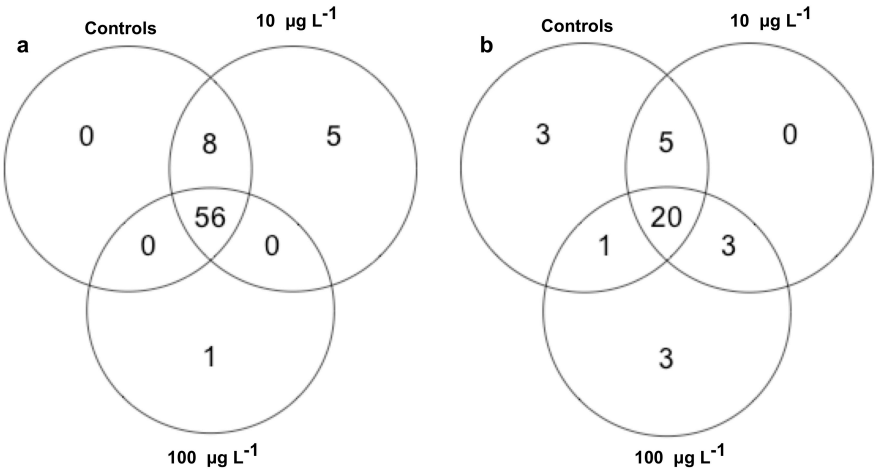

Figure 5.3. Venn diagrams showing the number of OTUs common and unique to each treatment at 45 days of biofilm development with AluI (a) and HhaI (b) tRFLP datasets.

The Venn diagrams in Figure 5.3 show the number of OTUs unique to each, and shared between, treatments at 45 days of biofilm development. These diagrams show that differences between the 10 and 100 μg L^{-1} treatments in the AluI dataset (Figure 5.3a) were largely due to the presence of 5 unique OTUs in the 10 μg L^{-1} treatment, while differences between the control and 100 μg L^{-1} treatments and the 10 and 100 μg L^{-1} treatments in the HhaI dataset were based on 5 OTUs common to the control and 10 μg L^{-1} treatments and 3 OTUs unique to the 100 μg L^{-1} treatment (Figure 5.3b). Overall, these diagrams highlight that the majority of OTUs were common to all three treatments after 30 days of ERT treatment (i.e., 80% in the AluI dataset and 57% in the HhaI dataset).

5.4. Discussion

Our results revealed both contrasting and consistent effects of ERT on bacterial community structure when using tRFLP datasets generated with AluI and HhaI restriction enzymes. Analysis of bacterial community structure using the AluI tRFLP dataset did not reveal effects that could be attributed to ERT at any time point. The only significant difference observed in community structure was between the 10 and 100 μg L^{-1} treatment after 30 days of ERT exposure, and there is thus no evidence to indicate that this was an ERT related effect (Table 5.5). Conversely, analysis using the HhaI tRFLP dataset indicated a significant effect of both ERT treatments after 9 days of exposure and of the 100 μg L^{-1} treatment after 30 days of exposure. Neither dataset provided evidence to suggest that a 30- day exposure to 10 μg L^{-1} ERT [nominal] would lead to significantly different stream biofilm bacterial communities (Table 5.5). Similarly, the only significant difference in the number of OTUs between treatments attributable to ERT was between the control and 100 μg L^{-1} treatment using the HhaI tRFLP dataset after 9 days of ERT exposure. Analysis of the abundance of 16S rRNA genes suggested that ERT did not have an effect on bacterial abundance. Collectively, these results provide no evidence to suggest that a 30-day exposure to ERT concentrations in the range of 10 μg L^{-1} (i.e., 7.3 ± 3.9 μg L^{-1}) would lead to changes in the bacterial community structure or overall bacterial abundance of stream biofilms, while they suggest that changes in bacterial community structure may occur at concentrations in the range of 100 μg L^{-1} (i.e., 87.2 ± 31.1 μg L^{-1}).

Kim et al. (2005) observed a decrease in erythromycin sensitive and an increase in erythromycin resistant gram-positive strains in stream sediments exposed to high concentrations (i.e., 20 mg L^{-1}) of erythromycin for 230 days, while effects on gram-negative strains were observed to be less pronounced. Although our data does not allow us to differentiate between effects on gram positive and gram-negative bacteria, the differences between treatments observed with the HhaI tRFLP dataset likely reflect - at least in part - changes in the presence/absence and abundance of gram-positive bacteria. The abundance of gram-positive bacteria in the benthos of stream ecosystems has been observed to be considerably lower than that of gram-negative bacteria across a range of environmental conditions (Gao et al. 2005). Consistent with this observation, our data shows that differences in bacterial

community structure between the 100 μg L^{-1} treatment and the control and 10 μg L^{-1} treatments after 30 days of ERT exposure were due to 5 OTUs common to the control and 10 μg L^{-1} treatments and 3 OTUs unique to the 100 μg L^{-1} treatment, all of which had low relative abundances and accounted for only 23% of the total number OTUs detected (Figure 5.3b). Our results are also in agreement with a recent study by Fan et al. (2009), who observed changes in the bacterial communities of experimental wastewater batch reactors exposed to 50 μg L^{-1} erythromycin-H_2O and 100 μg L^{-1} erythromycin.

The degradation rate of erythromycin in aqueous solutions is lowest at a pH of 7.3 and it increases faster towards acidic than towards alkaline conditions (Kim et al. 2004b), suggesting that the range of pH values throughout our experiment may have provided relatively stable conditions for ERT (Table 5.1). Other factors that influence the fate of ERT are microbial mineralization, the formation of clay complexes and organic matter content (Kim et al. 2004a; Kim et al. 2004c). Despite the observed loss of ERT vs. what would be expected of a conservative solute, the periodic injection of ERT into the microcosms helped maintain average concentrations of ERT within 27 and 13% of the nominal 10 and 100 μg L^{-1} treatments, respectively. This suggests that periodic releases of ERT during a 28 - day treatment period with Aquamycin® 100 under given physicochemical conditions may result in fairly stable ERT effluent concentrations.

Our results have limitations inherent to PCR based techniques of bacterial community analysis (Egert and Friedrich 2003; Blackwood et al. 2007). These limitations were addressed in part by using peak alignment and noise filtering methods that help remove PCR artefacts and provide an un-biased method to identify true T-RFs (Smith et al. 2005; Abdo et al. 2006). We additionally used two restriction enzymes for tRFLP, which helped increase the taxonomic resolution of our analysis. Therefore, although the patterns we describe may not represent minor members of the bacterial community, they do reflect true differences in the dominant bacterial taxa of stream biofilms. The use of recirculating model streams also allowed us to effectively isolate the effects of ERT while maintaining a good level of ecosystem representation, and therefore our results provide an environmentally meaningful assessment of the effects of ERT on the bacterial community structure of stream biofilms.

5.5. Conclusion

Assessing the environmental safety of antibiotics used in freshwater salmonid aquaculture is essential to ensure its sustainable expansion and prevent adverse effects on ecosystems and human health. Perhaps contrary to popular belief, freshwater salmonid aquaculture is not restricted to the world's temperate regions and has the potential to expand throughout a wide latitudinal range (Tello et al. 2010). Our results suggest that a 28-day exposure to ERT concentrations in the range of 10 μg L^{-1} is unlikely to affect the bacterial community structure of stream biofilms.

Chapter 6

Linking antibiotic pollution to antibiotic resistance: the use of MIC distributions to characterize selective pressures on bacteria

A shorter version of this chapter has been published in: Tello, A., Austin, B. and Telfer, T.C. 2012. Selective pressure of antibiotic pollution on bacteria of importance to publich health. Environmental health perspectives doi

A. Tello conceived and designed the study, performed the research and wrote the manuscript. B. Austin and T.C. Telfer had editorial input.

6.1. Introduction

Antibiotic pollution may favour the development and spread of antibiotic resistance (Martinez 2008). One of the difficulties of relating increased levels of resistance in the environment to antibiotic pollution, however, is the fact that antibiotic resistance genes can be co-released into the environment with antibiotic compounds (Kümmerer 2009b). The question then is whether an observed increase in resistance emerged as a result of the selective pressure of the antibiotic in the environment or if it emerged within the treated host.

Antibiotic resistance genes and antibiotic compounds are different pollutants that have different modes of action and are subject to different fate processes in the environment (Martinez 2009). They are also likely to respond differently to treatment processes designed to remove them from environmental compartments and from liquid and solid wastes (Pei et al. 2007). Estimating the relative contribution of pollution by antibiotic resistance genes and antibiotic compounds to increased levels of antibiotic resistance is important, as it may raise the effectiveness of counteractive measures.

Except for the dramatic concentrations of antibiotics and concurrent high levels of resistance found in streams receiving effluents from a drug production plant in India (Kristiansson et al. 2011), there is limited evidence as to whether environmental concentrations of antibiotics can enhance the development and spread of resistance in the environment (e.g., Knapp et al. 2008). Current guidelines on the environmental risk assessment (ERA) of medicinal products for human and veterinary use in the European Union, for example, do not explicitly address the effect of antibiotics on the prevalence of antibiotic resistance in the environment (EMEA 2006; EMEA 2008).

From an environmental health perspective, the selective pressure that antibiotic pollution may exert on bacteria of clinical importance is of particular concern. Several clinically relevant bacteria, such as *E. coli* and the enterococci, occur and are able to grow in different environments (Topp et al. 2003; Moriarty et al. 2008). In the presence of environmental concentrations of antibiotics, they may face a selective pressure leading to a gradual increase in the prevalence of resistance.

In this study we use bacterial species sensitivity distributions (SSDs) derived from a comprehensive set of minimum inhibitory concentration (MIC) distributions of

antibiotics to model bacterial sensitivities and characterize the selective pressure that antibiotic pollution may exert on bacteria of importance to public health in the environment. This is done under the premise that antibiotics will primarily increase the prevalence of resistance by favouring the selection of resistant phenotypes via the inhibition of sensitive ones. Although there is evidence to suggest that sub-inhibitory concentrations of antibiotics may indirectly favour resistance (Hoffman et al. 2005), the use of bacterial inhibition as an assessment endpoint provides a standardized response across taxa that can be directly linked to a selective pressure favouring an increase in the prevalence of resistance.

We derived SSDs for three antibiotics from publicly available MIC distributions and determined the fraction of inhibited bacterial taxa at antibiotic concentrations measured in different environments and used as action limits in ERA. To the best of our knowledge, this is the first study to put measured environmental concentrations of antibiotics in perspective with the antibiotic sensitivity of clinically relevant bacteria.

6.2. Methods

6.2.1. MIC distributions

MIC distributions for ciprofloxacin, erythromycin and tetracycline were obtained from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC and Zone diameter distribution website (http://www.srga.org/eucastwt/wt_eucast.ht; accessed November 2010; Kahlmeter et al. 2003). Distributions are based on data collated from more than 20,000 different worldwide sources and encompass the variability within species and between researchers, methods and geographical areas.

Ciprofloxacin, erythromycin and tetracycline were selected from among a list of approximately 150 compounds in the database for three reasons: 1) they represent 3 distinct classes of antibiotics of importance to human and veterinary medicine 2) the number of bacterial taxa represented in their MIC distributions was higher than in most of the other compounds in the database and 3) they have been measured in different environmental compartments.

6.2.2. Phylogenetic analysis

Bacteria represented in the EUCAST MIC distributions were placed in a wider phylogenetic context by conducting a brief phylogenetic analysis. 16S rRNA sequences from bacterial taxa represented in the MIC distributions were obtained from the All-Species Living Tree Project (LTP) (Yarza et al. 2008) March 2011 release and imported into the ARB software (Ludwig et al. 2004). Bacterial taxa represented in the MIC distributions of ciprofloxacin, erythromycin and tetracycline were selected to create a pooled 16S rRNA sequence alignment comprising the species represented in the MIC distributions of all three antibiotics. Some bacterial taxa were not represented in the LTP database, and therefore the alignment contained a subset of the taxa represented in each original MIC distribution (see Appendix, Table A1). This alignment along with the entire LTP alignment for the Domain Bacteria was imported into the R package ape v.2.6-2 (Paradis and Strimmer 2004), in which an evolutionary distance matrix was calculated for each alignment using Kimura's two-parameter substitution model (Kimura 1980). An unnrooted phylogenetic dendrogram was estimated from the evolutionary distance matrix of the pooled antibiotic alignment using the neighbour-joining method (Saitou and Nei 1987) and confidence was assessed by bootstrapping with 1,000 permutations. This tree was placed in the context of the entire LTP phylogeny by highlighting its corresponding branches in an unrooted, neighbour-joining tree of the entire LTP 16S rRNA alignment.

Mantel tests (Mantel 1967) were used to explore the correlation between evolutionary distances and pairwise differences in median MICs between taxa for each antibiotic dataset (i.e., do differences in antibiotic sensitivity increase with evolutionary distance?). Correlations were calculated excluding and including data beyond the wild-type cut-off value (CO_{WT}), which separates microorganisms with (i.e., non wild – type) and without (i.e., wild – type) acquired resistance mechanisms (Kahlmeter et al. 2003). The correlation structure of the data was then further explored using Mantel correlograms (Legendre and Legendre, 1998). Mantel tests and correlograms were performed with the R package *ecodist* v.1.2.3 (Goslee and Urban 2007) with 5000 permutations. Table A1 in the Appendix lists the bacterial taxa represented in the MIC and 16S rRNA datasets of each antibiotic.

6.2.3. Bacterial SSDs

6.2.3.1. Endpoint selection

SSDs were derived using the median MIC (MIC_{50}) and the no observed effect MIC (NOEC) of each taxon. MIC tests are performed using double-dilution steps of antibiotic concentrations and the data they generate is interval-censored. Therefore, the conservative MIC_{50} of each species was taken to be the antibiotic concentration immediately below the observed 50^{th} percentile. The NOEC was taken to be the antibiotic concentration immediately below the lowest MIC observed in each taxon. MIC_{50} and NOEC values were aggregated within cogeneric species by taking the arithmetic mean to minimize the lack of independence between individual observations (see Figure 6.4). Only pooled MIC_{50} and NOEC values from genera for which there was evidence to suggest that under certain conditions they could grow in an environmental compartment (e.g., soil, sewage, freshwater, etc.) were used to derive SSDs (see Table 1).

6.2.3.2. Linking endpoints to resistance

The MIC_{50} was calculated including data beyond the CO_{WT}. A comparison of MIC_{50} values with the wild-type MIC range of species in the EUCAST distributions indicates that it is an adequate estimate of the wild-type MIC (i.e., it falls within the wild-type MIC range in those species that have one), except for a few cases in which it falls above the wild-type MIC range. Concentrations of antibiotics $\geq$ the MIC_{50} are therefore likely to inhibit approximately half of the wild-type population. Assuming equal growth rates of wild-type and resistant populations, this causes an increase in the prevalence of resistance in the remaining active populations (Figure 6.1). Thus we assume that the prevalence of resistance may increase to less than 100% due to the inhibition of a fraction of the wild-type population. The NOEC, on the other hand, represents a minority of isolates across taxa whose MIC are sometimes below the MIC range representative of the wild-type population, and it was used as a means to assess the lower limit of antibiotic sensitivity represented in the MIC distributions.

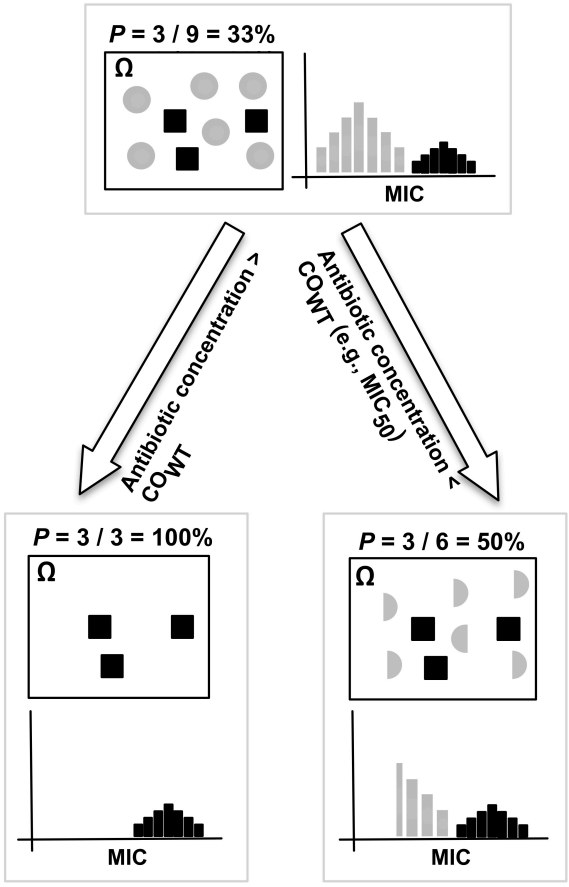

Figure 6.1. Conceptual link between the MIC_{50} and antibiotic concentrations above the CO_{WT} with resistance prevalence (*P)* in a universe (Ω) including resistant (black) and wild-type (gray) populations, and its relation to the MIC distribution. Antibiotic concentrations greater than the CO_{WT} will completely inhibit wild-types and resistance prevalence in the active (i.e., growing) population will be 100%. Concentrations of antibiotics ≤ the CO_{WT} , such as the MIC_{50} , will inhibit a fraction of the wild-type population (e.g., 50%). Assuming equal growth rates of wild-type and resistant populations, this will increase antibiotic prevalence in the active population.

6.2.3.3. Bootstrap regression

SSDs were derived by bootstrap regression (Grist et al. 2002). The MIC_{50} and NOEC vectors of each antibiotic were resampled 5,000 times. To each of these bootstrap resamples, a log-logistic model was fitted by maximum likelihood estimation of the distribution parameters and direct optimization of the log-likelihood function with the Nelder and Mead (1965) method using the R package

fitdistrplus v.0.1-4. The distribution parameters α (i.e., location) and β (i.e., scale) from each fitted curve were used to get 5,000 replicate estimates of antibiotic concentrations associated to a potentially affected fraction (PAF) between percentiles 0.01 and 0.99 at 0.01 step intervals. From these, the bootstrap estimate and 95% bootstrap confidence intervals were calculated. An R script to replicate the analysis is included in the Appendix.

We determined the PAF of bacterial genera by all three antibiotics at the aquatic and soil VICH phase I action limits (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products [VICH] 2000) and at MECs reported in the literature. This was complemented with a direct comparison of MECs and VICH action limits with the CO_{WT} of species represented in the MIC distributions of each antibiotic. Although ciprofloxacin is not approved for use in veterinary medicine, it is the major active metabolite of enrofloxacin in different species (Idowu et al. 2010). In the absence of data for enrofloxacin, it was used as a representative of the fluorquinolones when comparing PAFs with VICH phase I action limits. MECs of ciprofloxacin, erythromycin and tetracycline were obtained from Kolpin et al. (2002), Golet et al. (2002), from the data collated by Hamscher (2006) and from Luo et al. (2011). Antibiotic concentrations are expressed as ppb to facilitate analysis and comparisons.

6.3. Results

6.3.1. Phylogenetic and environmental overview of MIC distributions

Seventy-nine species from the ciprofloxacin, erythromycin and tetracycline MIC distributions were represented in the LTP 16S rRNA database (Figure 6.2) (see Appendix, Table 1). Major bacterial groups in Figure 6.2 – appearing in order from top to bottom in the dendrogram – include staphylococci, enteroccocci, streptococci, a few representatives of the Actinobacteria (e.g., Clostridium spp. and Mycobacterium spp.), Bacteroides, pseudomonads (e.g., Pseudomonas spp. and Burkholderia spp.) and the enterics (e.g., Escherichia spp., Enterobacter spp., Proteus spp., Klebsiella spp.). The range of evolutionary distances covered by these species spans the range of evolutionary distances represented in the entire LTP database, highlighting it as a rather diverse phylogenetic group (Figure 6.3).

Bacterial genera used to derive the SSD of each antibiotic represent commensal and pathogenic bacteria that occur – and may grow – to a larger or lesser extent in the environment (Table 1). Among the 27 genera included in the SSDs are some known to be widely distributed in the environment, such as Pseudomonas, Acinetobacter, Burkholderia and Chryseobacterium (Vandamme et al. 1994; Madigan et al. 2009), as well as others for which growth in the environment has either been reported or for which there is evidence to suggest that under certain conditions it is likely to occur. Hendricks (1972), for eample, showed that some *Enterobacteriaceae* could grow in water collected downstream of a municipal sewage facility at temperatures as low as 5°C. Gibbs et al. (1997) reported years later the re-growth of faecal coliforms and *Salmonella* in biosolids and soil ammended with biosolids. The populations of *E. coli* in manured soils can be very dynamic (Topp et al. 2003) and Inglis et al. (2010) recently showed that *Campylobacter* can persist for long periods of time in compost, which may suggest cryptic growth. Enterococci have been shown to grow in municipal oxidation ponds (Moriarty et al. 2008), and the facultative intracellular pathogen *Listeria monocytogenes* has been shown to be widespread in certain catchments (Lyautey et al. 2007) and able to grow in soil suspensions characteristic of certain organic fractions (Sidorenko et al. 2006). Staphylococci, including *Staphylococcus aureus*, have been isolated from marine water samples (Gunn and Colwell 1983), and methicillin – resistant *Staphylococcus aureus* was recently isolated from marine water and intertidal sand from beaches on the west coast of the USA (Soge et al. 2009). *S. aureus* has also been shown to be capable of growth in sterile soil (Liang et al. 1982), suggesting that it might be possible for it to grow in this environment under conditions of low competition.

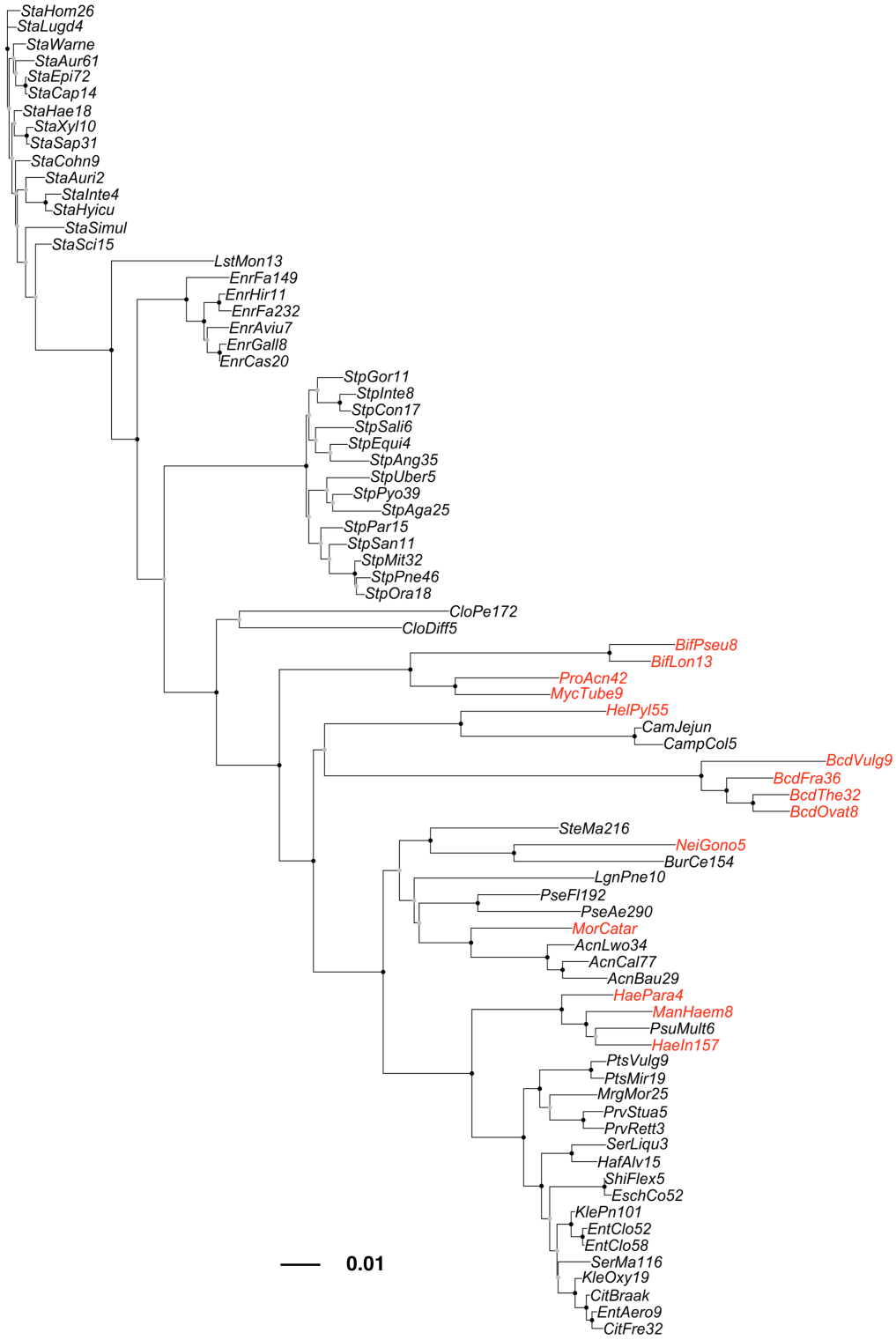

Figure 6.2. Unrooted neighbour-joining dendrogram of species represented in the pooled 16S rRNA alignment of ciprofloxacin, erythromycin and tetracycline. Species highlighted in red were not included in the species sensitivity distributions due to lack of evidence of growth in the environment. Scale bar units are number of nucleotide substitutions per site. Black nodes: ≥ 70% bootstrap support. Gray nodes: < 70% bootstrap support.

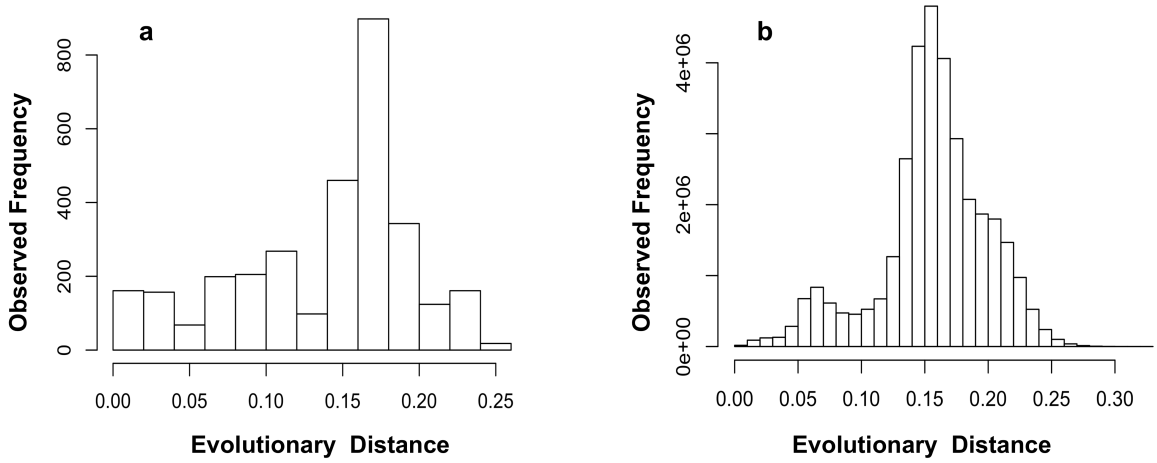

Figure 6.3. Histograms showing the range of pairwise evolutionary distances covered by (a) the pooled 16S rRNA alignment of species represented in the MIC distributions of all three antibiotics and (b) the entire LTP 16S rRNA alignment for the Domain *Bacteria*. Pairwise evolutionary distances in Figure 6.3a were calculated from the same 16S rRNA alignment used to make the dendrogram in Figure 6.2.

Ayyadurai et al. (2008) recently demonstrated that *Yersinia pestis* remained viable and fully virulent in humid sand for 40 weeks. *Clostridium difficile* is widely distributed in the environment (Al Saif and Brazier 1996), and it is not unreasonable to speculate that there may be niches that could support its sporulation. Collectively, these studies highlight there is a potential for growth in bacteria of clinical relevance in different environments and under varying biological and physicochemical conditions, even if only during a short temporal window. In the presence of antibiotics, wild-type populations may be inhibited to various extents, increasing the prevalence of resistance.

Table 6.1. Bacterial genera included in the SSD of each antibiotic. (+) and (-) indicate presence or absence of the genera in each dataset, respectively.

	Genus	Cipro	Eryth	Tetra	**Reference**
1	*Acinetobacter*	+	-	+	Madigan et al. 2009
2	*Alcaligenes*	+	-	-	Agerso et al. 2005; Madigan et al. 2009
3	*Burkholderia*	+	-	-	Madigan et al. 2009
4	*Campylobacter*	+	+	+	Brandl et al. 2004; Inglis et al. 2010
5	*Chryseobacterium*	+	-	-	Vandamme et al. 1994
6	*Citrobacter*	+	-	+	Madigan et al. 2009; Kitts et al. 1994
7	*Clostridium*	-	+	+	Al Saif and Brazier 1996; Madigan et al. 2009
8	*Enterobacter*	+	-	+	Hendricks 1972; Rattray et al. 1995; Hernandez et al. 1998
9	*Enterococcus*	+	+	+	Mundt 1961/1963; Desmarais 2002; Moriarty et al. 2008
10	*Escherichia*	+	-	+	Hendricks 1972; Gibbs et al. 1997; Topp et al. 2003; Zaleski et al. 2005
11	*Hafnia*	+	-	+	Janda 2006a
12	*Klebsiella*	+	-	+	Liang et al. 1982; Zadoks et al. 2011 (and references therein)
13	*Kluyvera*	+	-	+	Janda 2006b
14	*Legionella*	+	+	-	Fliermans et al. 1981
15	*Listeria*	+	-	+	Sidorenko et al. 2006; Lyautey et al. 2007
16	*Morganella*	+	-	+	Kitts et al. 1994
17	*Pasteurella*	+	+	+	Bredy and Botzler 1989
18	*Proteus*	+	-	+	Hendricks 1972
19	*Providencia*	+	-	-	Kitts et al. 1994
20	*Pseudomonas*	+	-	+	Madigan et al. 2009
21	*Raoultella*	+	-	+	Zadoks et al. 2011 (and references therein)
22	*Salmonella*	+	-	+	Liang et al. 1982; Gibbs et al. 1997
23	*Serratia*	+	-	+	Madigan et al. 2009
24	*Staphylococcus*	+	+	+	Liang et al. 1982; Gunn and Colwell 1983; Soge et al. 2009
25	*Stenotrophomonas*	+	-	+	Bollet et al. 1995
26	*Streptococcus*	+	+	+	Gledhill and Casida 1969
27	*Yersinia*	+	-	+	Sidorenko et al. 2006; Ayyadurai et al. 2008
	Total	**26**	**7**	**22**	

6.3.2. Antibiotic sensitivity and evolutionary distance

An implicit assumption in the use of SSDs is that taxa used to derive them represent independent observations drawn from a random distribution (Suter et al. 2002). In higher organisms, this assumption is rarely met because the response to chemical stressors between related taxa tends to be correlated due to the presence of conserved physiological processes and sites of toxic action (e.g., Fletcher et al.

1990). To minimize this lack of independence, several methods and researchers recommend combining the endpoints from co – generic species (see Sutter et al. 2002). In MIC datasets, however, it is questionable whether such a correlation between bacterial taxa might consistently hold, and determining whether it does in any given dataset is important to adopt measures to minimize the lack of independence between observations.

Two factors that are likely to introduce substantial variability in the antibiotic sensitivities within and between bacterial species – and hint at the possibility that, in some cases, MIC_{50} values between closely related species might indeed be independent – are the species definition of bacterial taxa and lateral gene transfer (LGT). The species definition for bacteria is much broader than for higher plants and animals (Staley, 1997) and genomics has shown that current bacterial species include strains which are "perhaps not homogeneous enough for species to be predictive of the phenotype and ecological niche of the strains they encompass" (Konstantinidis and Tiedje 2005). Lateral gene transfer, on the other hand, uncouples the evolution of phenotype from the evolution of the majority of the genome (Doolittle and Papke 2006) and can dramatically alter the antibiotic sensitivity of a bacterial strain when the genetic element being transferred confers resistance to one or more antibiotics.

To explore this in some detail we used Mantel test's to assess the correlation between evolutionary distance and pairwise differences in MIC_{50} values in our datasets, and Mantel correlograms to get a closer look at the correlation structure of the data. Figure 6.4 shows Mantel correlograms for each antibiotic including and excluding data beyond the CO_{WT}, along with the Mantel correlation coefficient for the entire dataset in the upper left hand side of each graph (i.e., $\mathbf{R_M}$). Mantel correlations between pairwise differences in MIC_{50} and evolutionary distances were positively and significantly correlated in the ciprofloxacin and erythromycin datasets when excluding data beyond the CO_{WT}, and in the ciprofloxacin datasets when including data beyond the CO_{WT}. Correlograms in Figure 6.4 describe the difference in MIC_{50} values between bacterial species at a series of evolutionary distances, with positive and negative points indicating small and large differences in MIC_{50} values, respectively. These correlograms show positive autocorrelation at small evolutionary distances and negative autocorrelation at large evolutionary distances as the main correlation structure in the data. This is apparent in the

correlogram for ciprofloxacin including and excluding data beyond the CO_{WT} (Figure 6.4a and 6.4d). This pattern indicates that, in this particular dataset, differences in the median anitibiotic sensitivity of phylogenetically close bacterial species is lower than would be expected by chance, whereas differences between more distant species are higher than would be expected by chance. The presence of significant large and small differences in MIC_{50} values at an evolutionary distance of approximately 0.12 in Figure 6.4c stresses that bacterial species can diverge or converge in their median antibiotic sensitivity at evolutionary distances that are not consistent with the main pattern in the data.

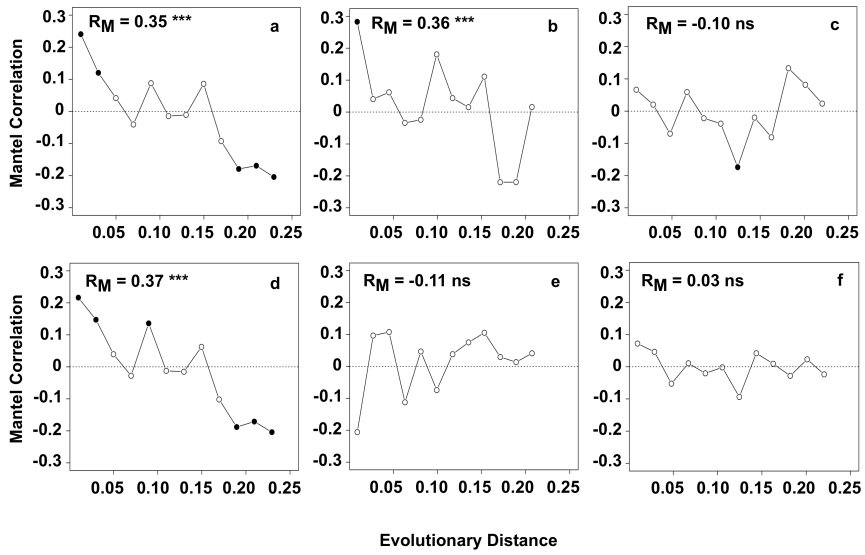

Figure 6.4. Mantel correlograms descrbing the correlation between MIC_{50} and evolutionary distance for ciprofloxacin, erythromycin and tetracycline excluding (a b c) and including (d e f) data beyond the CO_{WT}. Solid dots represent a significant correlation at the corresponding evolutionary distance ($\alpha = 0.05$). Significance tests are based on 5000 permutations.

The inclusion of data beyond the CO_{WT} had little impact on the overall Mantel correlation and correlation structure of ciprofloxacin and tetracycline, but had a pronounced effect on the erythromycin dataset. In erythromycin, data beyond the CO_{WT} comprised, on average, 25% of the observations (i.e., data points) for each species in the dataset, compared to 10 and 17% in the ciprofloxacin and tetracycline datasets, repectively. This effectively shifted the MIC_{50} vector of erythromycin – whose mean difference with the MIC_{50} vector excluding CO_{WT} data was an order of

magnitude higher than in the other datasets – and affected the correlation structure of the data by increasing the difference in antibiotic sensitivity between closely related species (Figure 6.4b vs 6.4e).

Collectively, these observations indicate that the correlation structure of MIC data may vary between datasets and that it may be affected by the inclusion of data from resistant phenotypes (i.e., data beyond the CO_{WT}) even when using robust measures such as the MIC_{50} as an endpoint. These results demonstrate that the independence assumption implicit in the derivation of SSDs may be met by certain MIC datasets, and therefore that a – priori grouping of endpoints from co – generic species is not justified. Given that we decided to include data beyond the CO_{WT} to derive SSDs, these results would recommend grouping data from co – generic species in the ciprofloxacin datasets to minimize the lack of independence, whereas there does not seem to be a statistical ground for doing so in the erythromycin and tetracycline datasets. In this study we decided to group co-generic species in all datasets in order to apply a consistent methodology in the derivation of SSDs for all three antibiotics that would facilitate the subsequent interpretation and discussion of results.

6.3.3. Inhibitory effects at environmental concentrations

The log-logistic model had a good fit to the NOEC and MIC_{50} vectors, explaining 90% or more of the variance in the original data (Figure 6.5). Table 2 shows that the PAF of bacterial genera at MECs of ciprofloxacin, erythromycin and tetracycline in water environments – including surface water, STP effluents, raw sewage and groundwater – is low, with upper 95% confidence intervals of 7% and 3.2% in raw sewage for ciprofloxacin using the NOEC and MIC_{50} SSD, respectively. At the very low range of concentrations measured in surface waters and STP effluents, the practical difference between PAF estimates using the NOEC and MIC_{50} SSDs is minimal. In erythromycin, the NOEC and MIC_{50} SSDs overlap at the very lower tail of the distributions, causing the MIC_{50} SSD to estimate a slightly higher PAF than the NOEC SSD. Given that the PAFs of both SSDs are well below 1% at this range of concentrations, this discrepancy was not significant for our assessment of effects.

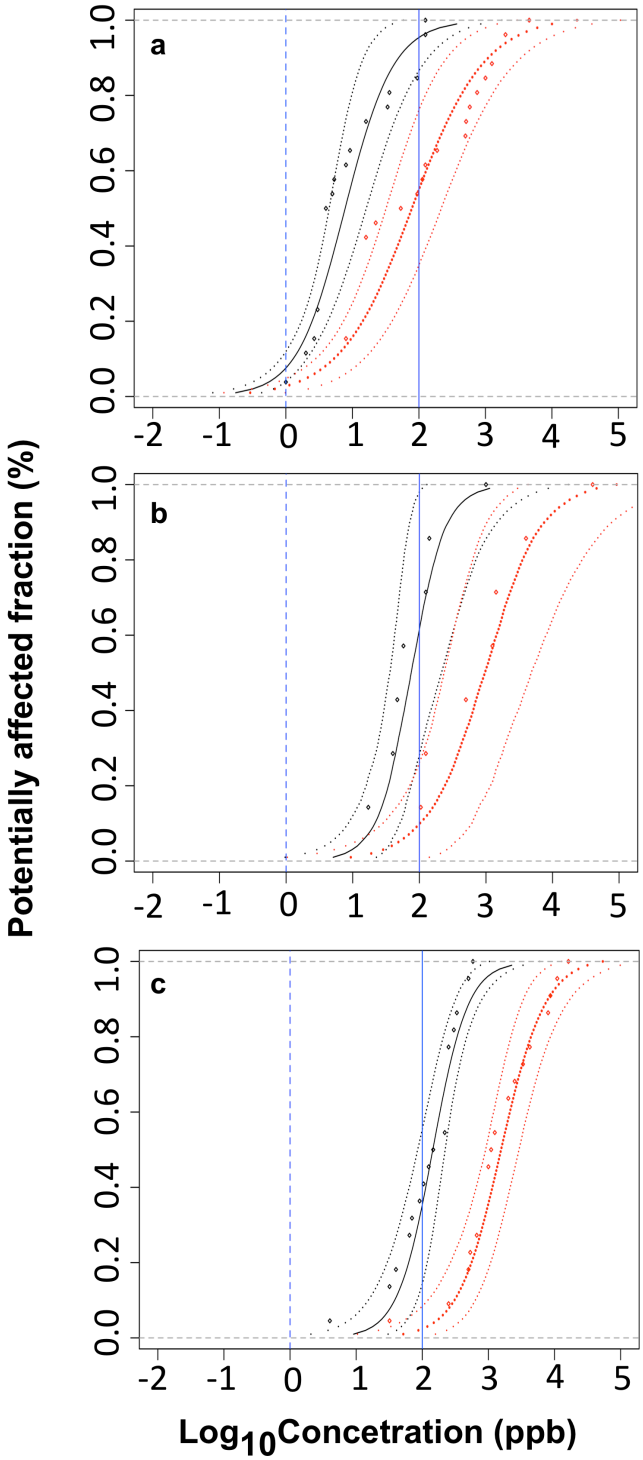

Figure 6.5. Species sensitivity distributions derived for the NOEC (black curve) and MIC_{50} (red curve) with overlayed empirical cumulative distributions (dots). Fitted curves represent the bootstrap estimate and 95% bootstrap confidence intervals for the log-logistic model. Solid and dashed blue vertical lines represent the VICH phase I soil and aquatic action limits, respectively. (a) Ciprofloxacin: NOEC (R^2= 0.90; p<0.0001), MIC_{50} (R^2= 0.91;

$p<0.0001$). Bootstrap estimate of model parameters: NOEC species sensitivity distribution (α= -2.1 , β= 0.37); MIC_{50} species sensitivity distribution (α= -1.0 , β=0.54). (b) Erythromycin: NOEC (R^2= 0.96; $p<0.001$), MIC_{50} (R^2= 0.97; $p<0.0001$). Bootstrap estimate of model parameters: NOEC species sensitivity distribution (α= -1.13 , β= 0.29); MIC_{50} species sensitivity distribution (α= -0.014 , β=0.48). (c) Tetracycline: NOEC (R^2= 0.97; $p<0.0001$), MIC_{50} (R^2= 0.98; $p<0.0001$). Bootstrap estimate of model parameters: NOEC species sensitivity distribution (α= -0.84 , β= 0.27); MIC_{50} species sensitivity distribution (α= 0.2 , β=0.33). The potentially affected fraction of bacterial genera at a given antibiotic concentration is read from the y-axis at the point in which the antibiotic concentration intersects with the species sensitivity distribution. For example, a concentration of 100 ppb (i.e., Log_{10}Concentration = 2) of ciprofloxacin inhibits approximately half of the wild-type population (i.e., red MIC_{50} curve) in 54% of the bacterial genera and at least some individuals (i.e., black NOEC curve) in 95% of the bacterial genera.

PAFs in river sediments, swine faeces lagoons, liquid manure and farmed soil are markedly higher than for aquatic compartments (Table 2). Concentrations of ciprofloxacin, erythromycin and tetracycline measured in river sediments are $\geq$ the MIC_{50} of up to 60%, 7.6% and 6% of the bacterial genera, respectively (i.e., upper 95% confidence intervals in Table 2). Concentrations of these three antibiotics in the sediments of a swine faeces lagoon are estimated to be $\geq$ the MIC_{50} of up to 92%, 21% and 56% of the bacterial genera. The extremely high concentration of tetracyline in liquid manure reported by Hamscher et al. (2006) is $\geq$ the MIC_{50} of practically 100% of the bacterial genera. The high concentration of tetracycline measured in farmed soil, on the other hand, is $\geq$ the MIC_{50} of up to 30% of genera. It is also estimated to inhibit at least some isolates in up to 90% of the bacterial genera (i.e., NOEC SSD).

Table 6.2. PAF for each antibiotic at MECs and ERA action limits using the NOEC and MIC_{50} SSDs.

Antibiotic	Environment	Concentration (ppb)	PAF (95% Confidence Interval)	
			NOEC SSD	MIC_{50} SSD
Ciprofloxacin	surface water	0.03 [a]	0.1 (0.008-0.4)	0.2 (0.03-0.4)
		0.36 [b]	2.3 (0.7-4.3)	1.2 (0.4-2.3)
	river sediments	48 [b]	89 (75-99)	40 (23-60)
	STP effluent	0.37 [b]	2.4 (0.7-4.4)	1.2 (0.4-2.3)
		0.062 [c]	0.3 (0.03-0.8)	0.3 (0.1-0.7)
		0.11 [c]	0.6 (0.08-1.3)	0.4 (0.1-1)
	raw sewage	0.313 [c]	2 (0.5-3.8)	1.1 (0.4-2)
		0.568 [c]	4 (1.6-7)	1.7 (0.6-3.2)
	swine faeces lagoon sediment	340 [b]	99 (95-100)	76 (59-92)
	VICH phase I limit (aquatic)	1	8 (4-12)	2.7 (1-4.7)
	VICH phase I limit (terrestrial)	100	95 (86-100)	54 (36-76)
Erythromycin	Surface water	0.024 [b]	0.0001 (0-0.02)	0.003 (0-0.09)
	river sediments	19 [b]	8.6 (0.5-23)	2 (0.03-7.6)
	STP effluent	0.07 [b]	0.0008 (0-0.07)	0.008 (0-0.2)
	swine faeces lagoon sediment	80 [b]	53 (21-92)	7.9 (0.4-21)
	VICH phase I limit (aquatic)	1	0.07 (0-1)	0.1 (0-1)
	VICH phase I limit (terrestrial)	100	62 (27-97)	9.7 (0.5-25)
Tetracycline	surface water	0.11 [a]	0.0006 (0-0.03)	0.0003 (0-0.01)
		0.42 [b]	0.006 (0-0.2)	0.002 (0-0.04)
	river sediments	73 [b]	24 (8-44)	1.6 (0.3-6)
	STP effluent	0.16 [d]	0.001 (0-0.05)	0.0005 (0-0.02)
		0.98 [d]	0.02 (0-0.4)	0.005 (0-0.09)
		0.09 [b]	0.0004 (0-0.02)	0.0002 (0-0.009)
	swine faeces lagoon sediment	1100 [b]	97 (92-99)	38 (22-56)
	liquid manure	66000 [d]	100 (100-100)	99 (97-100)
	farmed soil	443 [d]	86 (77-94)	15 (6.6-30)
	Groundwater	0.13 [d]	0.0008 (0-0.04)	0.0004 (0-0.01)
	VICH phase I limit (aquatic)	1	0.02 (0-0.4)	0.005 (0-0.09)
	VICH phase I limit (terrestrial)	100	35 (14-55)	2.4 (0.5-8.1)

[a]Occurrence data from Kolpin et al. (2002) (data are maximum measured concentrations).

[b]Occurrence data from Luo et al. (2011). Concentrations > limit of detection were averaged over sampling stations for sites 'Tributaries Water, Dec 2009', 'Tributaries Sediment Dec 2009', 'Pollution source water Dec 2009 S1 and S2' and 'Source Sediments Dec 2009 S3 and S4' from Table S6 of the article's supporting information. Surface water concentrations and river sediment concentrations are from corresponding sampling sites.

[c] Occurrence data from Golet et al. (2002) (data are mean measured concentrations).

[d] Occurrence data collated by Hamscher et al. (2006) (data are maximum measured concentrations).

We note that some environments, such as soil and sediments, are likely to contain more bacterial genera of clinical relevance than others. Thus, for example, a PAF of 30% for tetracycline in farmed soil may inhibit more bacterial genera than a PAF of 100% in liquid manure - whose bacterial composition will tend to reflect the microflora of the animal from which it is derived - and therefore have larger public health implications.

Figure 6.6 compares MICs ≥ the CO_{WT} of bacterial taxa represented in the MIC distribution of each antibiotic to MECs. Concentrations greater than the CO_{WT} of a given bacterial taxa completely inhibit the wild-type population, increasing the prevalence of resistance in the remaining active population to 100%. The MEC of ciprofloxacin in swine faeces lagoon sediment is above the CO_{WT} of 14 bacterial taxa belonging to 8 genera of predominantly enteric bacteria (Figure 6.6a). The concentration of tetracycline measured in liquid manure is above the CO_{WT} of all but one bacterial taxa and the concentration measured in swine faeces lagoon sediment is borderline with the CO_{WT} of Staphylococcus and Streptococcus (Figure 6.6c). MECs of erythromycin are below the CO_{WT} of all taxa (Figure 6.6b).

6.3.4. Inhibitory effects at VICH phase I action limits

PAFs at the VICH phase I aquatic action limit suggest that it is protective of major inhibitory effects on bacteria by all three antibiotics (Figure 6.5), although a minority of sensitive individuals could be inhibited in up to 12% of genera (i.e., upper 95% confidence interval in ciprofloxacin NOEC SSD) (Table 2). The 1 ppb aquatic action limit refers to an environmental introduction concentration (i.e., concentration in an effluent), and therefore exposure concentrations in receiving water bodies are further reduced by dilution.

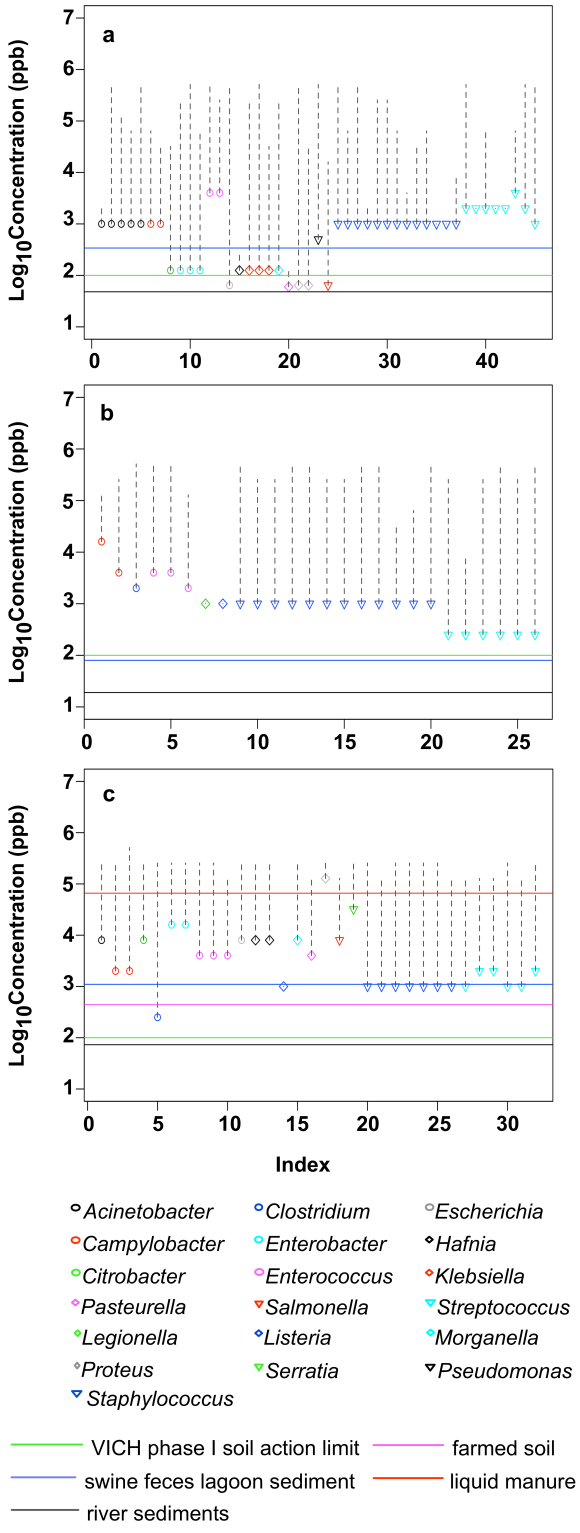

Figure 6.6. MICs ≥ the CO_{WT} for bacterial taxa in the MIC distributions of ciprofloxacin (a), erythromycin (b) and tetracycline (c). Colored symbols represent the CO_{WT} in different genera. Dashed vertical lines extend up to the maximum MIC beyond the CO_{WT}. Colored horizontal lines represent antibiotic concentrations as defined in the legend. The x-axis is an index representing the number of bacterial taxa in each MIC distribution. The y-axis indicates antibiotic concentrations.

Concentrations of ciprofloxacin and erythromycin at the VICH phase I soil action limit, on the other hand, are estimated to be ≥ the MIC_{50} of 76% and 25% of bacterial genera, respectively (Figure 6.5; Table 2). Figure 6.5 shows that the VICH phase I soil action limit is below the erythromycin and tetracycline CO_{WT} of all species, indicating that at this concentration these antibiotics are not expected to inhibit 100% of the wild-type population in any species. Conversely, the ciprofloxacin MIC distributions (Figure 6.6a) show that the 100 ppb soil action limit is above the CO_{WT} of 5 bacterial taxa and borderline with that of 9 other taxa.

It is also illustrative to consider these action limits in relation to the empirical MIC_{50} of individual species. The MIC_{50} for ciprofloxacin, for example, is 8 ppb in E. coli (n = 17,877), 8 ppb in E. cloacae (n = 2,354), 64 ppb in A. lwoffi (n = 262), 125 ppb in P. aeruginosa (n = 27,387) and 125 ppb in C. coli (n = 2,532). The MIC_{50} for erythromycin is 32 ppb in S. pneumoniae (n = 40,452) and 125 ppb in Sta. aureus (n = 36,038). The MIC_{50} for tetracycline is 32 ppb in C. difficile (n = 832) and 125 ppb in Str. pneumoniae (n = 13,813). These MIC_{50} are between 12.5 times lower to slightly higher than the 100 ppb soil action limit. Several species also have NOECs that are orders magnitude lower than the 100 ppb soil threshold.

6.4. Discussion

This study shows that environmental concentrations of antibiotics and concentrations representing action limits used in the ERA of veterinary medicines may be high enough to inhibit growth in bacteria of clinical importance that occur in different environments. By completely or partially inhibiting the growth of wild-type populations, antibiotics cause a selective pressure that will increase the prevalence of resistance. The PAFs for ciprofloxacin, erythromycin and tetracycline at MECs in river sediments, swine faeces lagoon sediments, liquid manure and farmed soil suggest that these environments are likely to be hot spots for the selection of resistance. In this regard, the comparison of MECs of ciprofloxacin and tetracycline with their respective CO_{WT} in different bacteria is striking (Figure 6.6), as it shows that wild-type populations of certain species are completely inhibited at these concentrations in-vitro. In swine faeces lagoons, liquid manure and soil amended with manure, concentrations of certain antibiotics can build up to levels that may act to extend the antibiotic selective pressure that started for some bacteria

within their treated hosts and exert a new selective pressure on other bacteria altogether. Interestingly, a remarkable study by Knapp et al. (2010) recently found a significant increase in tetracycline resistance genes in soil from the 1970s to the present that mimicked the use of tetracylines in agriculture in the Netherlands. Although studies have generally failed to find a significant effect of tetracyclines on resistance levels in soil (e.g., Agersø et al. 2006), some evidence suggests it could have contributed to the persistence and prevalence of resistance genes (Schmitt et al. 2006). Our results indicate that tetracycline concentrations in soil may build up to levels high enough to exert a significant selective pressure on clinically relevant bacteria.

The extrapolation of MIC data to the field has inherent limitations that must be considered in the interpretation of our results. Physicochemical and biological conditions of MIC tests, for example, are not representative of those generally encountered by bacteria in the environment. MIC tests are also acute tests, whereas the exposure to antibiotics in the environment is mainly chronic and will exert a constant selective pressure over extended periods of time (Sarmah et al. 2006; Kümmerer 2009a). Chronic exposure provides a longer temporal window for the selective enrichment of resistance and will favor stepwise transitions from low-level to high-level, clinical resistance. In this study we did not formally address the bioavailability of antibiotics in the environment. However, tetracyclines and macrolides have been shown to retain their bioactivity and inhibit bacterial growth even when tightly adsorbed by clay particles (Chander et al. 2005), and there is evidence to suggest that fluoroquinolones retain part of their activity when sorbed to solids (Córdova-Kreylo and Scow 2007). In the environment, bacteria are also likely exposed to multiple antibiotics and other substances such as metals and disinfectants, which will affect the selection of resistance. Synergistic and/or antagonistic interactions between combinations of antibiotics, for example, may significantly influence the evolution of resistance (Michel et al. 2008). Because many antibiotic resistance genes are associated to mobile genetic elements carrying multiple antibiotic resistance genes and genes conferring resistance to heavy metals and/or disinfectants (Chopra and Roberts 2001; Ciric et al. 2011), any of these factors may select for multi-drug resistance. An example relevant to this study is the broad host range transposon Tn1545, which encodes resistance to tetracycline, erythromycin and kanamycin (Clewell et al. 1995). In all, these factors emphasize

the complexity of relating antibiotic pollution to the prevalence of antibiotic resistance in the environment, and inevitably introduce a degree of uncertainty in our results. Despite these limitations, however, our results provide a means to grasp the potential effect of antibiotic pollution on the prevalence of resistance in clinically relevant bacteria in the environment by putting MECs in perspective with bacterial sensitivities.

The link between the MIC_{50} and resistance is based on the assumption that antibiotic concentrations ≤ the CO_{WT} may increase the prevalence of resistance to less than 100% by inhibiting a fraction of the wild-type population. Variation within the wild-type part of MIC distributions is normally in the order of 3-5 $\log_2$ MIC steps (Schön et al. 2009). While part of this variation reflects inherent variation in antibiotic sensitivity and other biological traits that influence the MIC, part of it also reflects method variability. In environmental compartments such as those discussed in this study, it is reasonable to expect some degree of inherent variation in the antibiotic sensitivity of wild-type populations. Environments such as sewage, river sediments and agricultural soil act as transient or permanent sinks in which wild-type populations of the same species, from different sources and with slightly differing sensitivities may physically converge. These environments are also likely to have micro gradients of physicochemical variables such as pH and nutrients, which are known to affect the MIC of bacteria in vitro (Bonfiglio and Livermore 1991; Butler et al. 2001). Collectively, these factors may provide enough variation in antibiotic sensitivity to enable the differential inhibition of wild-type populations under equal MECs of antibiotics.

The VICH phase I guidance document informs the ERA of veterinary medicines and has been implemented in the regulatory scheme in the EU, USA, Japan and Australia (de Knecht et al. 2009). Under VICH phase I guidance, the ERA of a veterinary medicine - except for parasiticides - stops if its environmental introduction concentration into the aquatic environment is < 1 ppb (i.e., aquatic action limit). For terrestrial environments, the ERA stops if the predicted environmental concentration in soil is < 100 ppb (i.e., soil action limit). Our results suggest that the VICH Phase I soil action limit for veterinary medicines is not protective of background antibiotic resistance levels. Concentrations below 100 ppb of certain antibiotics may inhibit a significant fraction of clinically relevant bacteria in the environment (Figure 5); the high PAFs for erythromycin and ciprofloxacin at

the 100 ppb soil threshold are a clear example of this. In a critique of action limits, Montforts (2005) used MIC data from 13 soil microorganisms and 22 antimicrobials to construct a substance/species sensitivity distribution, based upon which he determined that the aquatic and soil action limits should be set at 4×10^{-4} and 1 ppb if they were to be protective for all compounds. Similarly, our results suggest that VICH phase I action limits leave a rather ample margin for antibiotics to exert a selective pressure on bacteria of clinical importance in the environment.

Current knowledge on the presence and mechanisms of resistance in bacteria of clinical and environmental origin clearly indicate that the resistome of pathogens is and will continue to be inevitably linked with the environment (Martínez 2008; Wright 2010). Moreover, as this study shows, the prevalence of resistance in bacteria of importance to public health has the potential to be increased by antibiotic pollution in the environment. It therefore seems that the explicit consideration of resistance – or a proxy thereof – in the ERA of human and veterinary antibiotics is crucial to minimize the potential effect of antibiotic pollution on antibiotic resistance.

MIC distributions are at the centre of clinical microbiology. In conjunction with drug pharmacokinetics, they are used to establish clinical breakpoints for the effective treatment of infectious diseases (MacGowan and Wise 2001; Turnidge and Paterson 2007). Similarly, we suggest that MIC distributions can be used to explicitly link environmental concentrations of antibiotics with the prevalence of resistance, and can therefore provide a cogent framework to address the potential effects of antibiotics on antibiotic resistance in the initial phase of a risk assessment. Just as pharmacokinetics provides information on the fate of antibiotics in the body, environmental exposure assessment can be used to further refine the assessment of effects. If need be, MIC distributions may be used to set breakpoints to protect background resistance levels in the environment.

6.5. Conclusions

Concentrations of antibiotics measured in different environments due to their use in human and veterinary medicine and representing action limits used in ERA may be high enough to exert a significant selective pressure on clinically relevant bacteria. The PAF of bacterial genera at concentrations of antibiotics measured in

river sediments, liquid manure and farmed soil suggest that these environments are likely to be hot-spots for the development of resistance. The explicit consideration of antibiotic resistance in the ERA of antibiotics along with efforts to reduce their input into the environment - either by limiting their use and/or improving the treatment of liquid and solid wastes - are crucial to maintain background resistance levels.

Chapter 7

Prevalence of class 1 integrons in stream biofilms exposed to florfenicol and erythromycin thiocyanate: exploratory analysis

7.1. Background

The effect that the occurrence of human and veterinary antibiotics in the environment may have on the prevalence of antibiotic resistance has received increasing attention in recent years (e.g., Kümmerer 2009). Several studies have shown a link between the use of human and veterinary antibiotics and the prevalence of resistance determinants in the environment (e.g., Luo et al. 2010). In most cases, however, it is unclear whether an observed increase in prevalence occured due to pollution by antibiotic resistance genes and antibiotic resistant bacteria, or due to selection of these determinants in the environment by antibiotics. The latter is a critical question from an environmental risk assessment and management perspective, as the occurrence of concentrations likely to casue a selective pressure in the environment should be minimized.

Resistance to FFC is mediated by three different genes which code for proteins that belong to two different protein families. Genes *floR* and *fexA* belong to the Major Facilitator Superfamily and code for efflux proteins that export FFC out of the cell (Schwarz et al. 2004). A third gene, *cfr*, which has been shown to be an RNA methyltransferase, inhibits ribose methylation and thereby causes resistance to FFC, chloramphenicol and clindamycin (Long et al. 2006). *Cfr* belongs to the recently discovered radical SAM Superfamily of proteins (Sofia et al. 2001), and antimicrobial susceptibility testing has shown that *S. aureus* and *E. coli* strains expressing the *cfr* gene have high minimum inhibitory concentrations to a number of chemically unrelated drugs (Long et al. 2006). The gene *floR* was found to be part of transposon TnfloR from *E. coli*, *fexA* was identified in plasmid pSCFS2 from *S. lentus* and *cfr* was first identified in plasmid pSCFS1 from *S. sciuri* (Schwarz et al. 2000; Kehrenberg and Schwarz 2004; Doublet et al. 2005). To the best of our knowledge, these genes have not been reported in environmental samples.

Resistance to erythromycin is mediated by rRNA *erm* methylases that alter the ribosomal binding site for macrolides, by erythromycin *ere* esterases that hydrolize the lactone ring of macrolides, and by different macrolide efflux pumps (Leclercq and Courvalin 1993; Weisblum 1995; Roberts et al. 1999). Erythromycin resistance genes have been detected in different environments. Chen et al. (2007) detected six classes of *erm* genes in swine manure, swine feces lagoons and swine manure biofilter samples and Kim et al. (2005) detected *ereA* in sediment samples downstream of

effluents from freshwater aquaculture farms. Genes *ermB* and *ermE* were also recently reported to occur in river sediments subject to different types of anthropogenic disturbance (Graham et al. 2011). The *ermF* gene has been shown to have a wide host range accross gram-positive and gram-negative clinical bacteria, and it has the ability to transfer into and out of gram-positive and gram-negative donors and recipients (Chung et al. 1999).

In selected samples from the FFC experiment described in chapter 4 we did not detect the presence of *floR*, *fexA* or *cfr* genes. In selected samples from the ERT experiment described in chapter 5 we did not detect the presence of *ermA*, *ermB*, *ermC*, *ermF*, *ermT* or *ermX* genes. We did not probe for the presence of *ereA* or *ereB* genes, and this is something that will be addressed in the future. The stream from which water was collected to operate the microcosms drains a semi-forested agricultural landscape used predominantly for low-intensity grazing, and it is therefore not subject to major anthropogenic disturbance. Biofilms in the microcosms were also grown from stream water, whereas it might have been more likely to find these genes - if present - in the sediments. These factors may partly explain the absence of these resistance genes in our samples.

As an alternative marker that could potentially reflect a selective pressure of FFC and ERT, we decided to assess - in a largely exploratory approach - their effects on the prevalence of class 1 integrons. Integrons are bacterial genetic elements that can incorporate and express promotorless gene cassettes (Mazel 2006). Mobile gene cassettes are incorporated into integrons by site-specific recombination mediated by an integron-integrase (*intI*) that catalyzes recombination between a primary recombination site (*attI*) and a 59-base element site (*attC*) carried on gene cassettes (Hall and Collins 1995). Currently, two types of integrons have been described: mobile integrons located on transposons and which contain between two and eight gene cassettes conferring resistance to a range of antibiotics, and chromosomal integrons, which are non-mobile genetic elements that contain up to hundreds of gene cassettes of mostly unknown function (Guerin et al. 2009). Class 1 integrons are a class of mobile integrons found extensively in clinical bacterial isolates and their gene cassettes confer, collectively, resistance to a wide range of antibiotics (Mazel 2006). Recently, however, class 1 integrons without known antibiotic resistance gene cassettes and with a signature of lateral gene transfer (LTG) have been found in the chromosomes of non-pathogenic soil and freshwater *Betaproteobacteria*, suggesting

that *Betaproteobacteria* were the original source of class 1 integrons (Gillings et al. 2008). Collectively, class 1 integrons provide a vast genetic resource into which bacteria can 'tap into' in the face of selective pressures.

Previous research has shown that stream sediment samples from sites with considerable anthropogenic disturbance had a significantly higher abundance of class 1 integrase genes (*intI1*) than samples from agricultural or otherwise un-disturbed catchments (Rosewarne et al. 2010). Hardwick et al. (2008) also found that the abundance of *intI1* genes was negatively and significantly correlated with an index of general stream quality. Class 1 integrons carry diverse quaternary ammonium resistance determinants (Gaze et al. 2005; Gillings et al. 2010), and recent research suggests that selection by quaternary ammonium compounds is an important selective pressure for class 1 integrons in wastewaters and polluted surface waters (Gaze et al. 2011; Gillings et al. 2009).

Despite increasing interest in the link between environmental pollution and the prevalence of class 1 integrons in different environments, few studies have directly assessed the effect of antibiotic pollution on the abundance of *intI1* genes (e.g., Wright et al. 2008). The FFC resistance gene *floR* and the ERT esterase gene *ereA* have been associated to class 1 integrons (Arcangioli et al. 1999; Plante et al. 2003), suggesting that FFC and ERT selective pressures may - in certain cases - be reflected by changes in the prevalence of class 1 integrons. Wright et al. (2008) also showed that the abundance of *intI1* genes in freshwater microcosms exposed to 30 mg L^{-1} tetracycline for 7 days was significantly higher than in control microcosms, although no tetracycline resistance genes were recovered and class 1 integron gene cassettes conferring resistance to tetracycline have not been reported. Tetracyline is also not known to induce the SOS response in bacteria, which controls the rate of integron recombination (Guerin et al. 2009). However, several chromosomal integron cassettes with significant homology to resistance genes that may have the potential to express a resistance phenotype under the right selective pressures have been identified (Mazel 2006).

In this context, assessing changes in the prevalence of class 1 integrons in environmental samples exposed to antibiotics such as FFC and ERT may be used as an exploratory strategy to detect the presence of potentially novel resistance determinants and assess whether they confer a selective advantage under an environmentally relevant exposure scenario. For example, an observed increase in the

abundance of *intI1* genes in antibiotic treated samples would warrant further analysis to identify the gene cassettes associated with it. With this in mind, we assessed the prevalence of *intI1* genes in selected samples from the FFC (i.e., Chapter 4) and ERT (i.e., Chapter 5) experiments using qPCR. In this chapter we present and briefly discuss the results of these qPCR assays.

7.2. Methods

7.2.1. Prevalence of *intI1* genes

We assessed the abundance of *intI1* genes in samples from the ERT experiment taken from two flumes from each header - tank sink recirculation unit (i.e., 2 flumes per header tank - sink recirculation unit = 4 flumes per treatment) at each sampling time (i.e., before the start of ERT treatment at 15 days of biofilm development and after 9 and 30 days of ERT treatment at 24 and 45 days of biofilm development, respectively; Chapter 5). For the FFC experiment (i.e., Chapter 4) we assessed the abundance of *intI1* genes in samples from the control and the 10 and 100 μg L^{-1} treatments at 20 and 33 days of biofilm development and FFC exposure. The prevalence of *intI1* genes was calculated as the ratio of *intI1* genes to 16S rRNA genes. Abundance of 16S rRNA genes were corrected for multiple gene copies in bacterial genomes using a factor of 2.5 (i.e., the average 16S rRNA copy number in bacteria; Acinas et al. 2004). qPCR conditions for 16S rRNA and *intI1* genes were as described in Chapter 5 and Appendix 2

7.2.2. Statistical analysis

intI1 gene prevalence data was arcsin square-root transformed to minimize deviations from normality after checking quantile plots of each sample. Samples from the FFC experiment were analysed for each time point separately using a one-way ANOVA. Samples from the ERT experiment were analysed using a mixed-model ANOVA to assess the variation in *intI1* gene prevalence between ERT concentrations and between header tank - sink recirculation units within each treatment (i.e., factor 'header tank - sink recirculation unit' nested within factor 'ERT concentration').

7.3. Results and discussion

The prevalence of *intI1* genes across treatments ranged from 0.9% to 2.6% at 20 days of biofilm development and from 0.3% to 0.7% at 33 days of biofilm development in samples from the FFC experiment. In samples from the ERT experiment, prevalence of *intI1* genes ranged from 0.06% to 0.09% at 15 days of biofilm development, from 0.1% to 0.3% at 24 days of biofilm development and from 0.1% to 0.2% at 45 days of biofilm development. These values are within the range of those previously reported for biofilm and sediment samples from freshwater streams (Hardwick et al. 2008; Gillings et al. 2009; Rosewarne et al. 2010). Except for samples from the FFC experiment at 20 days of biofilm development, *intI1* gene prevalence was always below 1%, which is in agreement with previously reported values for stream sediment samples from pristine and agricultural catchments (Rosewarne et al. 2010). It is interesting to note, however, that while the average prevalence of *intI1* genes decreased by 68% between 20 and 33 days of biofilm development in samples from the FFC experiment, it increased by 88% between 15 and 45 days of biofilm development in samples from the ERT experiment. These contrasting patterns may reflect temporal variations in the abundance of *intI1* genes in the stream water used to grow the biofilms.

The prevalence of *intI1* genes in samples from the FFC and ERT experiment did not show significant differences that can be linked to the exposure of stream biofilms to these antibiotics. In samples from the FFC experiment at 20 days of biofilm development, there was a significant difference in the prevalence of *intI1* genes between treatments ($F_{2,21} = 5.7$, $p = 0.01$), yet Figure 7.1 and Tukey's a posteriori HSD test showed that this difference was due to the control having a significantly higher prevalence of *intI1* genes than the 100 μg L^{-1} treatment (adjusted p-value < 0.01). At 33 days of biofilm development there was also a significant difference in the prevalence of *intI1* genes between treatments ($F_{2,21} = 29$, $p < 0.0001$), yet this was due to the 10 μg L^{-1} treatment having a significantly lower prevalence compared to the control and 100 μg L^{-1} treatments (adjusted p-values < 0.0001 for both pairwise contrasts) (Figure 7.2). The prevalence of *intI1* genes between the control and 100 μg L^{-1} treatment was not significantly different.

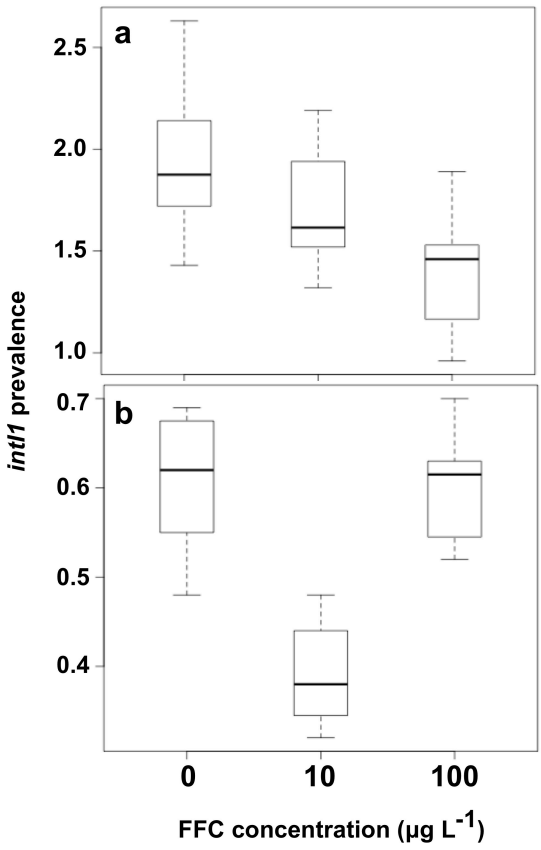

Figure 7.1. *intI1* prevalence in samples from the FFC experiment at (a) 20 days and (b) 33 days of biofilm development and FFC exposure.

In the ERT experiment and before the start of ERT exposure, there were no significant differences in the prevalence of *intI1* genes between samples taken from flumes belonging to different header tank - sink recirculation units ($F_{1,22}$ = 1.0, p = 0.33). After 9 days of ERT exposure, there were significant differences between samples belonging to the same ERT treatment but different header tank - sink recirculation units and no differences between ERT treatments (Table 7.1).

Table 7.1. Mixed model ANOVA table for *intI1* prevalence in samples from the ERT experiment after 9 days of ERT exposure. α = 0.05.

	Df	SS	MS	F	P
ERT concentration	2	0.0001	5.8×10^{-5}	2.4	0.12
Header tank (nested within ERT concentration)	3	0.0003	8.5×10^{-5}	3.5	0.04
Residuals	18	0.0004	2.4×10^{-5}		

After 30 days of ERT exposure, there were significant differences between samples belonging to the same ERT treatment and between treatments (Table 7.2). As can be seen in Figure 7.2, however, the difference between ERT treatments was driven by a higher prevalence of *intI1* genes in samples from the control treatment compared to the 10 and 100 µg L^{-1} treatments. An inspection of boxplots for *intI1* gene prevalence in individual header tank - sink recirculation units did not show any pattern consistent with ERT treatment or an effect of the triethanolemine buffer used as a carrier for ERT either (data not shown).

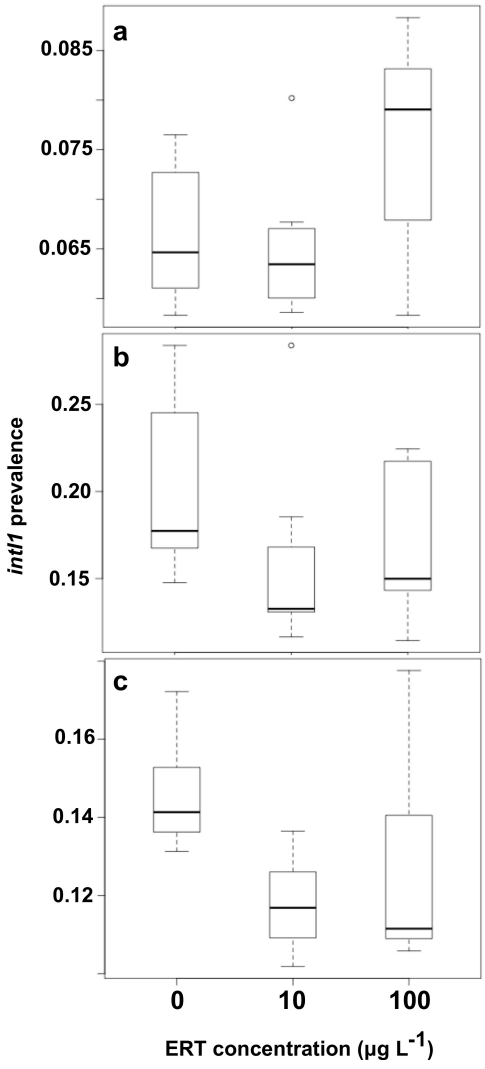

Figure 7.2. . *intI1* prevalence in samples from the ERT experiment (a) before ERT exposure at 15 days of biofilm development, (b) after 9 days of ERT exposure at 24 days of biofilm development and (c) after 30 days of ERT exposure at 45 days of biofilm development.

Table 7.2. Mixed model ANOVA table for *intI1* prevalence in samples from the ERT experiment after 30 days of ERT exposure. $\alpha = 0.05$.

	Df	SS	MS	F	P
ERT concentration	2	6.3×10^{-5}	3.2×10^{-5}	6.8	0.006
Header tank (nested within ERT concentration)	3	4.9×10^{-5}	1.7×10^{-5}	3.6	0.03
Residuals	18	8.3×10^{-5}	4.6×10^{-5}		

The absence of any pattern in the abundance of *intI1* genes consistent with the treatment of FFC and ERT suggest either the absence of gene cassettes involved in dealing with selective pressures caused by these antibiotics or that the concentrations tested were below those required to give them a selective advantage. In line with recent findings (Gillings et al. 2008; Gillings et al. 2009), however, my results are likely a reflection of the ubiquity of chromosomal class 1 integrons lacking any known resistance gene cassettes in aquatic environments. In this context, the patterns observed in the prevalence of *intI1* genes in my samples likely reflect random variation in the abundance of the minor members of the bacterial community that carry them. Future studies combining the experimental manipulation of aquatic bacterial communities with metagenomics are likely to provide relevant insights into the effects that antibiotic pollution has on the presence, abundance and distribution of novel antibiotic resistance determinants in aquatic ecosystems.

Chapter 8

General discussion

8.1. Discussion

This thesis showed that the implementation of effective benchmarks in the environmental risk assessment of freshwater aquaculture production is constrained by our current knowledge on the potential effects of veterinary medicines on aquatic ecosystems (i.e., Chapter 2). It also briefly highlighted the limitations of standardized tests to assess the effects of antibiotics on microorganisms and of current guidelines for the ERA of veterinary medicines in dealing with the potential environmental and public health implications of antibiotic pollution (i.e., Chapter 1). These observations defined the overarching issues addressed by this thesis using a combination of primary (i.e., Chapters 3, 4, 5 and 7) and secondary (Chapter 6) research.

To partly overcome some of the limitations of standardized tests, we built, optimized and evaluated a recirculating stream microcosm system to grow bacterial biofilms directly from stream water (i.e., Chapter 3). This experimental system provided a good compromise between replicability and environmental representation, and key information from preliminary experiments was used to guide the design of the experiments conducted to assess the effects of FFC and ERT on the bacterial community structure of stream biofilms.

The experiments discussed in chapters 4 and 5 of this thesis used the microcosms to assess the effects of FFC and ERT on the bacterial community structure of stream biofilms. The stability of FFC in the microcosms - determined in a preliminary experiment - allowed us to use a gradient of FFC concentrations and a regression approach to data analysis. This allowed us not only to detect the presence of effects, but to suggest a mechanism of action consistent with the trends observed in the data. The experiment conducted to assess the effects of ERT, on the other hand, provided important information regarding the environmental safety of ERT by narrowing down the concentrations that are likely to affect the bacterial community structure of stream biofilms during an exposure period conssistent with its recommended use to treat disease in freshwater salmonids. Our exploratory analysis on the abundance of class 1 integrons in stream biofilms exposed to FFC and ERT (i.e., Chapter 7), although preliminary, provides useful baseline data on the selective pressure of FFC and ERT at the concentrations tested.

Although further research is required to fully understand the effects of FFC and ERT on stream biofilm bacteria, these studies have effectively moved us a step forward in

linking the presence of veterinary medicines in aquaculture effluents with environmental effects. Importantly, our experimental streams allowed us to isolate the effects of the antibiotics while maintaining a good level of ecosystem representation in terms of hydrodynamics, physicochemical parameters and the dynamics of biofilm formation and succession itself. These studies therefore provide an enviornmentally meaningful assessment of the effects of FFC and ERT on stream biofilm bacteria under exposure scenarios relevant to freshwater aquaculture.

The methods and studies described in chapters 3,4,5 and 7 make a contribution to our understanding of the potential effects of antibiotic pollution on aquatic bacteria and aquatic ecosystems in general, and may be well suited to the higher-tier phases of an ERA, where refined information on environmental effects may provide further guidance for the marketing authorization of a veterinary medicine. In the initial phase of an ERA, however, decision makers require adequate information on the environmental safety of a product to decide whether or not the ERA should proceed to a more refined, higher-tier assessment of effects. A major shortcoming of current guidelines on the ERA of veterinary medicines is that they do not consider the effects of antibiotics on the prevalence of antibiotic resistance in the environment, despite its major implications for human health. This issue was addressed in chapter 6 by developing a conceptual framework to link antibiotic pollution to antibiotic resistance in the ERA of antibiotics, followed by an analysis that enabled us to put MECs of antibiotics from different environments and antibiotic concnetrations representing ERA action limits in perspective with the antibiotic sensitivity of clinically relevant bacteria. The use of MIC distributions based on data collated from thousands of worldwide sources gives our results a level of generalizability that is hard to achieve by a single study and, despite its limitations, makes a convincing case to include antibiotic resistance as an assessment endpoint in the ERA of antibiotics.

Collectively, the research presented in chapters 2, 3, 4, 5, 6 and 7 and briefly summarised in the preceeding paragraphs has fulfiled the objectives of this thesis, as outlined in section 1.5 of the General Introduction. The critical review presented in chapter 2 indentified key knowledge gaps regarding the environmental effects of veterinary medicines used in freshwater aquaculure. It also provides a conceptual characterization of effluents and ecological effects that is important to design and implement sound environmental policies for inland salmonid aquaculture. Chapters 3, 4, 5 and 7 have effectively furthered our understanding on the effects and

environmental safety of antibiotics used in freshwater aquacutlure by coupling the use of an experimental system with good ecosystem representation with relevant exposure scenarios. Finally, chapter 6 has developed a framework that effectively links antibiotic pollution to antibiotic resistance and that is suitable to assess the environmental risks of antibiotic pollution in the ERA process of antibiotics.

8.2. Future work

As a result of the research conducted throughout this thesis, the following key areas for future work have been identified.

1) The stimulatory effect of FFC on biofilm formation suggested by the results preseneted in chapter 4 must be further investigated under similar conditions to those used in this thesis. Because FFC presumably enhances biofilm formation by stabilizing transcripts coding for - among other genes - several adhesins (Blickwede et al. 2005), experiments may be designed to assess the effects of FFC on the gene expression of specific adhesins of freshwater bacteria and on total mRNA levels. Experiments may also be designed to measure changes in water column vs. surface adhered bacteria along gradients of FFC concentration. These studies are important given the implications that enhanced biofilm formation may have on the persistence of pathogens.

2) Antibiotics co-occur in aquaculture effluents with other compounds, including - but not limited to - suspended and dissolved solids, nutrients and disinfectants. Future work should assess how combinations of effluent constituents affect bacterial communities, including the prevalence of antibiotic resistance.

3) In the FFC and ERT experiments we observed a 'loss' of both antibiotics in the microcosms. Our studies, however, were not designed assess the fate of these antibiotics in the microcosms. Detailed modelling or empirical studies on the fate of FFC and particularly ERT after their use in freshwater aquaculture would be extremely valuable to refine assessments of effects and the design of experiments.

4) The analsys using MIC distributions presented in chapter 6 should be extended to consider the effects of antibiotics on the prevalence of multidrug resistance. This can be done by coupling the data from MIC distributions with data from bacterial genomes and metagenomes to estimate probabilities of resistance gene co-occurrences and the likelihood of co-selection of different antibiotic resistance gene combinations at different antibiotic concentrations. This work is currently under way.

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APPENDIX 1: Adittional evidence to support the vailidity of the results on the effect of FFC on bacterial commnity structure presented in chapter 4.

In the preliminary experiment described in chapter 3 we showed that bacterial communities were reproducible between different header tank - sink recirculation units. Strictly speaking, however, replicate flumes from each header tank - sink recirculation unit are pseudoreplicates, as they share the same water body (Hurlbert 1984). The regression approach adopted in the analysis of the data from the FFC experiment partly corrects for this, as a consistent trend in the response variables in relation to the imposed FFC concentration gradient is less likely to be due to random variation not related to FFC than are just 'signigicant differences' as assessed by e.g., an ANOVA - type approach to analysis. For all our response variables, we observed trends that were consistent with the imposed FFC concentration gradient, which makes us confident that the results observed are in fact due to FFC. To further confirm that the trends observed in bacterial community structure were due to FFC, however, we attempted to reproduce our results using the bacterial community dataset from all six header tank - sink recirculation units from the ERT experiment (Chapter 5) before the start of ERT exposure, at 15 days of biofilm development. We assigned each header tank - sink recirculation unit its corresponding FFC concentration used in the FFC experiment and assessed its significance and explanatory power with *adonis* using the same data transformations performed on the FFC dataset. This mock analysis allowed us to test whether there was a 'location'/'header tank' effect that could have caused the observed changes in bacterial community structure in response to FFC that we describe in chapter 4. tRFLP datasets from the ERT experiment were digested with two different restriction enzymes (i.e., AluI and HhaI), generating two tRLFP profiles per samples. *adonis* did not show significant effects with any dataset or data transformation, which provides an addittional measure of confidence on the results from the FFC experiment presented in chapter 4 (Table A.1). There were also no positive correlations between the mock FFC gradient and the number of OTUs or the Gini-coefficient, as we observed in the 'true' FFC experiment (i.e., Table 4.3).

Table A.1. Variance partitioning of the AluI and HhaI tRFLP datasets from the ERT experiment at 15 days of biofilm development using the FFC concentration gradient as a mock explanatory variable with *adonis*. There were no significant effects and the percent of explained variance in community dissimilarities (i.e., R^2) is much lower than that explained by FFC at 10 and 20 days of biofilm development (i.e., Table 4.5).

	AluI tRFLP dataset	**HhaI tRFLP dataset**
Relative peak height	$F_{1,22} = 1.9$; $R^2 = 0.08$; $p = 0.09$	$F_{1,22} = 1.5$; $R^2 = 0.06$; $p = 0.16$
Hellinger	$F_{1,22} = 2.0$; $R^2 = 0.08$; $p = 0.07$	$F_{1,22} = 1.7$; $R^2 = 0.07$; $p = 0.10$
Presence/absence	$F_{1,22} = 1.5$; $R^2 = 0.07$; $p = 0.20$	$F_{1,22} = 1.3$; $R^2 = 0.06$; $p = 0.28$

APPENDIX 2 : qPCR assay validation

A.2.1. Cloning and sequencing of 16S rDNA and Class 1 integron fragments for qPCR standards preparation

A 142 base-pair putative 16S rRNA fragment was amplified from an anonymous *Pseudomonas sp.* strain provided by the Institute of Aquaculture's Bacteriology Lab using primers BACT1369F and PROK1492R (Smith 2005). A 109 base-pair putative qintI1 gene fragment from a Class 1 integron integrase was amplified from biofilm DNA using primers qINT-3 and qINT-4 (Rosewarne et al. 2010). A single band was observed for products from both reactions in 2% agarose gel electrophoresis runs. PCR products were purified with the Qiagen® QIAquick PCR purification kit and cloned into the pGEM T-Easy vector (Promega® USA). pGEM T-Easy vectors are linearized vectors with a single 3'-thymidine at both ends that provide an overhang compatible with the 3'-adenine overhang of PCR products generated with Taq DNA polymerase. The molecular cloning site of the pGEM T-Easy vector is within the α-peptide coding region of the enzyme β-galactosidase, and insertional inactivation of the α-peptide enables identification of recombinants by blue/white screening on indicator plates. Ligation reactions were performed by mixing each PCR product with the cloning vector using an insert:vector ratio of 4:1 in half the volume recommended by the manufacturer, as described below:

2X Rapid Ligation Buffer, T4 DNA Ligase	2.5 µL
pGEM T-Easy Vector	0.5 µL
PCR product	Volume for a 4:1 insert:vector ratio
T4 DNA Ligase	0.5 µL
Milli-Q H_2O	Top up to total volume of 5 µL

Ligation reactions were mixed gently by pipetting and incubated at 4 °C overnight to achieve the maximum number of transformants. 50 µL of JM109 high efficiency competent cells were then transfered to the ligation reaction tubes. Cells were heat-shocked for 45 seconds at exactly 42 °C in a water bath, placed on ice for 2 minutes and then 950 µL of SOC medium were added to the reactions. They were then incubated at 37 °C with shaking at 150 rpm for 90 minutes. 100 µL of each

transformation culture was plated into LB/ampicillin (100 μg mL^{-1})/IPTG/X-Gal plates and incubated overnight at 37 °C. Twelve white colonies were selected from plates containing the BACT1369F/PROK1492R and qINT-3/qINT-4 fragments and screened for the presence of inserts using colony PCR with custom, plasmid specific primers TEASY-F (5'-AAAACGACGGCCAGTGAAT-3') and TEASY-R (5'-CGTTGGATGCATAGCTTGAG-3'), which flank the molecular cloning site of the pGEM T-Easy vector. Plasmid DNA was isolated from 4 mL LB broth/100 μg mL^{-1} ampicillin overnight cultures of positive colonies (i.e., which showed a band of the expected size in a 2% agarose gel electrophoresis) using the GeneElute™ Plasmid Miniprep Kit (Sigma-Aldrich® UK).

Sequencing was performed in 5 μL reactions using the GenomeLab™ Methods Development Kit Dye Terminator Cycle Sequencing (Beckman Coulter®) and primers TEASY-F/TEASY-R. 2.5 μL of the recommended amount of purified plasmid were mixed with 0.5 μL of 10 μM of each primer and 2 μL of GenomeLab™ sequencing mastermix. Separate forward and reverse sequencing reactions were performed to accomodate the different melting temperature (T_m) of primers TEASY-F (54.5 °C) and TEASY-R (57.3 °C). The sequencing reaction consited of 30 cycles of 96 °C for 20 sec, T_m of primer for 20 sec and 60 °C for 4 min. Sequencing reactions were stopped by adding 20 μL of a stop solution containing 2 μL of 3 M Sodium Acetate, 0.4 μL of 0.5 M EDTA, 1 μL of 20 mg mL^{-1} glycogen and 16.6 μL of Milli-Q H_2O. 60 μL of 95% (v/v) ice-cold ethanol were added immediately afterward. Reactions were gently mixed by pipetting, transfered to 1.5 mL tubes and centrifuged at 21000 x g at room temperature for 4 min. Pellets were rinsed once with 750 μL of 70% (v/v) ice-cold ethanol and centrifuged again at 21000 x g at room temperature for 4 min. Ethanol was removed from the tubes by pippetting and by placing them in a heat block at 40 °C for 5 min to evaporate any residual ethanol. Pellets were then dissolved in the GenomeLab™ formamide Sample Loading Solution (SLS) and sequenced using the standard Beckman Coulter CEQ 8800 program. Forward and reverse reads were assembled using the SeqMan software (DNASTAR® USA) and consensus sequences were blasted against the NCBI bacterial nucleotide collection (nr/nt) database with parameters optimized for bacterial taxa (taxid:2) and highly similar sequences. Blast results for both sequences indentified them as the targets of interest. Below are two sample top blast hits for each insert of interest:

```
>gb|JQ598792.1|  Pseudomonas sp. TI-8 16S ribosomal RNA gene, partial sequence
Length=1498

 Score =  257 bits (139),  Expect = 3e-66
 Identities = 141/142 (99%), Gaps = 0/142 (0%)
 Strand=Plus/Plus

Query  1     CGGTGAATACGTTGCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCA  60
             ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1357  CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCA  1416

Query  61    CCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGTTACCACGGTGTGATTCATGACTGGG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1417  CCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGTTACCACGGTGTGATTCATGACTGGG  1476

Query  121   GTGAAGTCGTAACAAGGTAACC  142
             |||||||||||||||||||||
Sbjct  1477  GTGAAGTCGTAACAAGGTAACC  1498


>gb|JN870909.1|  Pseudomonas sp. 4SN1 Class 1 integron integrase (intI1) gene,
partial cds
Length=944

 Score =  174 bits (94),  Expect = 2e-41
 Identities = 104/109 (95%), Gaps = 0/109 (0%)
 Strand=Plus/Plus

Query  1    TGCCGTGATCGAAATCCAGATCCTTGACCCGCAGTTGCAGGCCCTCGCTGATCCGCATGC  60
            ||||||||||||||||||||||||||||||||||||||  ||||| ||||||||||||||
Sbjct  455  TGCCGTGATCGAAATCCAGATCCTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGC  514

Query  61   CTGTTCCATAAAGAAGCTGGGCGAACAAACGATGCTCGCCTTCCAGAAA  109
            | |||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  515  CCGTTCCATACAGAAGCTGGGCGAACAAACGATGCTCGCCTTCCAGAAA  563
```

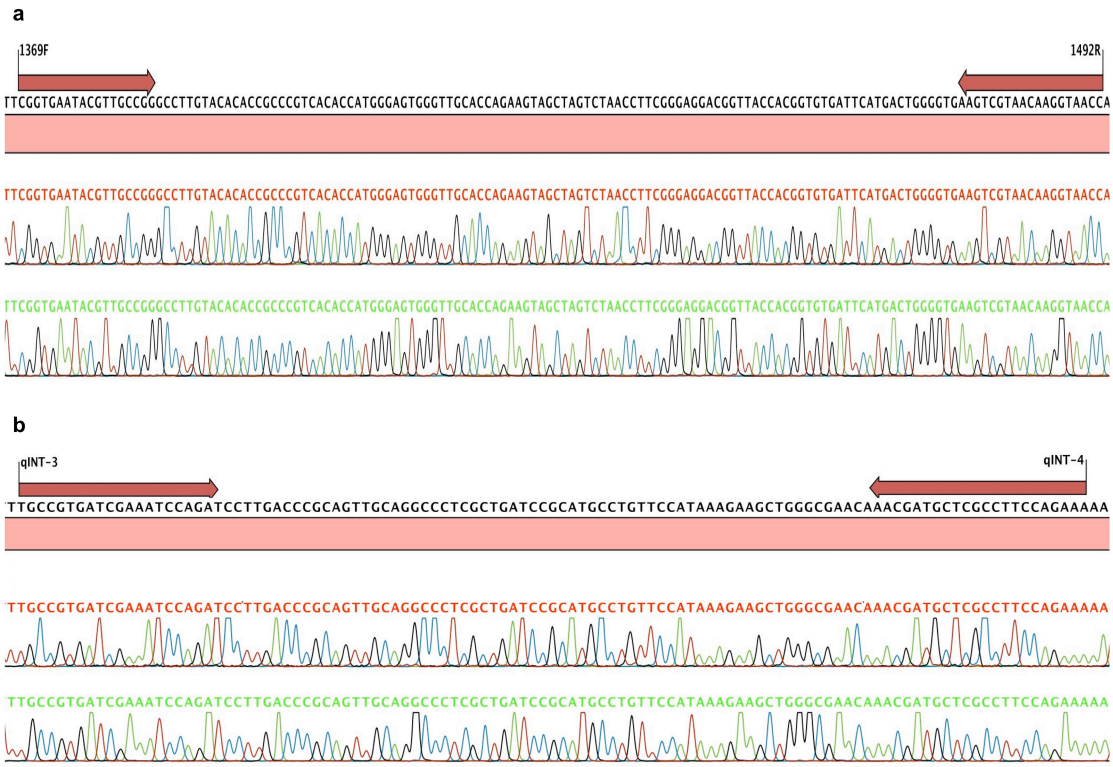

Figure A.2.1. Consensus sequence (black) and forward (red) and reverse (green) reads for the 142 bp 16S rRNA (a) and 109 bp Class 1 integron (b) inserts cloned into the pGEM T-Easy vector. Red arrows indicate the position of primers BACT1369F/PROK1492R and qINT-3/q-INT4.

After confirming the identity of the inserts, approximately 400 ng of purified plasmid were linearised with 40 U of Pst I restriction enzyme (New England BioLabs® UK), which cut the plasmid once and left the inserts intact. Plasmid DNA concentration and quality, and the presence of a single band for the linear form of the plasmid were checked spectrophotometrically and by agarose gel electrophoresis (Figure A.2.2).

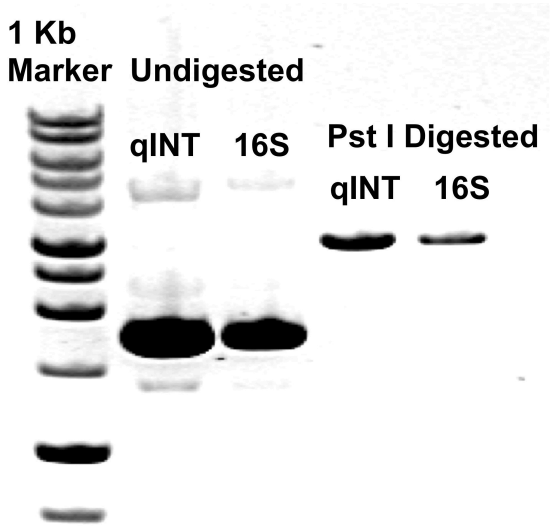

Figure A.2.2. Aagarose gel electrophoresis image showing undigested and Pst I digested, linear plasmid. Main band in undigested samples is circular plasmid, with uppermost and lowermost bands showing nicked and supercoiled forms of the plasmid, respectively. The linear forms of the plasmid carrying the 16S rDNA and Class 1 integron inserts corresponded with the expected size of ~3kb.

A.2.2. qPCR validation

The linearised plasmids shown in Figure A.2.2 were used to prepare standards for absolute quantification using qPCR. The concentration of the target template (i.e., the insert) in copies μL^{-1} of plasmid was determined using the equation:

$$copies/ul = \frac{6..023*10^{14}(Da/ng)*ConcetrationOfInsert}{RMM}$$

where RMM is the relative molecular mass of the insert and is equal to its length in base-pairs x 660 Da (Smith 2005). Standard curves covering the range 5 x 10^2 to 5 x

10^8 were freshly prepared by serial dilution of 10^8 stock aliquots kept at -20 °C and they were run in triplicate within the same plates used to quantify gene abundances in the samples.

A common issue in qPCR of 16S rRNA genes is that the threshold cycle (C_t) of the no-template control (NTC) is normally within the range of the lowest standards, and can therefore interfere in the accurate quantification of low copy numbers of 16S rRNA genes (Smith et al. 2006). In preliminary qPCR runs, we noticed that a fraction of the fluorescence contributing to the C_t value of the NTC was due to fluorescence generated by primer-dimers (i.e., primers binding to each other at their 3' ends). Based on this observation, we used a 4-step qPCR cycling program in which fluorescence detection was performed in a separate step at 80 °C after primer extension, which was above the T_m of the primer-dimers and prevented them from contributing to the measured fluorescence of the NTC.

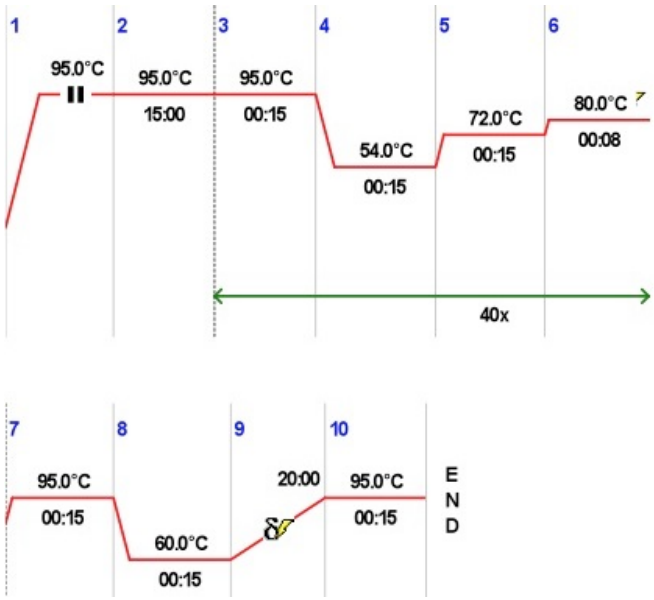

Figure A.2.3. 4-step hot-start qPCR cycling program used to quantify the abundance of 16S rRNA genes (steps 3, 4, 5 and 6). Steps 1 and 2 are an initial pause and a 15 min hot-start step. Steps 7, 8, 9 and 10 are the melting curve analysis.

With this 4-step qPCR cycling program we increased the C_t value of the NTC by more than 3.3 cycles (i.e., equivalent to a 10-fold dilution step) and the useful linear range of our 16S standard curve. Figure A.2.3 shows the 4-step qPCR cycling program used in the quantification of 16S rRNA genes using primers

BACT1369F/PROK1492R. The same cycling program was used to quantify the abundance of qintI1 genes using primers qINT-3/qINT-4, but with an annealing temperature of 60 °C. We excluded data from standard curves that had C_t values less than 3.3 cycles lower than the NTC (Smith et al. 2006).

We optimized qPCR conditions for the target genes of interest by performing primer concentration and temperature gradients with selected samples. Based on previous observations using conventional PCR, we performed a crossed primer concentration vs. temperature gradient of [0.25 µM / 0.3 µM / 0.35 µM / 0.4 µM] vs. [48 °C / 50 °C / 52 °C / 54 °C] for primers BACT1369F/PROK1492R and of [0.3 µM / 0.5 µM / 0.7 µM / 0.9 µM] vs. [58.1 °C / 60 °C / 62.6 °C / 64 °C] for primers qINT-3/qINT-4. Based on these crossed gradients, whose amplification plots and metling curves are shown in Figure A.2.4, subsequent qPCRs with primers BACT1369F/PROK1492R were performed using 0.4 µM of each primer and an annealing temperature of 54 °C. Subsequent qPCRs with primers qINT-3/qINT-4 were performed using 0.4 µM of each primer and an annealing temperature of 60 °C. The primer concentration/annealing temperature combination giving the lowest C_t value that did not show excess primers in a 2% agarose gel electrophoresis was selected as "optimal".

To assess the presence and effects of PCR inhibitors in our samples, we analysed serial dilutions of random samples from each time point and experiment using the optimized qPCR conditions. An appropriate dilution factor for each target and sample was selected based on the marginal improvements in efficiency - as assessed by reductions in C_t values - observed in the dilution series and on comparisons between the observed and theoretical ΔC_t values between subsequent dilutions. If perfect doubling occurs with each amplification cycle, the spacing between fluorescence curves follows the equation $2n$ = dilution factor, where n is the number of cycles between curves at the fluorescence threshold (i.e., the difference between C_t values = ΔC_t) (Bio-Rad 2006). By comparing observed vs. theoretical ΔC_t values, one can therefore assess the deviation in amplification with respect to the theoretical optimum at different ranges of dilution factors.

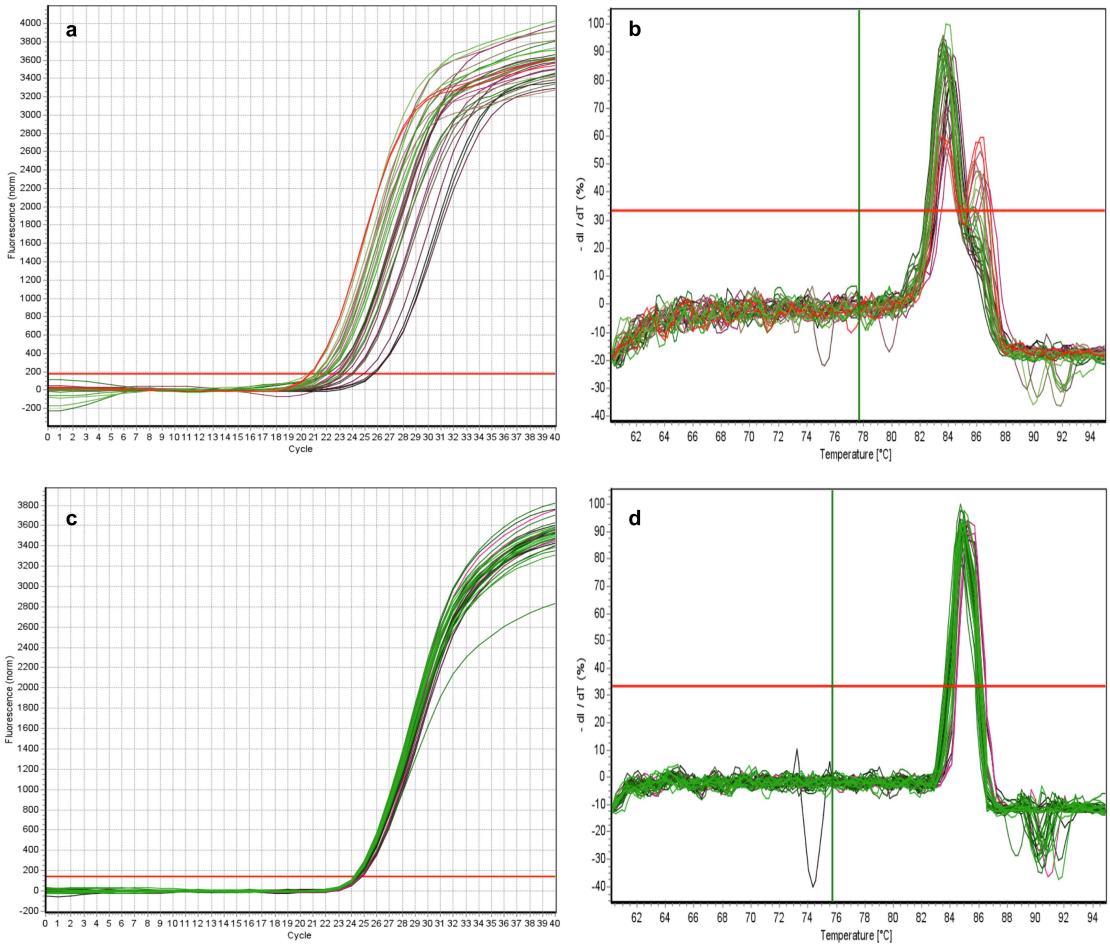

Figure A.2.4. Crossed primer concentration vs. annealing temperature gradients using primers BACT1369F/PROK1492R (a,b) and qINT-3/qINT-4 (c,d) performed during qPCR optimization.

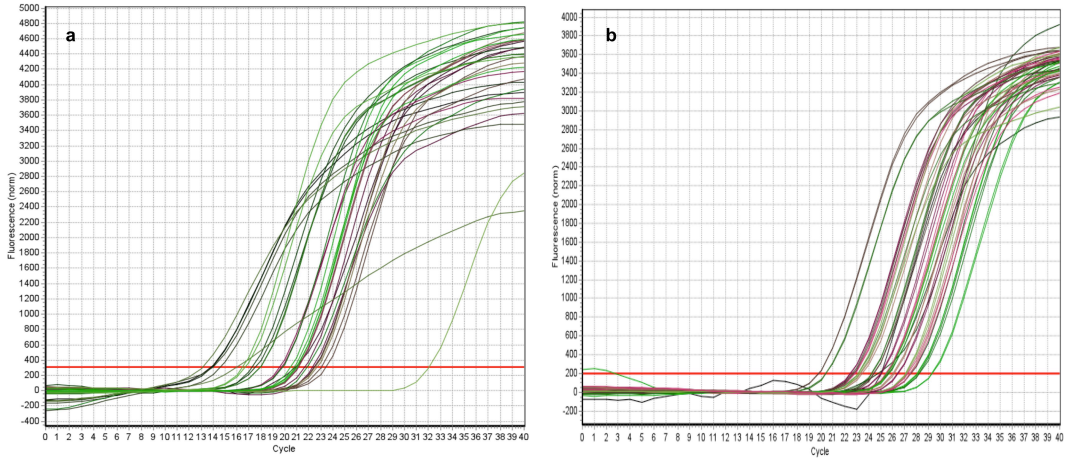

Figure A.2.5. Dilution plots from selected samples from the FFC (a) and EETT (b) experiment. Less dilute samples from the FFC experiment show signs of inhibition, as judged by the slope for their amplification plots (i.e., amplification plots furthest left in Figure 1a).

Based on this assessment, samples from the FFC experiment were assayed using a 1 in 200 and 1 in 10 dilution factor when targeting the 16S rRNA and intI1 genes, respectively. Samples from the EETT experiment were assayed using a 1 in 100 and 1 in 10 dilution factor when targeting the 16S rRNA and intI1 genes, respectively. Figure A.2.5 shows dilution plots for selected samples from the FFC and EETT experiment.

Figures A.2.6 and A.2.7 show the standard curve, amplification and melting curve plots for assays targeting the 16S rRNA and intI1 gene fragments.

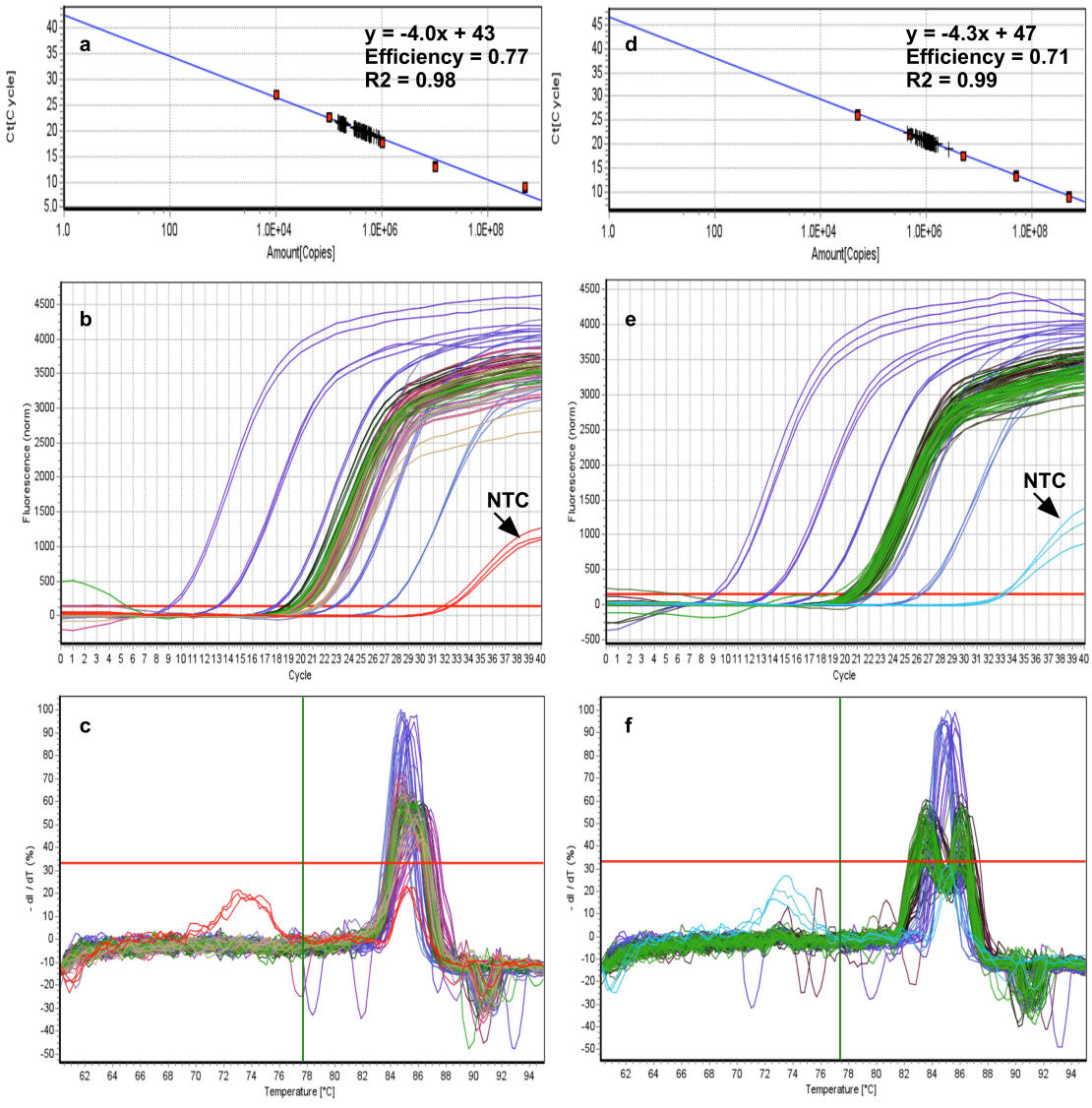

Figure A.2.6. Standard curve, amplification and melting curve plots for the 16S rRNA gene assay from samples belonging to the FFC (a,b,c) and EETT (d,e,f) experiment.

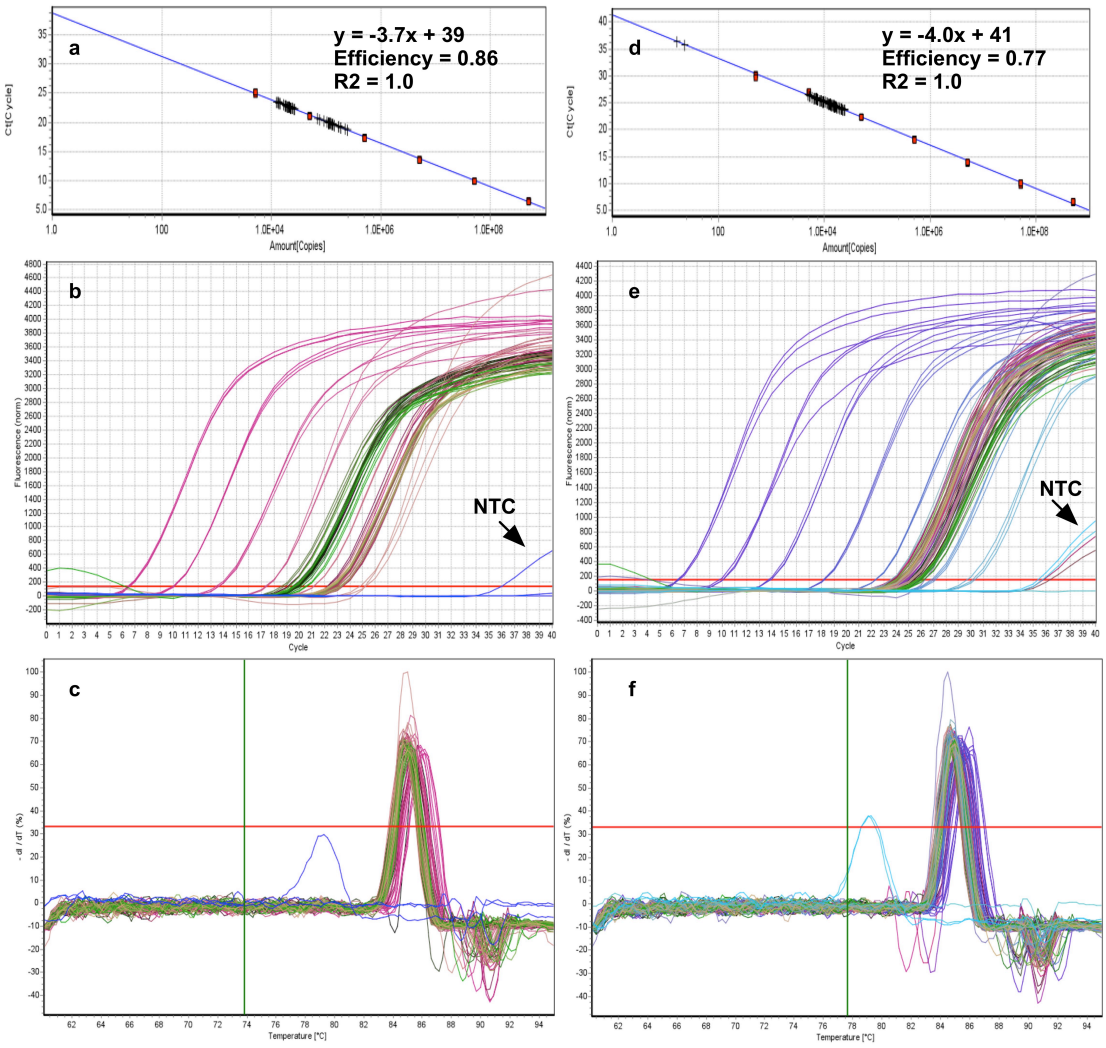

Figure A.2.6. Standard curve, amplification and melting curve plots for the *intI1* gene assay from samples belonging to the FFC (a,b,c) and EETT (d,e,f) experiment.

The melting curves of the 16S rRNA gene assay from the EETT experiment showed two peaks at approximately 83 and 86 °C. This was also observed in samples from the EETT experiment during optimization assays (Figure A.2.4b). Although the presence of two melting temperatures normally indicates contamination or the presence of non-specific amplification, the two melting temperatures observed in our samples were due to sequence variation in the 16S rRNA gene fragment. When amplifying fragments from the 16S rRNA gene in environmental samples, sequence variation between amplicons can lead to different T_m's in the same sample. Recently, for example, Blaschitz et al. (2011) showed that single nucleotide polymorphisms in the same region of the 16S rRNA gene we targeted in our assays caused significant differences in the T_m of *Mycobacterium tuberculosis*. To further explore this, we conducted in-silico PCR with primers BACT1369F/PROK1492R on five random 16S

rRNA genes that had perfect blastn matches to these primers and calculated the resulting theoretical T_m's of the amplicons using the Promega Biomath® calculator with default settings. The theoretical T_m of these amplicons spanned the range 82 - 85 °C, which matched - by 1 °C difference - the range observed in our assay (83 - 86 °C). The blastn outputs and estimated T_m for each in-silico amplicon are shown below.

>gb|JN825736.1| Flavobacterium columnare strain RDC-1 16S ribosomal RNA gene, partial sequence
Length=1478
>In-silico_amplicon1
TCGCTAGTAATCGCAGATCAGCCATGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGCCA
TGGAAGCTGGGGGTACCTGAAGTCGGTGACCGTAAGGAGCTGCCTAGGGTAAAACTGGTAACTAGGCT
PROMEGA BIOMATH CALCULATOR BASIC TM: 83 ºC

>gb|JQ269282.1| Bacterium WHC3-6 16S ribosomal RNA gene, partial sequence
Length=1473
> In-silico_amplicon2
ATCGCAGATCAGAATGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGG
TTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTG
PROMEGA BIOMATH CALCULATOR BASIC TM: 82 ºC

>gb|JF820825.1| Peptococcaceae bacterium enrichment culture NaFe56 small subunit ribosomal RNA gene, partial sequence
Length=1495
> In-silico_amplicon3
ATCGCAGGTCAGCATACTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAAAGTCTG
CAACACCCGAAGCCGGTGAGCTAACCGTAAGGAGGCAGCCGTCGAAGGTGGGGCCGATGATTGGGGTG
PROMEGA BIOMATH CALCULATOR BASIC TM: 85 ºC

>gb|JF824779.1| Uncultured alpha proteobacterium clone 930-C6 (GOMB51) 16S ribosomal RNA gene, partial sequence
Length=1453
> In-silico_amplicon4
TCGCAGATCAGCATGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGA
TTTACCCGAAGCCGGTGCGCTAACCTTTTTGGAGGTAGCCGTCCACGGTAAGTTCAGCGACTGGGGTG
PROMEGA BIOMATH CALCULATOR BASIC TM: 84 ºC

>gb|JF824771.1| Uncultured cyanobacterium clone 926-C12 (GOMB5) 16S ribosomal RNA gene, partial sequence
Length=1446
> In-silico_amplicon5
CGCTGGTCAGCTACACAGCGGTGAATTCGTTCCCGGGCCTTGTACACACCGACCGTCACACCATGGAAGCTGGT
TATGCCCGAAGTCGTTACGCTAACCTTTTTGGAGGCGGATGCCTAAGGTAGAATTAGTGACTAGGGTG
PROMEGA BIOMATH CALCULATOR BASIC TM: 82 ºC

We further confirmed the specificity of both the 16S rRNA and qINTI1 qPCR assay by running a 2% agarose gel electrophoresis of random samples selected from each plate (Figure A.2.7). A single band for the target of interest was observed in all samples. Average qPCR amplification efficiencies over repeated runs for the 16S rRNA and intI1 standard curves were 74 and 81%, respectively. The coefficient of determination of regression lines was always ≥ 0.98.

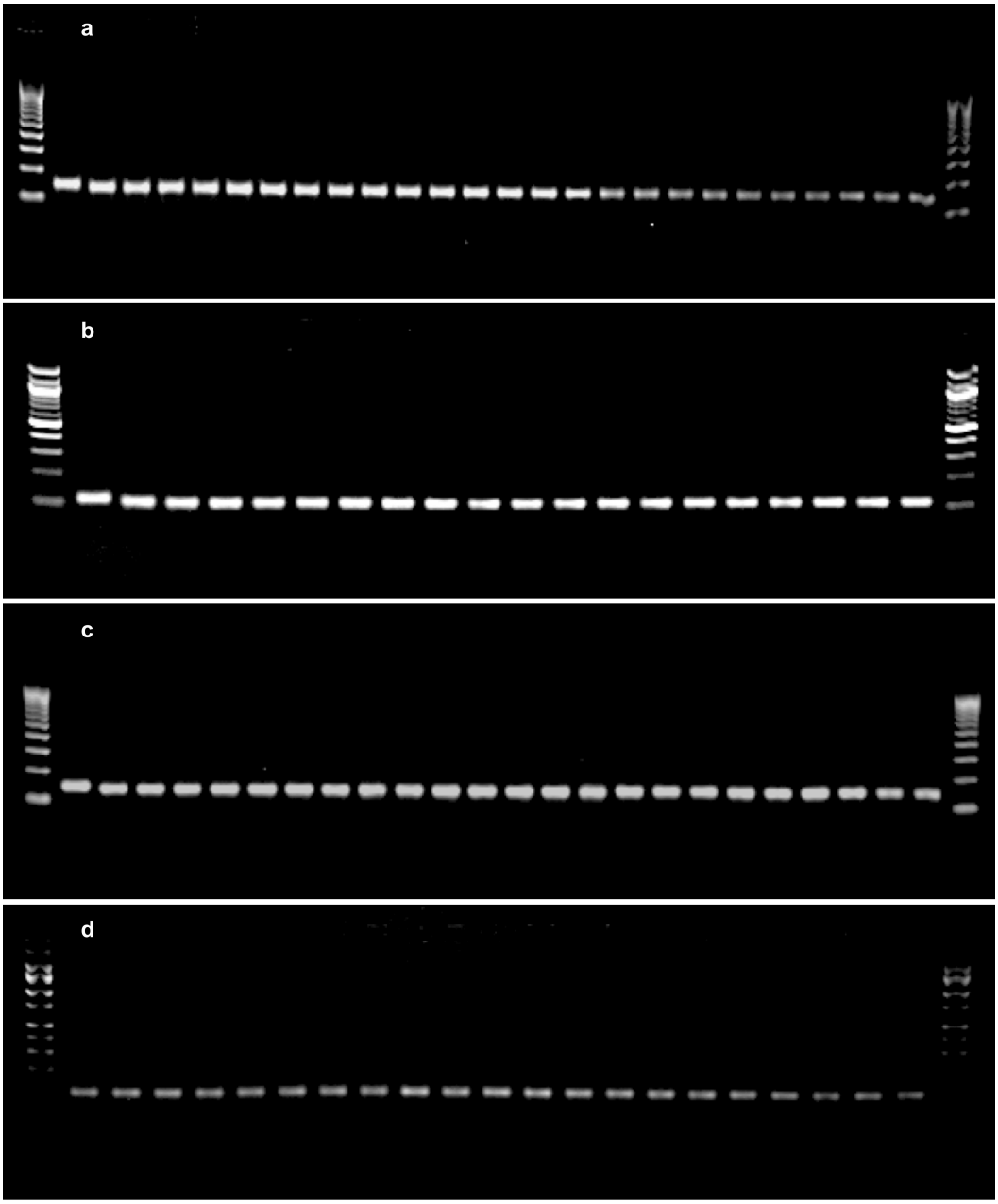

Figure A.2.7. 2% agarose gel electrophoresis of random amplicons from each qPCR assay. Figures A.2.7a and A.2.7b correspond to the 16S rRNA and intI1 assays from the FFC experiment. Figures A.2.7c and A.2.7d correspond to the 16S rRNA and intI1 assay from the EETT experiments. Marker is 100 bp ladder.

APPENDIX 3: Supplementary material for chapter 6.

Table A.3.1

Taxa represented in EUCAST MIC datasets. Cross (+) and dash (-) symbols indicate presence or absence of each entry in the given EUCAST MIC antibiotic dataset, respectively. The All-species Living Tree Project names of the subset of species which were represented in the LTP 16S rRNA database are given in the 'LTP Name' column. MIC data from co – generic species in each dataset were pooled to derive SSDs.

Species	Ciprofloxacin	Erythromycin	Tetracycline	LTP Name
Acinetobacter anitratus	+	-	-	
Acinetobacter baumanii	+	-	+	AcnBau29
Acinetobacter lwoffii	+	-	-	AcnLwo34
Acinetobacter calcoaceticus	+	-	-	AcnCal77
Acinetobacter spp	+	-	+	
Alcaligenes xylosoxidans	+	-	-	
Bacteroides fragilis	+	-	-	BcdFra36
Bacteroides fragilis group	+	+	-	
Bacteroides ovatus	-	-	-	BcdOvat8
Bacteroides thetaiotaomicron	-	-	-	BcdThe32
Bacteroides vulgatus	-	-	-	BcdVulg9
Burkholderia cepacia	+	-	-	BurCe154
Bifidobacterium longum	-	+	+	BifLon13
Bifidobacterium pseudolongum	-	-	+	BifPseu8
Bifidobacterium thermophilum	-	-	+	
Campylobacter coli	+	+	+	CampCol5
Campylobacter jejuni	+	+	+	CamJejun
Chryseobacterium meningosepticum	+	-	-	
Chryseobacterium spp	+	-	-	
Citrobacter braakii	-	-	+	CitBraak
Citrobacter freundii	-	-	+	CitFre32
Citrobacter koseri	-	-	+	
Citrobacter spp	+	-	+	
Clostridium difficile	-	+	+	CloDiff5
Clostridium perfringens	-	+	-	CloPe172
Enterobacter aerogenes	+	-	+	EntAero9
Enterobacter agglomerans	+	-	+	
Enterobacter cloacae	+	-	+	EntClo58
Enterobacter dissolvens	+	-	+	EntClo52
Enterobacter spp	+	-	+	
Enterococcus avium	-	-	-	EnrAviu7
Enterococcus casseliflavus	-	-	-	EnrCas20

Enterococcus faecalis	+	+	+	EnrFa149
Enterococcus faecium	+	+	+	EnrFa232
Enterococcus gallinarum	-	-	-	EnrGall8
Enterococcus hirae	-	+	+	EnrHir11
Escherichia coli	+	-	+	EschCo52
Haemophilus influenzae	+	+	+	HaeIn157
Haemophilus parainfluenzae	+	+	+	HaePara4
Hafnia alvei	+	-	+	HafAlv15
Helicobacter pylori	+	-	+	HelPyl55
Klebsiella oxytoca	+	-	+	KleOxy19
Klebsiella pneumoniae	+	-	+	KlePn101
Klebsiella spp	+	-	+	
Kluyvera spp	+	-	+	
Lactobacillus lactis	-	+	+	
Legionella pneumophila	+	+	-	LgnPne10
Listeria monocytogenes	+	-	+	LstMon13
Manhemia haemolytica	-	-	+	ManHaem8
Moraxella catarrhalis	+	+	+	MorCatar
Morganella morganii	+	-	+	MrgMor25
Mycobacterium tuberculosis	+	-	-	MycTube9
Neisseria gonorrhoeae	+	+	+	NeiGono5
Neisseria meningitidis	+	-	+	
Pasteurella multocida	+	+	+	PsuMult6
Peptostreptococcus spp	-	+	-	
Propionibacterium acnes	-	-	+	ProAcn42
Proteus mirabilis	+	-	+	PtsMir19
Proteus vulgaris	+	-	+	PtsVulg9
Proteus spp	-	-	+	
Providencia rettgeri	-	-	-	PrvRett3
Providencia stuartii	+	-	-	PrvStua5
Providencia spp	+	-	-	
Pseudomonas aureginosa	+	-	+	PseAe290
Pseudomonas fluorescens	+	-	-	PseFl192
Raoultella spp	+	-	+	
Raoultella spp+	-	-	+	
Salmonella enteritidis	-	-	-	
Salmonella paratyphi	-	-	-	
Salmonella typhi	-	-	-	
Salmonella typhymurium	-	-	-	
Salmonella spp	+	-	+	
Serratia liquefaciens	+	-	+	SerLiqu3
Serratia marcescens	+	-	+	SerMa116
Serratia spp	+	-	+	
Shigella sonnei	-	-	-	StaAur61
Staphylococcus aureus	+	+	+	
Staphylococcus aureus MRSA	+	+	+	
Staphylococcus aureus MSSA	+	+	+	
Staphylococcus auricularis	+	-	-	StaAuri2
Staphylococcus capitis	+	+	-	StaCap14

Staphylococcus coagulase negative	+	+	+	
Staphylococcus coagulase negative MRSE	+	+	+	
Staphylococcus cohnii	+	-	-	StaCohn9
Staphylococcus epidermidis	+	+	+	StaEpi72
Staphylococcus epidermidis MSSE	+	+	+	
Staphylococcus haemolyticus	+	+	+	StaHae18
Staphylococcus hominis	+	+	-	StaHom26
Staphylococcus hyicus	+	+	-	StaHyicu
Staphylococcus intermedius	+	+	-	StaInte4
Staphylococcus lugdunensis	+	-	-	StaLugd4
Staphylococcus saprophyticus	+	+	+	StaSap31
Staphylococcus sciuri	+	-	-	StaSci15
Staphylococcus simulans	+	-	-	StaSimul
Staphylococcus warneri	+	+	-	StaWarne
Staphylococcus xylosis	+	-	-	StaXyl10
Strenotrophomonas maltophila	+	-	+	SteMa216
Streptococcus acidominimus	+	-	-	
Streptococcus agalactiae	+	+	+	StpAga25
Staphylococcus anginosus	+	+	+	StpAng35
Streptococcus bovis	+	-	-	
Streptococcus constellatus	+	-	-	StpCon17
Streptococcus equinus	+	-	-	StpEqui4
Streptococcus gordonii	+	-	-	StpGor11
Streptococcus group C	+	-	-	
Streptococcus group G	+	+	+	
Streptococcus intermedius	+	-	-	StpInte8
Streptococcus milleri	+	+	-	
Streptococcus mitis	+	-	-	StpMit32
Streptococcus oralis	+	+	+	StpOra18
Streptococcus parasanguis	+	-	-	StpPar15
Streptococcus pneumoniae	+	+	+	StpPne46
Streptococcus pyogenes	+	+	+	StpPyo39
Streptococcus salivarius	+	-	-	StpSali6
Streptococcus sanguinis	+	-	-	StpSan11
Streptococcus thermophilus	-	+	+	
Streptococcus uberis	+	-	-	StpUber5
Streptococcus viridans	+	+	+	
Yersinia spp	+	-	+	
Total	**91**	**40**	**67**	**79**
16S rRNA sequences	**63 (70%)**	**28 (70%)**	**43 (64%)**	

README File and R script to derive SSDs by bootstrap regression

##
####README###
##
The folder containing this README.txt file should have,additionaly, the following files:

BacterialSSDs.R --> script to fit log-logistic model to MIC data using bootstrap regression
cipro.txt --> ciprofloxacin EUCAST MIC dataset
eryth.txt --> erythromycin EUCAST MIC dataset
tetra.txt --> tetracycline EUCAST MIC dataset

To run BacterialSSDs.R and BacterialPhylo.R you need a working version of R along with the following R
library:

fitdistrplus v.0.2-2

##
##

The use of BacterialSSDs.R requires some knowledge of R, especially if you wish to control the output formats, modify things to best suit your own needs and understand the operations being performed on the data. The notes I have included below assume some R knowledge and are intended to give you a few pointers to get started.The script is far from elegant code; it simply gets the job done and can help you with the initial data handling if you are already an R user.

The script can be run from the R prompt by running <source("BacterialSSDs.R")> or you can open the script as a text file in a text editor and then copy and paste the commands into the R prompt. Remember to first set your working directory and to have your datasets in it. You can set your working directory interactively by clicking on "Misc" and then on "Change working directory ...". The menu under which the change directory option is available will vary depending on your operating system (i.e., windows vs mac).

BacerialSSDs.R is set to run the ciprofloxacin dataset by default (cipro.txt). To fit SSDs to the other datasets use the 'Find and Replace' function of a text editor to Find <cipro> and Replace with e.g., <eryth>. The removal of genera from the datasets before SSD generation as described in the paper is specific to each dataset, as it depends on the index of the genera in the data frame. If you wish to replicate the analysyis for the other datasets, lines 49, 51 and 53 (marked on their right hand sides as '#1','#2' and '#3' in the script) need to be replaced for each dataset as follows:

#for eryth.txt:

```
agg.dat1<-agg.dat1[-c(1,2,6,7,9,10,12),] ##1
agg.dat2<-agg.dat2[-c(1,2,6,7,9,10,12),] ##2
agg.nobs<-agg.nobs[-c(1,2,6,7,9,10,12),] ##3

#for tetra.txt:

agg.dat1<-agg.dat1[-c(2,9,11,14,16,17,19,21),] ##1
agg.dat2<-agg.dat2[-c(2,9,11,14,16,17,19,21),] ##2
agg.nobs<-agg.nobs[-c(2,9,11,14,16,17,19,21),] ##3
```

When running the script, some outputs will be printed on the R prompt with a small description in capital letters. If the script runs Ok, a graph of the fitted model to the MIC50 and NOEC data with 95% bootstrap confidence intervals and overlayed empirical cumulative distributions of the original data will pop up in an R graphics display window. A .tiff file containing this same graph and named 'SSD1' will be saved to your working directory. Before running a second dataset rename this .tiff file so that it does not get overwritten by the following graph (...that is if you want to keep it). The script should take a couple of minutes to run, but this will depend on your computer power - it's the bootstrapping that takes a bit to run.

The potentially affected fraction (PAF) will only be returned for the VICH phase I action limits.Your results may vary very slightly each time you run the script unless you set the random number generator in R using "set.seed()". The PAFs you get when you run this script may also be very (and I mean VERY) slightly different than in the paper due to the same reason.

If you need any help feel free to email me at: atello.res@gmail.com.

```
##########################################################
##########################################################
```

```
#################################################################
########################################
###Bacterial Sensitivity Distributions using MIC Data Script
(Alfredo Tello - 2011) ###################
#################################################################
########################################
###Set working
directory#############################################################
############################
#setwd("/Users/Me/Desktop/MyData/")### set working directory
(can be set interactively in R) ###########
###Load required R
libraries#############################################################
########################
library(fitdistrplus)
#################################################################
########################################
###custom
functions#############################################################
################################
#################################################################
########################################
fx.logis<-function(x,a=1,b=1){1/(1+exp(-((log10(x)-a)/b)))} ##
logistic function
inv.logis<-function(p,a=1,b=1){a+b*log(p/(1-p))} ## inverse
logistic function
relatfreq<-function(x){x/sum(x)} ## returns relative frequency
of a given vector
#################################################################
########################################
###load eucast table and create data
set#####################################################################
#####
#################################################################
########################################
cipro.table<-read.table("cipro.txt",header=FALSE,row.names=1)
cipro.matrix<-data.matrix(cipro.table)
cipro.data<-apply(cipro.matrix[-
1,],1,function(x){rep(cipro.matrix[1,],x)}) ## generates
original MIC->
#dataset by replicating each MIC value by its observed
frequency in the table ##########################
print('NUMBER OF BACTERIAL TAXA IN MIC DATASET:')
print(nrow(cipro.matrix)-1)
#################################################################
########################################
###SSDs (median MIC and NOEC
endpoints)#############################################################
#############
#################################################################
########################################
medmic.cipro<-(as.numeric(lapply(cipro.data,median)))
medmic.cipro<-medmic.cipro/2
```

```
medmic.cipro[medmic.cipro==0.0625]<-0.064 ## fixes break in
MIC sequence from 0.125 to 0.064
lomic.cipro<-as.numeric(lapply(cipro.data,min)) ## this is the
lowest observed MIC for each strain
noec.cipro<-lomic.cipro/2 ## fixes break in MIC sequence from
0.125 to 0.064
noec.cipro[noec.cipro==0.0625]<-0.064
n.obs<-as.numeric(lapply(cipro.data,length))
frame1<-data.frame(TAXA=row.names(cipro.matrix[-
1,]),MIC50=medmic.cipro,NOEC=noec.cipro,NºObs=n.obs)
print('NON-AGGREGATED MIC50 AND NOEC VECTORS FOR BACTERIAL
SPECIES (MIC50 and NOEC VALUES ARE EXPRESSED AS PPM):')
print(frame1)
############################################################
#######################################
###Spp. aggregation by genera before endpoint vector
generation ######################################
#This step requires you to modify '#1'and '#3' below if you
would like to remove certain bacterial###
#genera from the analysis (e.g., genera that do not grow in
the environment) ##########################
#vectors specified in #1 and #2 are the indexes of bacterial
genera in agg.dat1 and agg.dat2#########
#default settings are those used for the cipro dataset and
they will not reproduce the results for the#
#other datasets.you will also need to modify '#3' for R to
print the data table######################
lab1<-row.names(cipro.matrix[-1,])
dat1<-medmic.cipro
dat2<-noec.cipro
dframe<-data.frame(lab1,dat1,dat2,n.obs,stringsAsFactors=F)
dframe$lab2<-factor(substr(dframe$lab1,1,7))
agg.dat1<-aggregate(dat1~lab2,data=dframe,FUN=mean)
agg.dat1<-agg.dat1[-c(3,11,13,18,20,21),] # 1
agg.dat2<-aggregate(dat2~lab2,data=dframe,FUN=mean)
agg.dat2<-agg.dat2[-c(3,11,13,18,20,21),] # 2
agg.nobs<-aggregate(n.obs~lab2,data=dframe,FUN=sum)
agg.nobs<-agg.nobs[-c(3,11,13,18,20,21),] # 3
medmic.cipro<-agg.dat1[,2]*1000 # multiplier converts to ppb
noec.cipro<-agg.dat2[,2]*1000 # multiplier converts to ppb
log.medmic.cipro<-log10(medmic.cipro)
log.noec.cipro<-log10(noec.cipro)
print('NUMBER OF BACTERIAL GENERA FOR SSD DERIVATION:')
print(nrow(agg.dat1))
print('AGGREGATED MIC50 AND NOEC VECTORS FOR BACTERIAL GENERA
(VALUES ARE EXPRESSED AS PPB)')
print(data.frame(Genera=agg.dat1[,1],MIC50=medmic.cipro,NOEC=n
oec.cipro,NºObs=agg.nobs[,2]))
############################################################
#######################################
###Percent variance of data explained by log - logistic model
#########################################
############################################################
#######################################
```

```
###medain MIC as
endpoint#########################################################
########################
freq.medmic.cipro<-as.numeric(table(medmic.cipro))
relfreq.medmic.cipro<-relatfreq(freq.medmic.cipro)
cum.freq.medmic.cipro<-cumsum(relfreq.medmic.cipro)
medmic.cipro.levels<-as.numeric(levels(factor(medmic.cipro)))
## mic concentration levels in medmic.cipro vector
fit1medmic.cipro<-fitdist(log.medmic.cipro,distr="logis")
param.medmic.cipro<-fit1medmic.cipro[[1]] ## parameters from
fitted distribution
a.medmic.cipro<-param.medmic.cipro[1] ## location param from
logistic dist
b.medmic.cipro<-param.medmic.cipro[2] ## scale param from
logistic dist
medmic.cipro.logis<-
fx.logis(medmic.cipro.levels,a=a.medmic.cipro,b=b.medmic.cipro
)
fitcheck.medmic.cipro<-
lm(cum.freq.medmic.cipro~medmic.cipro.logis)
print('PERCENT VARIANCE EXPLAINED BY FITTED MODEL (MIC 50
DATA)')
print(summary(fitcheck.medmic.cipro))
###noec MIC as
endpoint#########################################################
#########################
freq.noec.cipro<-as.numeric(table(noec.cipro))
relfreq.noec.cipro<-relatfreq(freq.noec.cipro)
cum.freq.noec.cipro<-cumsum(relfreq.noec.cipro)
noec.cipro.levels<-as.numeric(levels(factor(noec.cipro))) ##
mic concentration levels in noec.cipro vector
fit1noec.cipro<-fitdist(log.noec.cipro,distr="logis")
param.noec.cipro<-fit1noec.cipro[[1]] ## retrieve parameters
from fitted distribution
a.noec.cipro<-param.noec.cipro[1] ## get location param from
fitted logistic dist
b.noec.cipro<-param.noec.cipro[2] ## get scale param from
fitted logistic dist
noec.cipro.logis<-
fx.logis(noec.cipro.levels,a=a.noec.cipro,b=b.noec.cipro)
fitcheck.noec.cipro<-lm(cum.freq.noec.cipro~noec.cipro.logis)
print('PERCENT VARIANCE EXPLAINED BY FITTED MODEL (NOEC
DATA)')
print(summary(fitcheck.noec.cipro))
#################################################################
#########################################
###bootstrapping SSD
endpoints#######################################################
#####################
#################################################################
#########################################
###median
MIC#############################################################
#################################
```

```
resamples.log.medmic.cipro<-
lapply(1:5000,function(i)sample(log.medmic.cipro,length(log.me
dmic.cipro),replace=T))
fit.log.medmic.cipro<-
lapply(resamples.log.medmic.cipro,fitdist,distr="logis",start=
c(fit1medmic.cipro$estimate[1],fit1medmic.cipro$estimate[2]))
location.resamples.log.medmic.cipro<-
lapply(fit.log.medmic.cipro,function(x){x[[1]][1]})
scale.resamples.log.medmic.cipro<-
lapply(fit.log.medmic.cipro,function(x){x[[1]][2]})
location.log.medmic.cipro<-
as.numeric(location.resamples.log.medmic.cipro)
scale.log.medmic.cipro<-
as.numeric(scale.resamples.log.medmic.cipro)
quantiles<-as.list(seq(0.01,0.99,by=0.01))
invfit.log.medmic.cipro.vectors<-
lapply(quantiles,inv.logis,a=location.log.medmic.cipro,b=scale
.log.medmic.cipro)
medianFit.medmic.cipro<-
as.numeric(lapply(invfit.log.medmic.cipro.vectors,quantile,0.5
))
upperCI.medmic.cipro<-
as.numeric(lapply(invfit.log.medmic.cipro.vectors,quantile,0.9
75))
lowerCI.medmic.cipro<-
as.numeric(lapply(invfit.log.medmic.cipro.vectors,quantile,0.0
25))
###noec
MIC#############################################################
##################################
resamples.log.noec.cipro<-
lapply(1:5000,function(i)sample(log.noec.cipro,length(log.noec
.cipro),replace=T))
fit.log.noec.cipro<-
lapply(resamples.log.noec.cipro,fitdist,distr="logis",start=c(
fit1noec.cipro$estimate[1],fit1noec.cipro$estimate[2]))
location.resamples.log.noec.cipro<-
lapply(fit.log.noec.cipro,function(x){x[[1]][1]})
scale.resamples.log.noec.cipro<-
lapply(fit.log.noec.cipro,function(x){x[[1]][2]})
location.log.noec.cipro<-
as.numeric(location.resamples.log.noec.cipro)
scale.log.noec.cipro<-
as.numeric(scale.resamples.log.noec.cipro)
quantiles<-as.list(seq(0.01,0.99,by=0.01))
invfit.log.noec.cipro.vectors<-
lapply(quantiles,inv.logis,a=location.log.noec.cipro,b=scale.l
og.noec.cipro)
medianFit.noec.cipro<-
as.numeric(lapply(invfit.log.noec.cipro.vectors,quantile,0.5))
upperCI.noec.cipro<-
as.numeric(lapply(invfit.log.noec.cipro.vectors,quantile,0.975
))
lowerCI.noec.cipro<-
```

```
as.numeric(lapply(invfit.log.noec.cipro.vectors,quantile,0.025
))
##############################################################
##########################################
###Affected fraction at VICH Phase I to Phase II concentration
threshold#################################
##############################################################
##########################################
conc<-c(1,100)
fit.cipro.env.noec<-
lapply(conc,fx.logis,a=location.log.noec.cipro,b=scale.log.noe
c.cipro)
fit.cipro.env.medmic<-
lapply(conc,fx.logis,a=location.log.medmic.cipro,b=scale.log.m
edmic.cipro)
est.noec.aqua<-lapply(fit.cipro.env.noec,median)
lci.noec.aqua<-lapply(fit.cipro.env.noec,quantile,0.0275)
uci.noec.aqua<-lapply(fit.cipro.env.noec,quantile,0.975)
LowerCI_NOEC<-round(as.numeric(lci.noec.aqua),5)*100
Estimate_NOEC<-round(as.numeric(est.noec.aqua),5)*100
UpperCI_NOEC<-round(as.numeric(uci.noec.aqua),5)*100
print('PAF% AT VICH ACTION LIMITS (NOEC). CONC IS PPB')
print(data.frame(conc,LowerCI_NOEC,Estimate_NOEC,UpperCI_NOEC)
)
est.medmic.aqua<-lapply(fit.cipro.env.medmic,median)
lci.medmic.aqua<-lapply(fit.cipro.env.medmic,quantile,0.0275)
uci.medmic.aqua<-lapply(fit.cipro.env.medmic,quantile,0.975)
LowerCI_MIC50<-round(as.numeric(lci.medmic.aqua),5)*100
Estimate_MIC50<-round(as.numeric(est.medmic.aqua),5)*100
UpperCI_MIC50<-round(as.numeric(uci.medmic.aqua),5)*100
print('PAF% AT VICH ACTION LIMITS (MIC50). CONC IS PPB')
print(data.frame(conc,LowerCI_MIC50,Estimate_MIC50,UpperCI_MIC
50))
##############################################################
##########################################
###SSD
plot########################################################
##################################
##############################################################
##########################################
plot(medianFit.noec.cipro,quantiles,type="l",cex.axis=0.8,cex.
lab=1,xlim=c(-2,5),xlab="Log10PPB",ylab="PAF (%)")
plot.ecdf(medianFit.medmic.cipro,add=TRUE,cex=0.2,lty=0,col="b
lue")
plot.ecdf(upperCI.noec.cipro,pch=16,cex=0.2,lty=0,add=TRUE)
plot.ecdf(lowerCI.noec.cipro,pch=16,cex=0.2,lty=0,add=TRUE)
plot.ecdf(upperCI.medmic.cipro,pch=16,cex=0.2,lty=0,add=TRUE)
plot.ecdf(lowerCI.medmic.cipro,pch=16,cex=0.2,lty=0,add=TRUE)
plot.ecdf(log10(noec.cipro),cex=0.4,add=TRUE,lty=0,pch=23) ##
overlays empirical ecdf noec MIC
plot.ecdf(log10(medmic.cipro),cex=0.4,add=TRUE,lty=0,pch=23)
## overlays empirical ecdf median MIC
abline(v=log10(1),col="red",lty=2);
abline(v=log10(100),col="red",lty=1)
```

```
tiff("SSD1.tiff",units="cm",height=14,width=18,res=700)
plot(medianFit.noec.cipro,quantiles,type="l",cex.axis=0.8,cex.
lab=1,xlim=c(-2,5),xlab="Log10PPB",ylab="PAF (%)")
plot.ecdf(medianFit.medmic.cipro,add=TRUE,cex=0.2,lty=0,col="b
lue")
plot.ecdf(upperCI.noec.cipro,pch=16,cex=0.2,lty=0,add=TRUE)
plot.ecdf(lowerCI.noec.cipro,pch=16,cex=0.2,lty=0,add=TRUE)
plot.ecdf(upperCI.medmic.cipro,pch=16,cex=0.2,lty=0,add=TRUE)
plot.ecdf(lowerCI.medmic.cipro,pch=16,cex=0.2,lty=0,add=TRUE)
plot.ecdf(log10(noec.cipro),cex=0.4,add=TRUE,lty=0,pch=23) ##
overlays empirical ecdf noec MIC
plot.ecdf(log10(medmic.cipro),cex=0.4,add=TRUE,lty=0,pch=23)
## overlays empirical ecdf median MIC
abline(v=log10(1),col="red",lty=2);
abline(v=log10(100),col="red",lty=1)
dev.off()
##################################################################
##########################################
###end############################################################
##########################################
```