RESPONSES OF THE FRESHWATER SNAIL, *LYMNAEA PEREGRA*, TO POLLUTANTS AS AN INDICATOR OF ECOLOGICAL WATER QUALITY.

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by

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DECLARATION

This thesis has been composed in its entirety by the candidate and no part of this work has been submitted for any other degree.

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ABSTRACT

Initially the *L. peregra* bioassay developed for use under laboratory conditions demonstrated reduced ingestion and egestion rates of *L. peregra* at sublethal exposure concentrations to aqueous solutions of cadmium and copper. The two organic compounds tested, the polyaromatic hydrocarbon pyrene and the pyrethroid insecticide lambda-cyhalothrin, did not inhibit ingestion or egestion at sublethal concentrations. Post-exposure median ingestion and egestion values (EC_{50}) for copper were 15 and 13 μg/L and for cadmium were 0.05 and 5.6 μg/L respectively. The lethal effect values for *L. peregra* exposed to copper and cadmium were 115 μg/L (estimated laboratory derived LC_{50}) and 2500 μg/L (LC_{50} from literature) respectively were both higher than the estimated EC_{50} values. This indicates the relevance of using these post-exposure sublethal endpoints for *L. peregra* exposed to these heavy metals. Investigation of the uptake of cadmium by *L. peregra* revealed that the major route was through aqueous solution with only minor contributions from contaminated food.

The *in situ* bioassay was developed further under semi-natural (in microcosms) and field conditions. *L. peregra* showed reduced egestion following aqueous exposure to zinc in microcosm studies (at 0.5 μg/L), but again did not show a response to either of the organic contaminants lambda-cyhalothrin and the herbicide linuron.

The *L. peregra* field bioassay was deployed at 9 historically contaminated sites throughout Scotland and Northern England. Post-exposure egestion rates were shown to be significantly reduced two metal contaminated sites. The inconsistency

of results gained from the remaining sites may have been due to decreased sensitivity brought about by wide variation in egestion rates.

The *L. peregra* bioassay is useful tool for the investigation of the effects of single stressors such as heavy metals. However, the lack of consistent results gained from the *in situ* deployments suggests that the *L. peregra* bioassay should be used, at this time, as one of a suite of tests to investigate the quality of freshwaters rather than as a discrete test.

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CHAPTER 1.

INTRODUCTION.

1.1 GENERAL INTRODUCTION.

The maintenance of good water quality and the restoration of degraded worldwide freshwater resources are rapidly becoming global concerns. With escalating modernisation and industrial development, some degree of harm to these freshwater resources will be associated with various existing activities and processes. It would be unrealistic to maintain that contamination of aquatic systems will not occur either knowingly or as a consequence of industrial processes. With this in mind, tools and procedures must be developed to more accurately monitor contaminants with a view to minimizing pollution in freshwater and the subsequent harm caused to organisms, populations, communities and ecosystems.

Aquatic toxicologists, in general terms, reflect these concerns by developing techniques to determine the potential or actual effects of toxic substances (manufactured chemicals and other anthropogenic and natural materials and activities) on the structure and function of aquatic ecosystems (Forbes and Forbes, 1994). However, the complexity of evaluation at ecosystem level makes the study of individual organisms or their component parts require a more pragmatic approach. Either in the laboratory or *in situ*, the use of assays, which employ single or relatively few species and test methods are valuable tools in the evaluation of risk, pollution monitoring or assessment of recovery.

1.2 FRESHWATER MONITORING IN THE U.K.

Monitoring is an important component of environmental management; it is essential for determining the impact of contaminants on natural ecosystems and for assessing the efficacy of pollution control measures (Maltby *et al.*, 2002). Throughout the United Kingdom (U.K.), regulatory bodies routinely monitor rivers at intervals of between one and six months (Abel, 2000), for the purposes of compliance and surveillance. Compliance monitoring is carried out to ensure contaminant dischargers are meeting regulatory criteria or to oversee long-term water quality. Surveillance is carried out to assess the condition of water bodies in order to check that water resource management programmes or conservation measures are working, or to gauge the impact of non-point source pollution (Rosenberg and Resh, 1993). Monitoring of water bodies in the U.K. is carried out mainly thorough assessment of community structure, with little chemical testing.

Biological monitoring has been used to assess water quality since the early 20th century, and for the most part involves sampling benthic macroinvertebrate community structure. Benthic macroinvertebrates are a highly diverse group inhabiting lakes and streams, where they are important in moving energy through food webs. Different groups of macroinvertebrates vary in their tolerance to pollution, which lead to the creation of indices of water quality based on the presence or absence of certain species. The indices most often used in the U.K., are those based on the Biological Monitoring Working Party (BMWP) score system (Clarke *et al.*, 2002). Indices are derived by scoring the perceived tolerance of each family present in a sample. Significant deviation from a previously recorded

condition or an unusually low score may indicate a pollution incident or a failure of implemented water resource management programs. However, scores for individual sites will vary due to differences in their physical attributes (Wright *et al.*, 1993) making direct comparisons between locations problematic.

This approach has been further developed in the U.K., where multivariate methods employing site-specific environmental information to predict community structure have been employed. The River Invertebrate Prediction and Classification System (RIVPACS) (Wright *et al.*, 2000) was developed for use in the U.K. and provides a framework for assessing the ecological quality of river sites. This method compares the difference between the predicted biotic score (derived from reference site data including physical and chemical measurements), and the actual biotic score.

Defining the biological quality of rivers using the resident benthic macroinvertebrate community has its limitations. Sampling community structure is costly and time consuming and species distribution may be affected by seasonal variations in dispersal. Additionally, when impacts on community structure have been determined, the damage has already been done, as community level measures are insensitive to sub-lethal levels of stress in the short term (Maltby, 1999).

Other biological methods of water quality assessment exist such as the Mean Trophic Ranking (MTR), which uses the trophic tolerance of aquatic macrophytes to generate a score, which reflects the assemblage of plants present at a site (Holmes *et al.*, 1998). River water quality may also be assessed on the basis of fish abundance, habitat suitability and fish abundance relative to habitat quantity. One

such system is the Environment Agency's Fisheries Classification Scheme (FCS), which classifies river reaches in terms of excellent, good, fair etc. according to the abundance of fish within different salmonid and coarse fish groups (Raven *et al.*, 1998).

Chemicals occurring in receiving waters are evaluated mainly through highly specific measurements of single substances. The release of these single substances into the aquatic environment in the U.K. is controlled through use of laboratory defined Environmental Quality Standards (EQS). EQS are generally based on the results of exposure and toxicity data reported in the published literature or measured in laboratories. The integration of these measurements into a definition of water quality generally comes as a result of laboratory-based effects testing. This compound specific approach may fail to take into account natural geographic variation in water chemistry such as pH and dissolved oxygen (Norris and Thoms, 1999), as well as any additive, synergistic or antagonistic effects.

The discharge of effluent in the United States has been regulated through the use of Whole Effluent Toxicity tests (WET) for over a decade (Maltby *et al.*, 2000). A similar approach is being developed in the U.K. WET tests involve the application of whole effluent assessment as an alternative or supplement to a substance specific approach (Gert-Jan de Maagd, 2000), and are generally performed under controlled experimental conditions using standard species and the results extrapolated to field situations. Currently these tests are increasingly used to monitor compliance of consented discharges. The WET approach measures toxicity directly, but is unable to identify the individual toxic components of the effluent (Maltby *et al.*, 2000). In

addition, the collection and transport of water samples from the field to the laboratory may alter toxicity, resulting in poor agreement between field and laboratory results.

For some time now, researchers have recognised the need to combine traditional monitoring techniques with toxicity testing to produce a more comprehensive strategy for the protection of freshwater resources. Through the implementation of the Water Framework Directive (WFD), the EU has committed to attaining "good water status" for all surface and ground waters. This legislation incorporates a combined approach to controlling pollution at source through the setting of EQS and Emission Limit Values (ELV). The WFD provides water quality objectives (WQO) for rivers, lakes, coastal waters and groundwater. The implementation of the Water Framework Directive aims to improve the quality of the water environment through the control of hazardous substances and of water quantity, leading to the sustainable use of aquatic ecosystems. The implementation of this legislation has made it possible for European scientists and government to explore new monitoring techniques and establish innovative tools for evaluating water quality and the impact of contaminants on aquatic ecosystems.

1.3 GENERAL INTRODUCTION TO BIOASSAYS.

Generally bioassays are carried out as a means of determining the concentration of a chemical in the environment that will produce no adverse effects (Cairns and Pratt, 1989). These effects levels are predicted following the exposure of a living test organism (usually a single species) to certain chemical concentrations for defined

periods of time. The cladoceran *Daphnia magna* is currently the most frequently used and well known test organism (Cairns and Pratt, 1989; Maltby and Calow, 1989). Other standard laboratory species include marine copepods (eg. *Acartia tonsa*), marine bivalves (eg. *Crassostrea gigas, Mytilus edulis*), freshwater and marine fish (eg. *Oncorhynchus mykiss, Pimephales promelas, Cyprinodon variegatus*), earthworms (eg. *Eisenia foetida*) and birds (eg. *Anas platyrhynchos, Colinus virginianus*) (Calow, 1998). These tests estimate the LC_{50} or LD_{50} of a chemical singly or in a mixture, generally employing mortality as an endpoint. One of the perceived advantages of laboratory-based toxicity tests is that if adverse effects occur they can be ascribed solely to the contaminant under study as all other confounding variables have been controlled.

While the outcomes of these tests are superior to predictions made on the basis of chemical or physical measurements alone, questions still remain with regard to the extrapolation of results from laboratory to field situations. Single species laboratory tests have been criticised for oversimplifying response (Forbes and Forbes, 1994), and overestimating the magnitude and duration of exposure relative to that observed in the field (Pratt, 1991), which may lead to an overestimation of toxicity. Under laboratory conditions test organisms do not experience natural changes in abiotic factors that may affect toxicity and so are open to criticism for lack of realism as well as lack of correlation to field conditions.

1.3.1 *IN SITU* BIOASSAYS AND SUBLETHAL ENDPOINTS.

In situ bioassays use a selected organism to provide an estimate of toxicity and relative potency of a toxicant or pollutant under field conditions. Organisms exposed to contaminants under natural conditions will provide a more realistic picture of the potential for toxicity, through the removal of artifacts associated with laboratory testing (Chappie and Burton, 1997) and by taking into account naturally occurring factors that may modify toxicity such as chemical, physical and biological processes (Rand *et al.*, 1995). Results obtained using *in situ* bioassays are therefore appreciably more relevant to the natural situation than those of laboratory experiments (Schulz and Liess, 1999).

In situ testing strategies include a wide range of experimental designs, from simple indoor multi-species assemblages to field studies (Sanchez and Tarazona, 2002). *In situ* single species bioassays traditionally employ resident or transplanted organisms, and measure endpoints based on lethal or sublethal responses (Crane *et al.*, 1996). Recently, studies using caged transplanted animals have become increasingly popular. These tests use either laboratory reared organisms or animals collected from 'clean' sites which are placed in cages and transplanted to the contaminated site for a defined period of time.

A number of recent studies have also highlighted comparisons between *in situ* testing and laboratory results. Ringwood and Keppler (2002) conducted sediment toxicity assays with juvenille *Mercenaria mercenaria* to compare results from laboratory and field deployments. They reported that the laboratory-based studies

underestimated potential toxicity at degraded sites when compared to results from *in situ* tests. Good agreement between *in situ* and laboratory testing was reported by Pereira *et al.* (1999), at most acid mine drainage sites and control sites using *Daphnia magna* and *Ceriodaphnia dubia.* However, at the intermediately polluted sites, *in situ* toxicity was generally higher than in classical laboratory-based tests. In this case some aspects of sample collection, transport or preservation may have altered actual toxicity. Peeters *et al.* (2001) reported that laboratory bioassays using *Chironomus tentans* and *Daphnia magna* were more weakly correlated with the in *situ* macroinverebrate composition than chemical measurements. Tucker and Burton (1999), reported differences in the toxicity of urban and agricultural runoff under field and laboratory conditions to *Chironomus tentans* and *Hyalella azteca.* In this study agricultural runoff was more toxic under field conditions while urban runoff was more toxic to organisms in the laboratory. Caution must be used when extrapolating results from the laboratory to field situations. Laboratory results may not adequately reflect the species' response in the field nor the risk posed by a specific contaminant in aquatic ecosystems. These studies also indicate the need to incorporate bioassays, laboratory studies and monitoring programs into risk assessment procedures as they reveal different and often complementary information on ecotoxicological effects.

With any single-species approach, the question of which species to use always arises. Table 1.1 outlines a number of recent studies, which have reported results of *in situ* bioassays on a variety of organisms. The choice of test species should be determined by the question being addressed, but, in general, test species should be

sensitive and ecologically relevant (Maltby, 1999) and easily cultured or collected from the field.

Single species *in situ* bioassays can be used as early warning indicators of water quality as effects measured at the individual level will often be manifested more rapidly (hours to days) than resulting changes in community structure (months to years) as measured by benthic macroinvertebrate surveys (Maltby, 1994). By selecting ecologically relevant species and endpoints, it is possible to use the results of single-species *in situ* bioassays as short-term predictors of more long-term effects on populations and communities.

Under natural conditions organisms are not typically exposed to high acutely toxic concentrations of contaminants and as chemicals disperse, dilution will occur, so a greater number of organisms will be exposed to sublethal concentrations (Maltby, 1999). This does not mean that organisms exposed to lower than lethal concentrations of contaminants will not be harmed. For this reason toxicity tests employing death as an endpoint will not be adequately sensitive to measure the effects of a sublethal contaminant concentration which may have far reaching consequences for a population. Sublethal endpoints are acknowledged as more sensitive, as often the first reaction of an organism to stress is a physiological one (Gerhardt, 1996).

Sublethal concentrations may ellicit one of three classes of effects: histological, biochemical/physiological and behavioural (Shehan *et al.*, 1984). Histological effects are manifested through the modification of tissue and organ function while biochemical and physiological effects appear as changes in proteins, haematology

Table 1.1: Examples of test organisms used for *in situ* bioassays and endpoints under study.

Organism	Endpoint	Reference
Mercenaria mercenaria	Death and growth	Ringwood and Keppler, 2002
Corbicula fluminea	Death	Soucek *et al.*, 2000
Daphnia magna	Feeding rate	McWilliam and Baird, 2002b
	Feeding rate	Barata *et al.*, 2002
Ceriodaphnia dubia	Death	Ireland *et al.*, 1996
	Death, growth, fertility and reproduction	
Gammarus duebeni	Pleopod beat frequency and swimming endurance	Lawrence and Poulter, 1998
Gammarus pulex	Death and feeding rate	Crane *et al.*, 1995
	Death	Schulz and Liess, 1999
	Feeding rate	Maltby *et al.*, 2002
Anodonta cygnea	Glutathion-S-transferase activity	Crane *et al.*, 1995
Chironomus riparius	Emergence	Crane *et al.*, 1995
Chironomus tentans	Death and growth	Sibley *et al.*, 1999
	Death	Tucker and Burton, 1999
	Death	Chappie and Burton, 1997
Limnephilus lunatus	Death	Schulz and Liess, 1999
Lumbriculus variegatus	Death and growth	Sibley *et al.*, 1999
Pimephales promelas	Growth	Siwik *et al.*, 2000
Hyalella azteca	Death	Tucker and Burton, 1999
	Death	Chappie and Burton, 1997

and respiration. Behaviour represents an integrated response, corresponding to complex biochemical and physiological functions, so chemically induced changes in behaviour may reflect effects on internal homeostasis making them sensitive indicators of sublethal effects (Shehan *et al.*, 1984). Behavioural effects include locomotion and swimming, feeding, attraction/avoidance, prey/predator relationships, aggression and territoriality, and learning (Rand *et al.*, 1995).

In order for a sublethal response to be useful as an indicator of pollutant effects, it is important to establish normal background variability against which toxicant-induced changes can be detected (Maltby, 1999). This requires the measurement of a repeatable and reproducible endpoint, which is affected by sublethal concentrations.

1.3.2 FEEDING AS AN ENDPOINT.

Feeding inhibition is a general stress response exhibited by a wide variety of organisms and has been used as an endpoint in a number of studies e.g. Ferrando *et al.*, 1993; Malbouisson *et al.*, 1995; Jacobsen and Forbes, 1997; Leppanen and Kukkonen, 1998; Jensen *et al.*, 2001; Mendez *et al.*, 2001; Maltby *et al.*, 2002; McWilliam and Baird, 2002a. Reduced feeding rate is one mechanism that can potentially explain reductions in survival, growth and reproduction occurring in toxicant-exposed organisms (Jensen *et al.*, 2001) and in the case of grazers, to indirect effects on algal community structure (McCormick and Stevenson, 1989).

Many authors have investigated the effect of contaminants on feeding rates and behaviour, using organisms from different trophic levels ranging from *Daphnia* sp. to fish. Feeding inhibition can be affected though impairment of chemoreception

altered behaviour (such as foraging strategies) and through direct poisoning. There are a number of studies examining the effects of various contaminants on feeding inhibition in *Daphnia* sp. eg. Ferrando and Andreu, 1993; Fernandez-Casalderrey *et al.*, 1994; Allen *et al.*, 1995; Taylor *et al.*, 1998 and McWilliam and Baird, 2002a. Further examples of animals demonstrating altered feeding behaviour in the presence of contaminants include the woodlouse *Porcellio laevis*, which as reported by Odenaal and Reinecke (1999) showed marked preference for uncontaminated oak leaves when offered a choice between those and leaves contaminated with a sublethal concentration of cadmium. (Woodward *et al.*, 1995) reported that rainbow trout (age-0), when fed a diet of invertebrates collected from a metal contaminated site, exhibited feeding inhibition as compared with the control population. This was due to a more than 50% reduction in feeding activity measured as prey strikes per minute over an 88-day exposure period. Some animals may show an initial preference for contaminated food, as found by Stewart *et al.* (1992) with *Gammarus* sp. During a 48 hour trial *Gammarus* sp. exhibited a preference for food contaminated with a mixture of polychlorinated biphenols (PCBs), cobalt, copper, cadmium, nickel, mercury, uranium and zinc. However in a longer 7 day trial the contaminated food was avoided. These and other studies outlined in table 1.2, demonstrate the usefulness of reduced feeding as a sensitive endpoint in toxicity testing, which has been shown to be effective when using a range of organisms with a variety of contaminants.

The acquisition and allocation of energy determines growth and developmental rate, fecundity and survival, all of which are important components of fitness and determinants of population structure and dynamics (Maltby, 1994). At the

individual level only two types of effects are of interest: survival and reproduction, which will ultimately determine population dynamics (Kooijman, 1993). Depressed food intake will mean reduced growth and as smaller animals take in less food therefore less energy is available for reproduction. These short-term changes in food intake at the individual level can lead to long-term changes in population dynamics, which have the potential to alter community structure and function. Information gained from short-term bioassays may provide insight into the mechanisms of long-term community structure alterations, or give warning of potential contaminant-induced impacts at the community level (McWilliam and Baird, 2002b).

Table 1.2: Examples of studies employing changes in feeding rate and/or feeding behaviour as an endpoint.

Organism	Contaminant	Endpoint	Author
Daphnia magna	Copper	Behaviour	Ferrando and Andreu-Moliner, 1993
Daphnia magna	Pesticides	Behaviour	Fernandez-Casalderey *et al.*, 1994
Daphnia magna	Cadmium vanadium	Feeding rate	Allen *et al.*, 1995
Daphnia magna	Cadmium	Feeding rate	Taylor and Soares, 1998
Daphnia magna	Various	Feeding rate	McWilliam and Baird, 2002a
Gammarus pulex	Lindane	Feeding rate	Malbouisson *et al.*, 1995
Gammarus pulex	Various	Feeding rate	Maltby *et al.*, 2002
Radix peliculas	Cadmium	Feeding rate	Lam, 1996
Potamopyrgus antipodarum	Cadmium	Feeding rate	Jensen *et al.*, 2001
Capitella sp.	Fluoranthene	Feeding rate	Mendez *et al.*, 2001
Nassarius festivus	Heavy metals	Behaviour	Cheung *et al.*, 2002
Chaoborus sp.	Cadmium	Feeding rate	Munger and Hare, 2000
Lepomis macrochirus	Copper	Feeding rate	Sandhinrich and Atchison, 1989
Lepomis macrochirus	Cadmium	Behaviour	Bryan *et al.*, 1995
Cyprinus carpio	Nickel chromium	Feeding rate	Thantheyus, 1992
Oncorhynchus mykiss	Various	Behaviour	Woodward *et al.*, 1995

1.4 MOLLUSCS AND CONTAMINANTS.

Pond snails have been used to evaluate the toxicity of various types of pollution. They have been used to monitor and evaluate the toxicity of metals such as cadmium (Gomot, 1998), copper, chromium, mercury, zinc, nickel and silver (Khangarot and Ray, 1988) and aluminium (Elangovan *et al.*, 1997; Desy *et al.*, 2002). They have also been used to test the toxicity of pesticides (Bluzat and Seuge, 1983), insecticides (Woin and Bronmark, 1992) and polyhalogenated biphenyls (Wilbrink *et al.*, 1992).

A considerable body of literature exists regarding the use of molluscs to monitor levels of metal pollution. Heavy metal concentration in the tissue is generally correlated with the concentration of the metal in the environment (Loeb, 1994). The actions and interactions of a large set of environmental factors controlling bioavailability and chemical speciation will influence the degree of metal transfer from the surrounding media to the organism. Most metals taken up by lymnaeids are stored intracellularly or sequestered by vesicle-bound granules and cytosolic proteins (Baudrimont *et al.*, 1997). These have been shown to play an important role in controlling the kinetics of bioaccumulation and the expression of toxic effects through the reduction of the metal's accessibility to functional binding sites (Baudrimont *et al.*, 1997).

Elangovan *et al.* (1997) reported a significant accumulation of aluminium in the whole soft tissues, gut, digestive gland and kidney of *L. stagnalis* over a 10 day exposure. When transferred to clean water rapid loss of aluminium from the gut

and soft tissues was observed, with approximately 90% remaining in the digestive gland. They concluded from this study that the most likely route of entry for aluminium was the gut. There is, however, a paucity of information on the effects that metal accumulation might exert upon gastropod metabolism and physiology.

Very few studies have investigated the occurrence of xenobiotic-metabolising enzymes and the effects of environmental contaminants on their activities in freshwater molluscs. Wilbrink *et al* (1991) have reported the activity of glutathione S-transferase detoxification mechanism in the digestive gland of the pond snail *L. stagnalis* (Wilbrink *et al.*, 1991). Baturo and Lagadic (1996) also reported the presence of glutathione S-transferase as well as benzo[a]pyrene hydroxylase in *L. palustris* when exposed to atrazine and hexachlorobenzene. The low inducibility of mollusc xenobiotic-metabolizing enzymes by chlorinated compounds was re-confirmed by this study.

Few studies exist which consider the sublethal effects of contaminants on the behaviour of lymnaeids and the possible consequences for natural populations and the subsequent alteration of river function. A bioassay for use in both the laboratory and the field measuring sublethal effects using *L. peregra* over a short time period would be a useful tool for possible integration with monitoring programmes and would provide some insight into the effect of contaminants on the wandering snail.

1.5 TEST ORGANISM.

1.5.1 GENERAL.

The gastropod family Lymnaediae constitute a large and widely distributed group, which has been examined extensively with cited literature dating to over a century ago (Van der Schalie and Berry, 1973).

The species *Lymnaea peregra,* (Basommatophora; Pulmonata; Gastropoda) is widely distributed throughout the British Isles and may be the most common freshwater snail in Europe (Fitter and Manuel, 1986) found in ditches, ponds, streams and lakes. Lymnaeidae are amphibious, living in the littoral regions of streams and rivers. A thin, fragile shell with an elongated spire protects the soft body of *L. peregra.* The body whorl is large with a big aperture and no operculum. They have a large, highly vascularised mantle cavity in which air is renewed by periodically venturing to the water surface and opening the pneumatostome into the external atmosphere (McMahon, 1983). The head has a single pair of non-invaginable tentacles with basal eyes, as do all Basommatophora (McMahon, 1983). These snails are morphologically variable and their taxonomy is unclear (Islam *et al.*, 2001); there are often small variations in shell forms between populations and it is generally acknowledged as a plastic species in this regard. Many authors have described the various shell shapes of aquatic gastropods from both natural and laboratory populations. Despite small differences between populations in separate habitats, anatomical dissimilarities in characteristics of the forms *L. ovata, L. involuta* and *L. p. burnetti* have been shown not to justify their specific separation

from *L. peregra* (Boycott *et al.*, 1930; Hubendick, 1978; Ward *et al.*, 1997; Islam *et al.*, 2001). Arthur, 1982 reported that the differences in the shell shape of two populations of Irish *L. peregra* were mainly due to direct environmental effects. He also noted that the similarity between the shell shape of two laboratory cultured populations was caused by a shift in the shell morphometrics of the lotic population to the lentic phenotype, while the shell shape of the natural pond population was retained in the culture, presumably approximating lentic conditions. Three shell forms have been recognised as occurring in laboratory manipulated populations by Evans (1989), sub-involuta, involuta and normally-spired. In particular, the forms *peregra* and *ovata* distinguished by shell shape are often considered variants of the same species (Paul *et al.*, 1997).

1.5.2 Reproduction.

L. peregra is a simultaneously hermaphroditic gastropod which at birth measures approximately 0.8 to 1 mm (Brendelberger, 1997a), and will grow up to about 20 mm (Dussart, 1997). *L. peregra* is often described as iteroparous under natural conditions (Calow, 1978), with a concentrated egg-laying period and only one or two generations in a year (Skoog, 1976).

As a self-fertilising functional hermaphrodite (Diver *et al.*, 1925), *L. peregra* can store viable sperm for several months after copulation for use in cross-fertilisation (Boycott *et al.*, 1930). Cross-fertilisation occurs preferentially over self-fertilisation whenever allosperm is available (Boycott *et al.*, 1930). Eggs are laid in gelatinous capsules containing batches of up to several hundred eggs that hatch from late

spring to autumn (Jarne and Delay, 1990), with wide variation in number of eggs per capsule reported in the literature. Under field conditions, Islam *et al.* (2001) observed the maximum eggs/snail/ season was 320.74 ± 56.43, which within the same range but lower than the number recorded by Calow (1978) under laboratory conditions at 300-1100 eggs/snail/season.

1.5.3 FOOD AND FEEDING.

L. peregra is a grazer, crawling over surfaces pushing material into their mouths, taking a 'bite' 24 to 60 times/min (Dillon, 2000). During feeding the proboscis is extended and folded over the section of substrate to be grazed, the jaws open and the radula is protruded. The radula is a ribbon with hundreds of fine teeth mounted on a tongue-like mass called an odontophore. As a patch of substrate is scoured the odontophore is withdrawn and the jaws are closed. A thick cuticle on the jaws facilitates the breakdown of large particles or filaments with a gizzard at the entrance to the stomach, which serves a grinding function (Dillon, 2000). Sand grains feature prominently in the stomach of *L. peregra* as found by Calow (1970) and Storey (1970). The contribution made by sand grains to the nutrition of *L. peregra* is not fully understood. According to Storey (1970), sand grains in the diet enhance the growth of *L. peregra.* The generally accepted view is that they help grind food in the gizzard and may also provide micronutrients.

Only the smallest particles (0.4μm or less) pass from the stomach to the digestive diverticulae, where both phagocytosis and extracellular digestion have been reported (Dillon, 2000). Undigested material passes out of the stomach and is

compacted into faecal pellets in the upper intestine. Three different types of faeces have been recognized in *L. peregra*, the gizzard string (containing indigestible material), the caecal string (a mucoid cementing string) and the liver string with the bulk of digestion and assimilation taking place in the liver (Calow, 1970). Faeces are moved through the intestine to the anterior, where they are expelled from the right mantle edge. Brendelberger (1997b) has documented voluntary and regular coprophagy in *L. peregra*, but the possible benefit has not yet been determined. As some diatoms and green algae may pass through the gut undigested (Underwood and Thomas, 1990), coprophagy may be a method to further degrade this material.

For such a common and widespread grazer, surprisingly little is known about its natural diet aside from a general preference for periphyton and detritus. In a study of gut content, Calow (1970) identified approximately 70% diatoms, 25% filamentous green algae and 5% other algae. Analyses of the gut contents of various populations of *L. peregra* revealed them to be indiscriminate grazers with studies on food choice and preference often reaching conflicting conclusions.

L. peregra has been shown to grow and reproduce on a wide variety of diets including macrophytes, filter paper, rotting bark, rotting leaves (Storey, 1970), blue-green algae (Storey, 1970; Skoog 1978), diatoms (Calow, 1970; Skoog, 1978) and spinach (Skoog, 1978). Presently many researchers culturing lymnaeids find that they grow effectively on a diet of fish food and lettuce.

1.5.4 IMPORTANCE IN THE FOOD WEB.

Herbivory can be a key process in regulating the structure and function of primary producers in many ecosystems (McCormick and Stevenson, 1998) usually through the reduction of algal standing crops and rates of productivity and by altering the composition of periphyton communities (Mulholland *et al.*, 1991; Lamberti *et al.*, 1995). Grazers can also benefit host macrophytes by removing epiphytes that would otherwise compete with them for nutrients and light (James *et al.*, 2000). Different types of herbivores may have varying effects on the biomass and structure of periphyton, due to differences in grazer feeding structures, ingestion rates, habitat preferences, or behaviour (Hill and Knight, 1988).

Herbivores may offset the effect of low external nutrient supplies available to stream periphyton communities by releasing nutrients assimilated by algae back to the water in an available form. Increased nutrient levels can cause a shift from communities dominated by diatoms to communities dominated by filamentous green algae (Fairchild *et al.*, 1985). Nutrient addition also accelerates the successional process by causing more rapid replacement of species (Fairchild and Lowe, 1984).

Grazing can also have the opposite effect. Grazed communities are often dominated by either prostrate species (which adhere tightly to the substrate), or by small under storey species which can avoid being grazed by virtue of their size (Sumner and McIntire, 1982). Increased turnover rates have been demonstrated through measurement of the carbon: nitrogen ratio (Hunter, 1980) and oxygen evolution

(Lamberti and Resh, 1983) in highly grazed periphyton, which is also characteristic of rapid turnover during early succession. This demonstrates that grazing might also maintain periphyton communities in earlier successional states.

Snail density and distribution patterns will also be affected by predators such as fish, crayfish and leeches to a greater extent than by competition (Lodge, 1986). Dillon (2000), reviews the many predators of molluscs from mammals to invertebrates and gives evidence of the effect of predation on population size, life history and community effects. Dillon (2000), comments that most studies regarding the relationship between mollusc distribution and predation seem to suggest negative correlations although positive correlations or none at all, are often reported.

1.6 CRITERIA FOR THE CREATION OF A BIOASSAY

Attention to the following criteria during the development of a new ecotoxicity test will produce a more robust procedure, the results of which will be useful for regulatory purposes.

RELEVANCE

Relevance of a test may be addressed by focusing on the following three questions:

- To what extent do results obtained from the chosen test relate to adverse changes in the ecological system under study (Calow, 1996)?

- Is the test species chosen sensitive and does it contribute towards the functional integrity of a community or represent a particular trophic group (Calow, 1989)?
- Is the test endpoint ecologically relevant? If so it should be possible to extrapolate effects to other trophic levels (Cairns and Pratt, 1989).

RELIABILITY

Calow (1996) defines reliability as the ability to make observations in a controlled way and with confidence. Reliability infers that the results can be repeated and reproduced with little variability i.e. the same test performed with the same substance should give comparable results. This is important for regulatory purposes as tests must be sufficiently standardized and simple that they are easily carried out in laboratories of widely varying capabilities (Cairns and Pratt, 1989). The use of a readily available test organism and established methods to ensure quality of individuals may go partially toward the standardization and simplification of the procedure. Additionally background variation in the response should be kept to a minimum to increase detection of an impact.

SENSITIVITY

The response should be sufficiently sensitive to avoid excessive Type II errors (false negatives). False negatives can be kept to a minimum by ensuring apt statistical power. The sensitivity of the test will ultimately be dictated by the responsiveness or susceptability of the chosen organism to toxicant action and how this relates to adverse ecological effects (Cairns and Pratt, 1989).

COST AND TIMING

A simple test procedure i.e. one that keeps time, space and level of expertise to a minimum will result in a test with little associated costs. The creation of a simple test procedure may involve some compromise between variables keeping the cost and timing to a minimum and those maintaining that will produce the best possible results.

1.7 AIMS.

The aim of this study was to create a novel bioassay to assess the effects of changes in water quality on macroinvertebrate grazer response. The bioassay was initially developed for use in the laboratory, with the eventual aim of adapting it for *in situ* deployment. The work presented in this thesis is part of the European Union (EU) funded Integrated Assessment Tools to Gauge Local Functional Status Within Freshwater Ecosystems (TARGET). The central aim of TARGET was to develop a novel approach to the establishment of ecologically based standards for river water quality based on ecosystem function. A set of rapid bioassessment tools were developed to determine the ecological quality of rivers under the European Commission's Water Framework Directive, which could be applied (with minor modifications) across the European Union. These tools or bioassays are ultimately intended to facilitate management decision making, by supplying information on the state of key ecological processes.

The main aims of this thesis were as follows:

1. To develop a method for the *L. peregra* bioassay under laboratory conditions (Chapter 2) including mode of feeding as well as baseline feeding and egestion rates.
2. To demonstrate the use of post-exposure reduction of egestion as an endpoint under laboratory conditions when exposed to various contaminants (Chapters 3 and 4)
3. To determine the main uptake route of metals to *L. peregra*: food or water? Is uptake related to toxic response and is growth affected by cadmium exposure (Chapter 5)?
4. To develop the laboratory bioassay for deployment under in situ conditions and demonstrate the ecological relevance of the test in relation to other bioassays deployed in microcosms (Chapter 6).
5. To deploy the bioassay at upstream (control) and downstream (polluted) field sites, historically contaminated with various types pollutants, to assess the use of the test in the field (Chapter 7).

CHAPTER 2.

GENERAL MATERIALS AND METHOD DEVELOPMENT

The following chapter gives details of general methods for the collection, culture and maintenance of the pulmonate gastropod *L. peregra*. This chapter will also consider the characterisation of ingestion and egestion rates for the development of a bioassay using *L. peregra,* and the determination of optimal conditions for its use in the laboratory. More specific protocols regarding experimental test systems, the use and deployment of the bioassay and chemical analyses are provided in future chapters.

2.1 LABORATORY CULTURE AND MAINTENANCE OF *LYMNAEA PEREGRA*.

A necessary component of any laboratory toxicity test is the ability to reproduce and repeat both experiments and results (Baird *et al.*, 1989). Animals used in standard toxicity testing generally have defined culture procedures in order to ensure reproducibility and repeatability between laboratories. *Daphnia* sp. culture is perhaps the best example of an attempt to standardise culture techniques within and between laboratories. However, even with the implementation of rigorous culturing and testing protocols for *Daphnia* sp., as outlined by the American Society for Testing and Materials (ASTM) (1980), and American Public Health Association/American Water Works Association/Water Environment Federation (APHA-AWWA-WEF) (1995) reproducibility between laboratories is not guaranteed.

Despite the amount of literature regarding the use of lymnaeids in laboratory experiments, there is a lack of specific information addressing their culturing techniques. The ability to culture successive generations of *L. peregra* in the laboratory has been reported by many authors (i.e. Van der Schalie and Berry, 1973; Van der Steen *et al.*, 1973; Calow, 1981). These authors include comments concerning optimum culture temperature, food and water quality for lymnaeid cultures kept in the laboratory. For example, Calow (1981) suggests a water temperature of between 16 and 22°C and a pH of approximately 8 for maximum growth, survival and fecundity of *L. peregra* in culture. Despite the apparent ease with which many scientists maintain and culture Lymnaeids under laboratory conditions, no specific set of parameters defining optimal conditions have been put forward. There are, however, numerous suggestions in the literature, not all of which are applicable under every circumstance. For example, several authors have successfully cultured *L. stagnalis* using aerated tap water. However, this has not worked in every culturing system as evidenced by the range of media formulations found in the literature. With this in mind, optimal culturing conditions and husbandry of *L. peregra* were investigated over a period of approximately 8 months and the conclusions are described in the following section.

The aim of this section was to describe general techniques for culture and maintenance of *L. peregra* throughout the duration of the project. Additionally, a possible re-circulating system for culturing *L. peregra* is described.

2.1.1 MEDIUM.

The formula for the artificial medium used to maintain all *L. peregra* cultures throughout the duration of this work was obtained from Dr. Heidi Smith (Staffordshire University) in June 2000. Medium was prepared and stored in 10 L plastic carboys, using 5 inorganic salts dissolved in distilled water. The salts used are outlined in Table 2.1.

Table 2.1: Stock concentrations of Analar grade inorganic salt solutions used to prepare snail medium and volume of each added to 10L of distilled water.

Salt	Concentration (g/L)	Volume used to make 10 L (ml)
$KHCO_3$	11.1	3.3
KNO_3	15.0	3.3
$MgSO_4$	96.1	3.3
$NaHCO_3$	79.9	6.7
$CaCl_2 \cdot 2H_2O$	294.1	10.0

The medium had pH between 7.7 and 8.2 and water hardness of 205 mg/L total hardness, 192 mg/L $CaCO_3$ hardness.

2.1.2 FOOD.

Under maintenance culture conditions animals were fed on a diet of Tetramin fish food (Tetrawerk, Germany), supplemented with boiled, pureed baby spinach. Spinach was boiled vigorously at 1 g wet weight spinach to 1 ml snail medium for 5 minutes, then cooled and macerated using a hand blender (Moulinex, Turbomix) for 2 minutes.

2.1.3 *L. PEREGRA* CULTURE AND MAINTENANCE.

Snails were collected as required from one of two sites. During the experimental period March 2000 until June 2001, animals were collected from the margin of the River Devon (NN989043) Figure 2.1. From June 2001 until the completion of experimental work in March 2003, animals were collected from Howietoun Fishery (NS78308845) Figure 2.1, Stirling, Scotland by sweeping under and around submerged macrophytes using a pond net (1 mm mesh).

Table 2.2: Water quality parameters courtesy of the Scottish Environmental Protection Agency (SEPA) database for spring 2002, from two sites used for snail collection.

Parameter	River Devon	Howietoun
Dissolved Oxygen (mg/L)	12.3	10.6
pH	7.3	7.5
Suspended Solids (mg/L)	2.1	3.73
Temperature (°C)	7.7	12.1
Conductivity (µs/cm)	74.1	93
Alkalinity	28.8	40.67
Nitrites (mg/L)	0.002	0.021
Nitrates (mg/L)	0	0.177
Ammonia (mg/L)	0.012	0.137
Phosphates (mg/L)	0.008	0.08

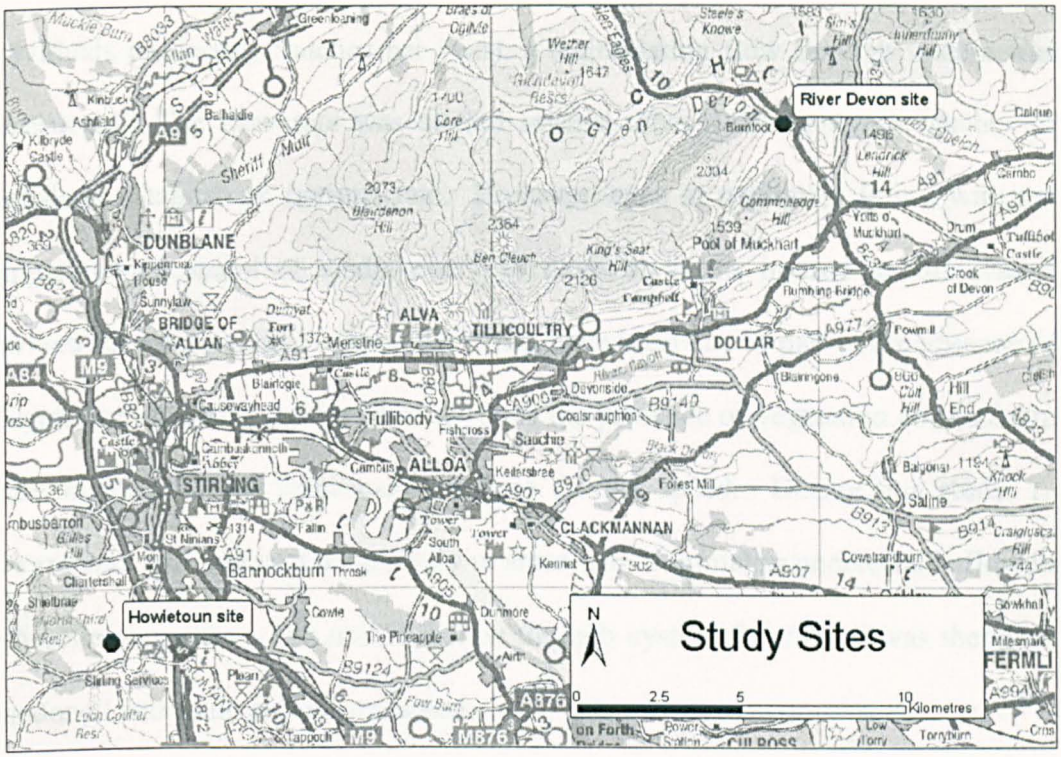


Figure 2.1: Location of sites used for collection of *L. peregra*.

Upon return to the laboratory, collected animals were maintained at 20°C (±1°C) constant room temperature, with 16 hours light to 8 hours dark photoperiod. Cultures comprising 10 to 15 animals were kept in 6 L plastic aquaria containing 5 L of media. Individual tanks had a rigid plastic perforated lid to prevent escape while still permitting surfacing. Aeration was provided by an Algarde aquarium air pump, model 5000S, with airline and air stones attached which fed into each tank. The level of aeration in each tank was controlled through an adjustable tap attached to the airline feeding each aquarium. Approximately 0.5 g of Tetramin fish food (Tetrawerk, Germany) and 6 ml of boiled, macerated spinach was supplied to each tank every 5 to 7 days. Aquaria were cleaned and artificial freshwater medium (see Table 2.1) was exchanged every 6 to 7 days.

In order to increase the culturing capability (i.e. to increase the number of individuals producing clutches per year), a recirculating flow through system was constructed. The flow rate was altered several times with numerous batches of animals to establish an optimal rate. However, even at negligible flows, with the complete replacement of media every 14 days, no snails housed in this system survived for longer than 21 days. This may have been due in part to the absence of refugia, as even in the fastest flowing rivers the presence of vegetation and rocks in a stream channel can significantly reduce current speed. During this study, *L. peregra* was found almost exclusively in areas with minimal or undetectable flow in both collection sites. The use of a flow through system for culture was therefore abandoned and static cultures resumed.

2.2 OPTIMISING CULTURE CONDITIONS – OXYGEN REQUIREMENTS.

Lymnaeids can remain submerged for long periods of time. However, under conditions of low dissolved oxygen or high temperature they recharge their pulmonary bubble using atmospheric oxygen (Berg and Ockelman, 1959). *L. peregra* will move to the air/water interface to obtain oxygen, or follow an environmental oxygen gradient in the water. Either or both of these patterns may be used in the two-stage search mechanism previously described for scent-following in pulmonates (Green *et al.*, 1992).

When the population density increases and access to the surface is restricted through a size limitation of the air/water interface, some snails will leave the water as a consequence of pheromonal or behavioural interactions (Green *et al.*, 1992). The

major factors contributing to leaving the water are: temperature, population density, distance from the water surface and to a lesser extent dissolved oxgen content of the water. *L. peregra*, as reported by Green *et al.* (1992), surfaced at rates which appeared to be relatively independent of dissolved oxygen. Some populations studied by Russell-Hunter (1953) surfaced at mean intervals of 18 minutes; however animals from deeper lake waters survive throughout the entire life cycle on purely cutaneous respiration.

The aims of this section were to assess whether or not *L. peregra* cultured in the laboratory, from field collected animals, could survive fully submerged (i.e. without surfacing) for up to 5 days in aerated media. This information was necessary for input into the design of the laboratory and field bioassays as snails would be fully submerged for up to 3 days.

2.2.1 Materials and Methods.

Second generation *L. peregra* collected from the River Devon site were used in this study. The water surface of 6 tanks each containing 5 individuals was covered with nylon netting (mesh size < 1mm) to prevent surfacing. One control tank (as described above for normal culturing conditions) containing 5 snails was also included in this investigation. Each tank was aerated as described in section 2.1 and animals were kept under constant environmental conditions (20 °C, 16 H/8H Light:Dark). The number of *L. peregra* surviving after 5 days was assessed and oxygen levels in the media were recorded using a YSI model 57 oxygen meter (Yellow Springs Insturmental Co. Inc, Ohio).

2.2.2 RESULTS.

All animals in both the control and submerged groups survived for the full duration of the test (5 days). Dissolved oxygen in all aquaria did not fall below 96.5% saturation at any time during the 5 day period.

2.3 DEVELOPMENT OF THE *L. PEREGRA* INGESTION AND EGESTION BIOASSAY.

In order to create a reproducible and repeatable bioassay, factors influencing variation in baseline rates (ingestion and egestion in this case) must be identified and minimised. However, pragmatic choices must be made when defining the parameters of a bioassay. Practicality and cost effectiveness concerning field deployment and laboratory person hours required to run the test must be kept in mind. Sample size, for instance, must be large enough to ensure that the statistical analysis of data generated will detect changes in measured rates, but low enough to be practicable. The standardisation of experimental conditions and definition of control rates also provides confidence when delineating changes in ingestion and egestion rates attributed to toxic stress from those linked to variation in baseline values.

As the endpoints chosen for the *L. peregra* bioassay were ingestion and egestion, control rates for both must be established to evaluate baseline variation.

2.3.1 FACTORS AFFECTING BASELINE INGESTION AND EGESTION RATES.

As discussed in section 1.4.3, *L. peregra* is a seemingly indiscriminate grazer, growing and reproducing on a wide variety of diets. However, this does not preclude the possible influence of either intrinsic or extrinsic factors on the amount of material ingested and assimilated. Intrinsic factors might include body size and parasite load whereas extrinsic factors include temperature, pH and the quantity of food given.

Variables used in the calculation of ingestion and egestion rates, indicate factors likely to affect baseline ingestion and egestion rates during the bioassay.

$$E_r or I_r = \frac{(f_1 - f_0) - C_f}{TL}$$

Equation 1

Where: I_r = Ingestion rate (mg/mm/d)

E_r = Egestion rate (mg/mm/d)

f_1 = Weight of filter paper containing spinach after feeding or filter paper containing filtered faeces (mg)

f_0 = Weight of filter paper containing dry spinach mat or weight of prewashed and dried filter paper (mg)

C_f = Correction factor for filter paper weight (mg)

T = Time in days (d)

L = Shell length (mm)

Kooijman (1993) identifies the three main factors that determine feeding rates as body size, food availability and temperature. By keeping food availability and

temperature constant in each experiment, these two variables may be eliminated as factors that may affect ingestion or egestion rates. As defined by Equation 1, the time spent feeding also contributes to the calculation of ingestion rate. The ingestion and egestion rates were measured 'per day' as the bioassay ran over discrete periods of 24 hours. This meant that time was also a constant value in each trial. The calculation of both ingestion and egestion rates also includes a correction factor (C_f), which takes into account any influence experimental parameters (such as salts in the media) might have on the difference between the initial and final weight of the filter papers.

Consequently, the main influencing factor for both ingestion and egestion rates in Equation 1 was body size. Therefore an important consideration was how body size and food intake were related and subsequently which measure of body size to use. Since energy reserves contribute to weight and are sensitive to feeding conditions, body wet weight usually shows much more variability, in comparison to length measurements (Kooijman, 1993).

As feeding is defined as behaviour it will be particularly erratic when compared with other processes involved with energetics (Kooijman, 1993). Under natural conditions lymnaeids must move to locate food and the rate of movement is proportional to their length. So measuring food intake as a function of length for a grazer generally gives good relationships. However, for a bioassay in which the grazer has no need to travel to search for food, the inclusion of body size will simply be a tool to standardise the amount eaten against some measure of body size. As discussed in various chapters of his book, Kooijman (1993) describes the

proportional relationship of ingestion rate and body size for individual species as well as the comparative interspecies relationship between ingestion rate and a length measure.

Generally snails move over surfaces pushing material into their mouths, depleting one patch and moving on to the next. Little consideration is given in the literature to daily feeding patterns, however Runham (1975), states that *Lymnaea* feed throughout the whole day and night. As concluded by Kohn (1983), gastropod feeding responses are subject to modification by experience, including habituation, sensitisation, satiation and associative learning. Therefore past experiences as well as motivation play important roles in feeding behaviour as opposed to daily feeding rhythms.

In order to reduce the influence of density on feeding behaviour and to minimise the occurrence of diversionary activities such as copulation, individuals were isolated during this bioassay. *L. peregra* has also been shown to exhibit coprophagy, but demonstrates a preference for older (5 days old), over younger faeces (1 day old) (Brendelberger, 1997b). The 24 hour time period over which the assay runs was sufficiently brief to minimise this behaviour.

Lymnaeids serve as hosts for various parasites such at trematodes (i.e. fasciolids), paramphistomes, echinostomes, strigeids, and plagiorcids. Physiological alterations reported in snails as a result of their infection include changes associated with growth, fecundity, life span, heart rate and respiration and thermal tolerance (Malek,

1980a). The two main areas affected by parasitic infection in snails are the digestive and reproductive glands and processes.

By far the most affected organ of the gastropod is the digestive gland (Malek, 1980a). Infection with either daughter sporocysts or redia cause toxic alterations and degenerative changes due to mechanical pressure of the larvae on the host tissues (Malek, 1980a). Additionally, host food reserves are utilised by the developing larvae, therefore causing changes due to starvation effects (Malek, 1980b).

There are contradictory reports of the effects of trematodes on the growth of snails (Wright, 1971). Enhanced as well as reduced growth has been reported (Malek, 1980a). However reports of enhanced growth were mainly based on an observable increase in the size and thickness of the snail shell, and this is not necessarily accompanied by an increased growth of soft body (Malek, 1980b).

Direct attack on the reproductive system is rare (Wright, 1971) but there is general agreement that larval trematodes affect the fecundity of the snail and the presence of a redial stage in the life cycle often results in castration of the snail (Malek, 1980b). They bring about general reductions in fecundity, which may vary, from a complete and irreversible stoppage of egg production, a complete stoppage and subsequent resumption or merely a reduction in the number and size of egg-masses (Wright, 1971). A drop in number may also mean a reduction in viability of the eggs. Despite this the majority of trematode infections cause little physical damage to the host's reproductive system (Wright, 1971).

In spite of the parasitic stress on the snail caused by larval trematodes, it has been observed that infected individuals live for periods equal to, or slightly less than, uninfected ones if maintained under favourable conditions (Malek, 1980a).

An important consideration in an assay measuring ingestion or egestion rate is that food quantity is not a limiting factor. One way to establish the amount of food required over a specified time interval is to determine the functional feeding response of the animal under study. The functional response can be determined by measuring the feeding rate at increasing concentrations of food. Figure 2.2 illustrates the shapes of three possible responses as Holling types 1, 2 and 3 (Chow-Fraser and Sprules, 1992). Previous work using various herbivores such as bivalves (Sprung, 1984; Crisp *et al.*, 1985; Yule *et al.*, 1985; Macdonald, 1988) and daphnids (Porter *et al.*, 1982; Chow-Fraser and Sprules 1992;) show the rate of ingestion increasing as food concentration increases up to a threshold level termed the incipient limiting concentration (ILC, after Rigler 1961). Beyond the ILC, the maximum ingestion rate (MIR, after Chow-Fraser and Sprules, 1992) remains constant and may even decline.

The Holling type one curve (Figure 2.2) describes a rectilinear increase in ingestion rate with food concentration up to a plateau. The type one curve has a very sharply defined turning point which does not often occur in nature. To counteract this, the type 2 model uses non-linear regression to depict a curvilinear increase of ingestion rate with food concentration. Type 3 describes a sigmoidal increase, where the response is dependent on food concentration.

Many studies of functional feeding response have been carried out in the laboratory. This eliminates many of the possible determinants of the functional feeding response under natural conditions (Abrams, 1982). While the functional feeding response of *L. peregra* in the laboratory may be described by one of the three curves, the inclusion of time spent foraging and changes in resource density may result in a different categorisation under natural conditions. Under the controlled conditions of this laboratory study, the food supply was consistent (no patchiness), so food searching or foraging was not a consideration. For the purposes of this study it was necessary only to define the functional feeding response of *L. peregra* under laboratory conditions.

The main aims of this section were to develop and standardise the laboratory component of the *L. peregra* bioassay. In addition, conditions under which bioassay parameters would produce minimal variation in feeding and egestion rates as well as establishing the functional feeding response of *L. peregra* were explored under control conditions through determination of the following parameters:

- Feeding method and standard exposure conditions.
- Calculation of ingestion and egestion rates.
- Variation in ingestion and egestion rates.
- Determining a suitable measurement of size.
- Food concentration (functional feeding response).

The number of replicates required to produce sufficient statistical power to detect a minimum difference between treatments was also explored.

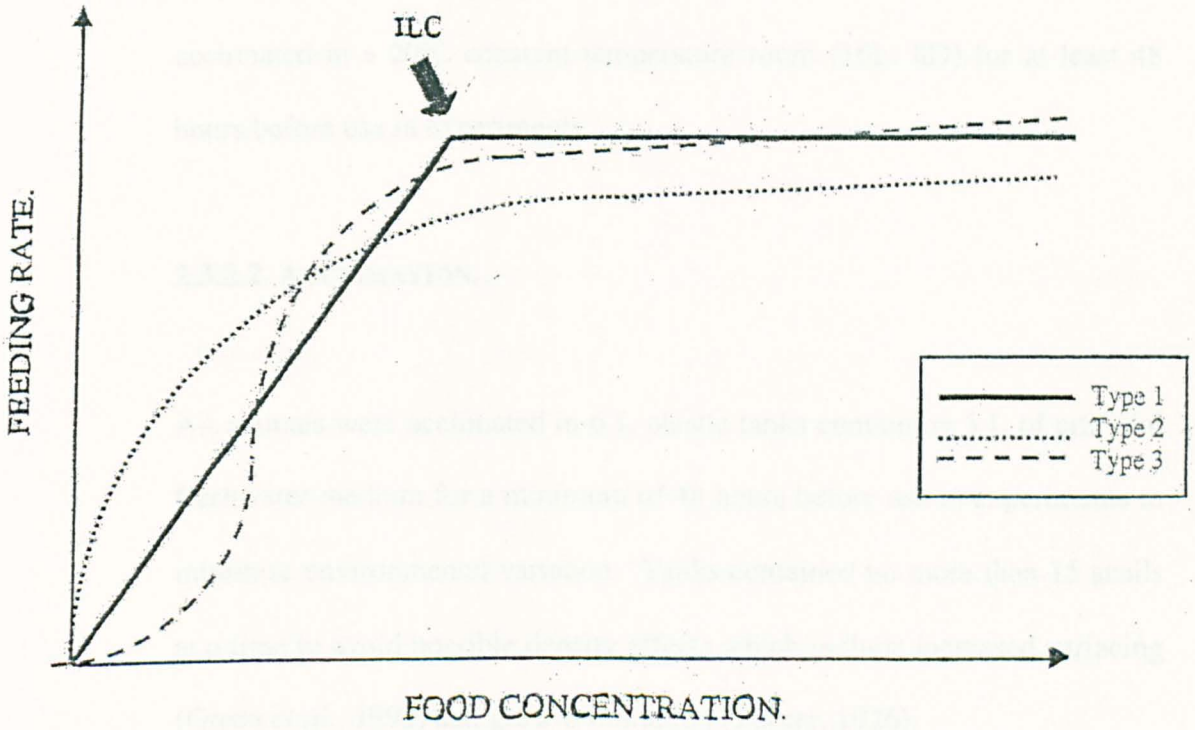


Figure 2.2: Diagramatic representation of the Type 1, 2 and 3 mathematical models used to describe the functional feeding response (adapted from Chow-Fraser and Sprules, 1992).

2.3.2 MATERIALS AND METHODS.

2.3.2.1 ANIMALS.

L. peregra were collected from the River Devon site. Animals were acclimated in a 20°C constant temperature room (16L: 8D) for at least 48 hours before use in experiments.

2.3.2.2 ACCLIMATION.

All animals were acclimated in 6 L plastic tanks containing 5 L of artificial freshwater medium for a minimum of 48 hours before use in experiments to minimise environmental variation. Tanks contained no more than 15 snails at a time to avoid possible density effects which include increased surfacing (Green *et al.*, 1992) and growth limitation (Turner, 1926).

2.3.2.3 FOOD AND COLLECTION OF FAECES.

Food was supplied to individual snails in the form of a 'spinach mat'. This method of feeding attempts to simulate a diatom mat. Spinach mats require approximately one day to generate whereas diatom mats can take up to 17 days to produce using a previously established culture. Prepared spinach solution (see Section 2.1.2) was diluted 1 part to 10 parts culture medium. Fifteen ml of this dilution was filtered onto a pre-washed, dried and weighed filter paper (47 mm Fisher GFGC) and dried at 60°C for 12 hours. Spinach

mats were then re-weighed (± 0.1mg) to calculate the weight of food supplied.

Individual *L. peregra* were placed randomly into jars, each containing a spinach mat as a food source, and were securely covered with mesh (<1 mm) using an elastic band. The jars were then placed in 6 L aquaria containing 5 L of medium for the specified feeding period (24 or 48 hours).

Following the feeding period, faecal pellets were collected from the surface of each spinach mat using a 3 ml plastic pipette. Faeces from each individual were then filtered onto a pre-soaked, dried and pre-weighed filter paper (47mm Fisher GF/C) Filter papers were then dried at 60°C for 24 hours after which time the dry weight was measured to the nearest 0.1 mg. Any spinach remaining was also removed from the filter paper, dried at 60°C for 48 hours and weighed for the eventual calculation of ingestion rate.

2.3.2.4 CALCULATING INGESTION AND EGESTION RATES.

Ingestion rate (I_r mg dry weight spinach/mm shell length/day) and egestion rate (E_r mg dry weight faeces/mm shell length/day) of each surviving *L. peregra* was calculated using equation 1 as outlined in section 2.3.1.1

2.3.2.5 SHELL LENGTH AS A MEASURE OF SIZE.

Sixty snails of various sizes were collected from the River Devon site. It was not necessary to acclimate these animals to laboratory conditions, as they were not used in an experimental capacity. Fourteen different measures of shell size were taken from each snail. The wet weight of the snail with shell and the dry tissue (without shell) weight were measured for each animal. Non-linear regression analysis was then performed using Minitab version 12.1, in order to establish the measure of shell length with the strongest relationship to both wet and dry weight. Graphs were generated using Sigma Plot version 7.0.

2.3.2.6 BASELINE INGESTION AND EGESTION RATES AND RELATIONSHIP WITH BODY SIZE.

Standardisation of an assay aims to minimise variation under control (unstressed) conditions. This section considers the variability of ingestion and egestion and the influence of body size on these rates.

Fifty snails with a range of shell lengths 5.9 mm to 13.5 mm were collected from the River Devon site to look at several aspects of ingestion and egestion in *L. peregra*:

- Baseline ingestion and egestion rates and their variability.
- Influence of shell length on ingestion and egestion.
- The relationship between amounts ingested and egested

Animals were acclimated to laboratory conditions for 48 hours and then placed in jars and fed as described in section 2.3.2.3. Faeces and uneaten spinach were collected from each animal every 24 hours; these were then dried and weighed as described in section 2.3.2.3.

Non-linear regression was performed using Sigma Plot Version 7.0 to establish the relationship between shell length and both ingestion and egestion. Power equations can be useful to describe the relationship between body size and another of its characteristics. According to this equation, body size will change as some power of ingestion and egestion. Feeding is proportional to body length and increases exponentially up to a point after which it becomes linear.

2.3.2.7 NUMBER OF REPLICATES.

An effective bioassay should use sufficient sample size (i.e. number of replicates) to allow the detection of a specified minimum difference between treatment means through statistical analysis. The power of a statistical test $(1 - ß)$, is defined as the probability that the test will correctly reject the null hypothesis when it is false, avoiding type II errors (ß) (Zar, 1999). By increasing the power of a statistical test, the chance of committing a type II error is diminished. This can be achieved through increasing the number of replicates used. Traditionally a power level of 0.8 is used in statistical testing.

The aim of this experiment was to establish the power (at the α = 0.05 significance level) and percentage difference detected between 2 or more means when using an ANOVA that could be detected using a sample size of 10, 12 and 15 individuals. Fifty individuals were collected from the River Devon site and allowed to acclimate to laboratory conditions for 48 hours. Egestion rate was then calculated according to section 2.3.2.3.

Bootstrapping was used to randomly assign individual egestion rates to a set of replicates with a sample size of 10, 12 or 15. The average power of each sample size was obtained by resampling each set of replicates 100 times.

2.3.2.8 FUNCTIONAL RESPONSE.

The functional feeding response of *L. peregra* was determined in order to ensure that food was not limiting during a 24-hour feeding period.

Five different food levels were prepared by adding 0 ml, 1 ml, 10ml, 15 ml and 20 ml of macerated spinach to 150 ml of snail media (see section 2.3.2.3.). Fifteen spinach mats were made from each of the 5 concentrations according to section 2.3.2.3. Sixty *L. peregra* collected from the River Devon site were randomly assigned to the 5 treatments and allowed to feed for 24-hours, after which time the wet weight, dry weight and length of each animal were determined.

The type II equation (Figure 2.2), based on the Michaelis-Menten equation of enzyme kinetics was fitted to feeding rate data using non-linear regression in Sigma Plot, Version 7.0. The equation defines an hyperbolic change in substrate utilisation with a change in substrate concentration and is given as:

$$F = \frac{F_{max}[C]}{K + [C]}$$

Where:

F = Feeding rate (mg/mm/day) as a function of food density

F_{max} = Maximum feeding rate (mg/mm/d)

[C] = Spinach concentration (mg/L)

K = Saturation coefficient or Michaelis constant

2.3.3 RESULTS.

Of the 14 shell measurements taken, the longest axis (see Figure 2.3) showed the strongest relationship with both wet and dry weight. Figures 2.4a and 2.4b (Table 2.5) show the non-linear relationships between shell length (measured through the longest axis), and both wet and dry weights of *L. peregra.*

Average ingestion and egestion rates for snails in this experiment were 0.51 ± 0.17 mg/mm/d and 0.19 ± 0.062 mg/mm/d respectively. When snails with a shell length greater than 8mm were used the average ingestion rate became 0.48 ± 0.17 mg/mm/d, whereas the egestion rate becomes 0.21 ± 0.05 mg/mm/d. The coefficient of variation remained essentially the same for spinach ingested when calculated with all data recorded using all snails and those with a shell size greater

than 8 mm (see table 2.3). However, the coefficient of variation of egestion rate decreases when snails with a shell size of less than 8 mm were excluded. A shell size of 8 mm was chosen as this is average length at which *L. pere*gra become reproductively active (Calow and Lam, 1989).

Table 2.3: Coefficients of variation (cv) for ingestion and egestion rates calculated using snails of all shell lengths and when shell lengths below 8mm are excluded.

	Ingestion rate cv%	**Egestion rate cv%**
All shell lengths	33	35
Greater than 8mm only	33	23

Figure 2.5a and 2.5b (Table 2.6) shows the nature of the relationship between ingestion and egestion rate and increasing body length. As mentioned earlier, feeding rate changes as a function of body size. There is a markedly stronger relationship between egestion rate and body length, with 63% of variability explained by the model as compared with ingestion rate at 40%.

The amounts ingested and egested by *L. peregra* over a 24 hour period show a significant relationship as demonstrated in Figure 2.6 (Table 2.7). Regression analysis for this data gives the equation $y = 0.26 + 0.81x$, with an r^2 value of 0.53. With increasing shell length, the spread of data points also increased.

The functional feeding response exhibited by *L. peregra* was characterised by a type II curvilinear increase in feeding rate with spinach concentration (Figure 2.7, Table

2.8). The coefficient of determination was r^2=0.62 with a maximum feeding rate of 3.9 mg/mm/d. The variation in feeding rate, seen as the spread of data around the curve, is large, but remains fairly constant as food concentration increases. The coefficient of variation indicates that 62% of the variation in feeding rates could be described by the model.

Table 2.4 shows the changes in the power of ANOVA performed on egestion rate data collected following a 24-hour period of feeding, with replicates of 10, 12 and 15, when the minimum detectable difference between treatments was specified at 20% (at α=0.05 significance level). Power increased as the number of replicates increased. No number of replicates used reached the desired power of 0.8.

Table 2.4: Power achieved by 10, 12 and 15 replicates detecting a minimum difference of 20% between treatment means (α = 0.05).

Number of replicates	Power (1 – ß)
10	0.30
12	0.42
15	0.50

Table 2.5: Results of non-linear regression fitted to wet and dry weight of *L. peregra* tissue versus shell length (see Figure 2.4). Equation of the line follows $y = ax^b$.

Experiment	a	b	r^2	F	df	p
Wet weight vs shell length	0.47	2.70	0.87	413.3	49	<0.001
Dry weight vs shell length	0.09	2.24	0.56	75.27	49	<0.001

Table 2.6: Results of non-linear regression fitted to weight of spinach eaten and weight of faeces produced versus shell length (see Figure 2.5). Equation of the line follows $y = ax^b$.

Experiment	a	b	r^2	F	df	p
Spinach eaten	0.26	1.29	0.40	31.5	49	<0.001
Faeces produced	0.03	1.75	0.63	81.31	49	<0.001

Table 2.7: Regression results of weight of egested material vs ingested material over a 24 hour period (see Figure 2.6)

	a	b	r^2	F	df	p
Egested vs ingested	0.26	0.81	0.53	39.5	49	>0.001

Table 2.8: Fitted values of the Type II functional feeding response curve for *L. peregra* (see Figure 2.7). F max = maximum feeding rate, k = half saturation constant (at half the maximum feeding rate).

Fmax (mg/mm/d)	K (mg/cm^2)	r^2	p
3.9	1.9	0.62	<0.001

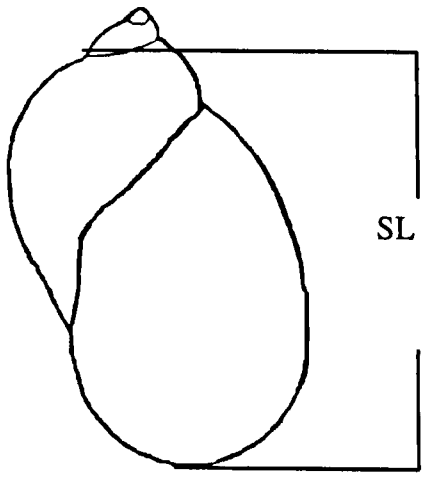


Figure 2.3:. Shell dimension used as measurement of length (SL). This was the longest axis through the shell excluding the final two whorls or 'spire'.

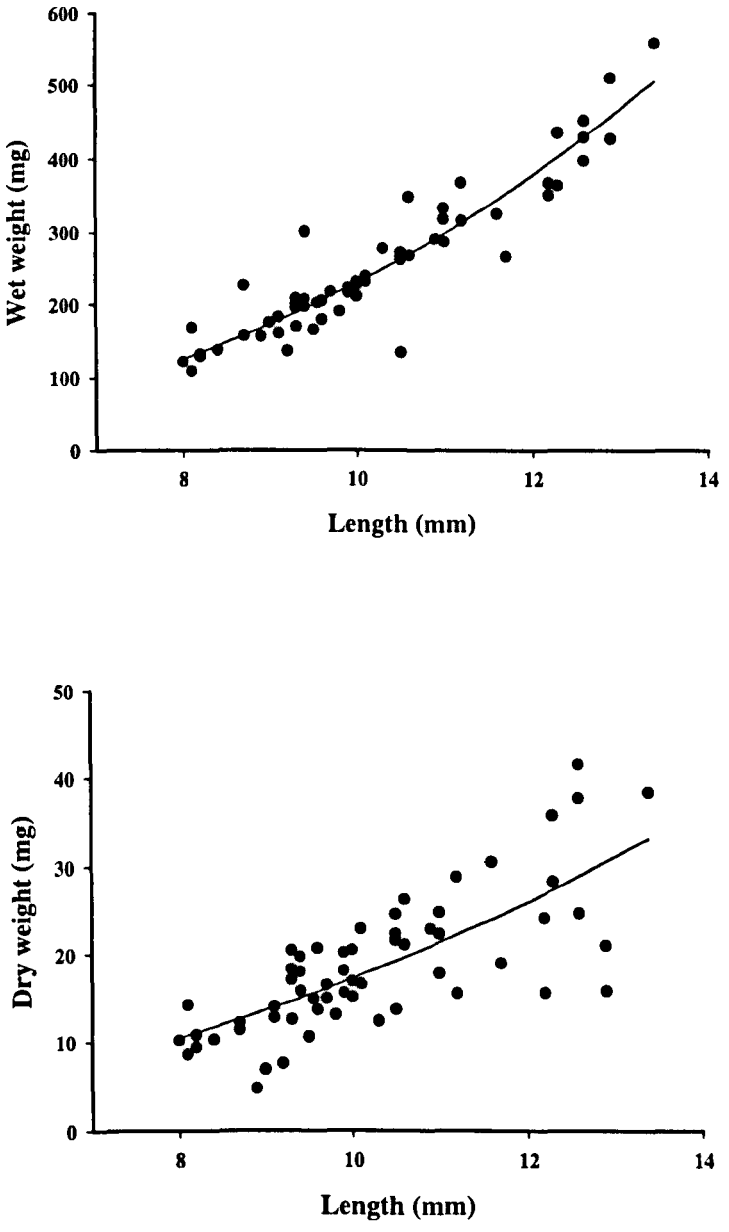
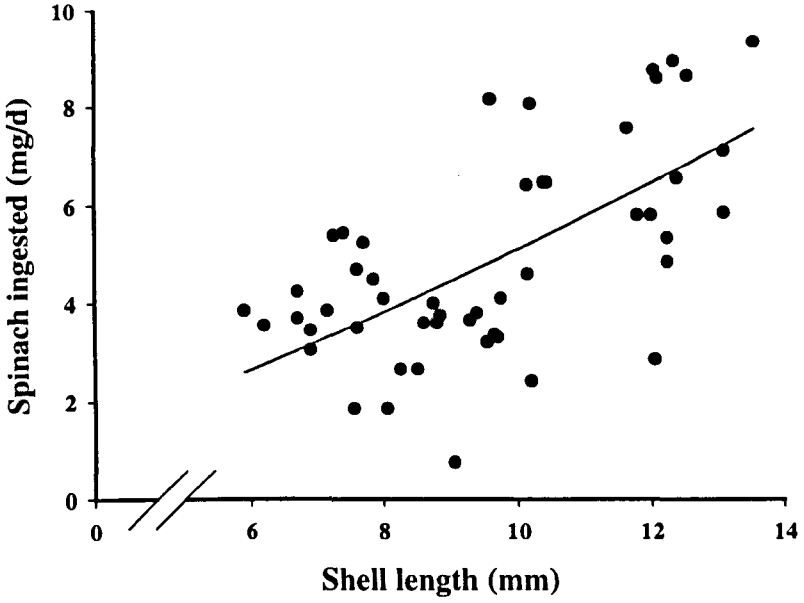


Figure 2.4: The non-linear regression for *(a)* wet weight and shell length ($r^2 = 0.87$; $n = 60$; $p < 0.001$)); *(b)* shows dry weight of tissue only with shell length ($r^2 = 0.56$; $n = 60$; $p < 0.001$). The measure of shell length used here was the longest shell axis excluding the final two whorls, or spire (see Figure 2.4.). For all measures of shell length, wet and dry weight see Appendix 1.



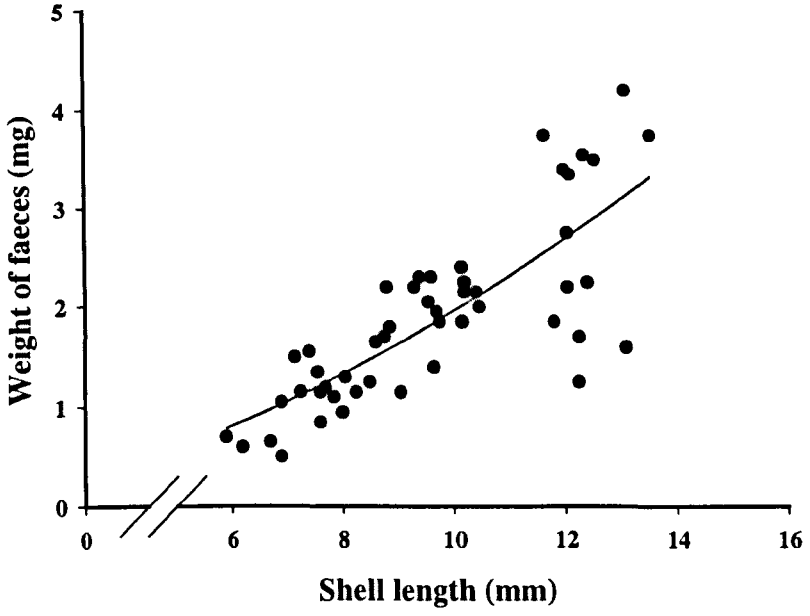


Figure 2.5: Non-linear regression of a) weight of spinach eaten per day against increasing shell length (r^2 =0.40; n = 50; p < 0.001) b) faeces weight produced per day against increasing shell length, (r^2 = 0.63; n = 50; p<0.001). Shell length was measured as shown in Figure 2.4.

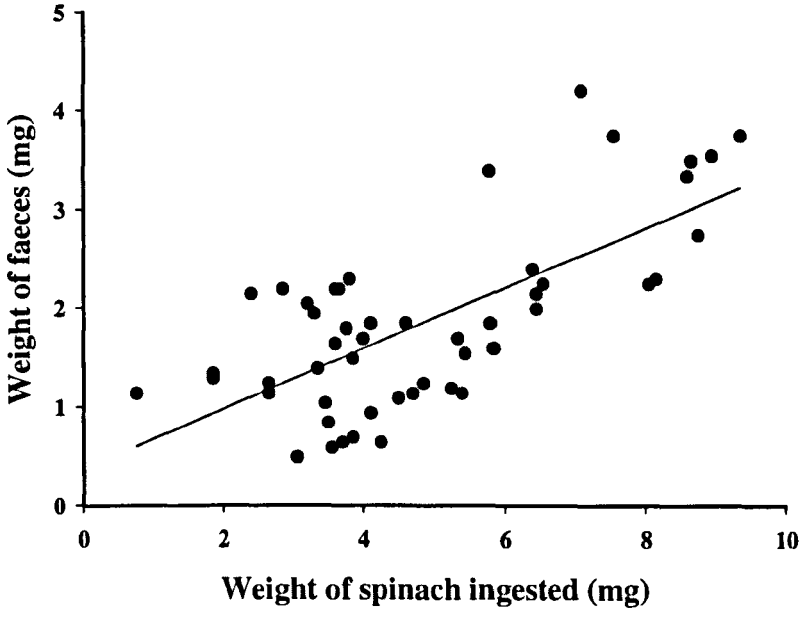


Figure 2.6: Weight of egested material (mg) taken over a 24-hour period against weight of spinach ingested over 24-hour period ($r^2 = 0.53$; n = 50; $p<0.001$).

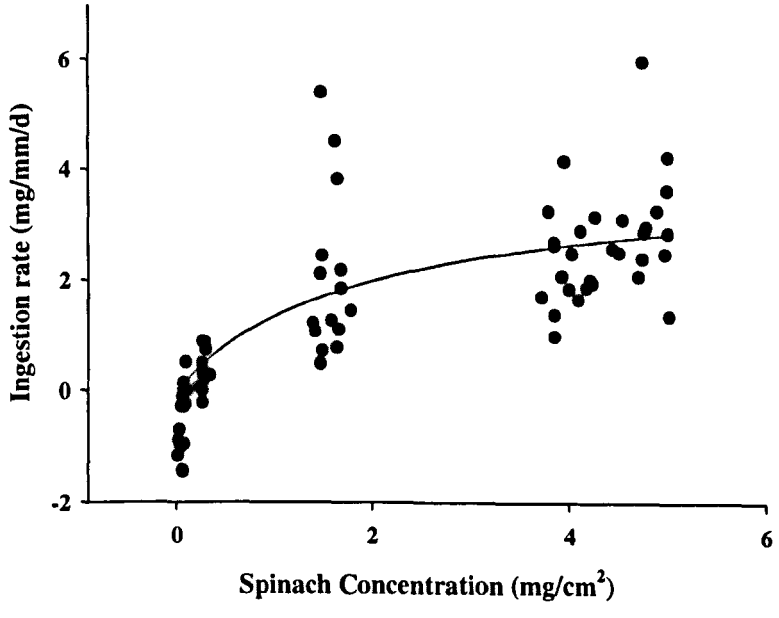


Figure 2.7: Feeding rate response of *L. peregra* to food concentration, with model fitted using the type 2 Michaelis-Menten equation ($r^2 = 0.62$; n = 75; $p<0.001$).

2.4 INTRODUCTION - LABORATORY CULTURED VERSUS FIELD COLLECTED ANIMALS.

As stated by Forbes (1995), Gaddum (1933) was among the first to recommend that the genetic variability among individuals used in toxicity bioassays should be minimised. His recommendation was based on the idea that increasing the homogeneity of response in a test population would improve the precision with which the population response could be estimated. It has become common practice to use highly inbred strains in toxicity bioassays, under the assumption that genetic uniformity decreases the phenotypic variability. This philosophy has been extended to the design of tests for ecotoxicological assessment of manufactured chemicals. It has been suggested that a single clone of the cladoceran, *Daphnia magna*, be used throughout the European Union in internationally standardized test protocols to assess the risk of new chemicals (Forbes and Forbes, 1994). However, opinions vary concerning the relevance of culturing single clones for use in the laboratory.

There are many studies on selfing and inbreeding depression in *Lymnaea sp.* (Colton and Pennypacker, 1934; Cain, 1956; Charlesworth and Charlesworth, 1987; Jarne and Delay, 1990; Coutellec-Vreto *et al.*, 1997; Coutellec-Vreto *et al.*, 1998). However no literature was found on the performance of animals kept in the laboratory for successive generations when compared to those collected from wild populations. The aims of this section were to compare the egestion rate of snails cultured in the laboratory for several generations with those collected from wild populations.

2.4.1 MATERIALS AND METHODS.

2.4.1.1 ANIMALS.

Two sets of experimental animals were used in this study. Firstly, *L. peregra* from a laboratory culture (third generation) started initially from animals collected from the River Devon site. Secondly, *L. peregra* collected from the River Devon site and acclimated in the laboratory under constant conditions (20 °C, 16 L: 8 D) for 48 hours prior to use in this study.

2.4.4.2 STATISTICAL ANALYSIS.

A repeated measures ANOVA was performed after using a homogeneity of variance test (Minitab, 2000) on y-square root transformed data in order to assess whether or not egestion rates for laboratory cultured *L. peregra* versus field collected animals were significantly different.

2.4.2 RESULTS.

Calculated egestion rates of 3rd generation lab cultured *L. peregra* versus field-collected animals are shown in Figure 2.8 (Table 2.9). Data was y-square root transformed to satisfy assumptions for one-way ANOVA. Homogeneity of variance was tested using Bartlett's and Levene's tests (transformed data gave test statistics = 1.087 and 0.012, P = 0.69 and 0.914 respectively). ANOVA results reveal that a significant difference in egestion rate exists between laboratory cultured and field-

collected animals ($F = 15.2$, $p < 0.001$), measured every 24 hours over 11 days. For all days with the exception of day 3, the field-collected population exhibited a higher rate of egestion than the laboratory-bred snails.

Table 2.9: Results of one way ANOVA to compare the egestion rates of field collected and laboratory reared *L. peregra.*

Source of error	Sum of squares	df	F-value	p
Treatment	0.431	1	15.2	<0.001
Error	6.000	185		
Total	6.494	186		

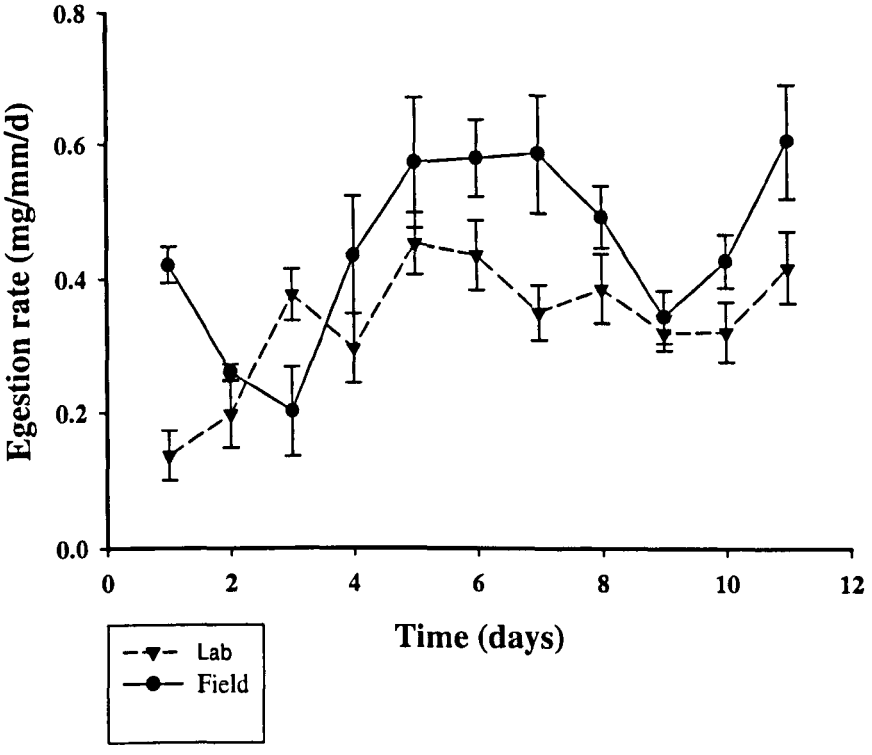


Figure 2.8: Egestion rates (±s.e.) of field collected and third-generation laboratory reared *L. peregra.*

2.5 DISCUSSION.

The development of a bioassay involves the manipulation of parameters and the minimisation of factors that may contribute to variation in the chosen endpoint. The aim of this chapter was to establish optimum methods for culturing *L. peregra*, as well as outlining the development of the laboratory based section of the bioassay through the measurement of baseline ingestion and egestion rates. In the *L. peregra* bioassay, ingestion and egestion rate variability may be affected by factors such as body size, number of replicates, functional feeding response, time spent feeding, temperature and parasite load. These factors are discussed, along with the apparent disparity in egestion rates between field-collected and laboratory cultured animals.

As discussed in section 2.1, there is a considerable amount of literature on lymnaeid culture. Various culturing methods and guidelines for media preparation have been proposed, from aerated tap water (Brendelberger, 1997a) to complex formulations (Dussart and Kay, 1980). Despite this, no standard culturing procedure has emerged. For the present work, the basic culturing and maintenance protocol was obtained from personal communication in 2000 with Dr. Heidi Smith from the University of Staffordshire who had successfully cultured many generations of *L. peregra.*

Many authors disagree on the subject of water leaving behaviour and time between surfacing events (Russell-Hunter, 1953; Berg and Ockelmann 1959; Green *et al.*, 1992). Section 2.2 illustrates that *L. peregra* can survive fully submerged for up to and perhaps longer than 5 days, provided the oxygen concentration of the water is

sufficiently high. This indicates that a period of imposed submergence would not adversely affect *L. peregra*, unless the dissolved oxygen concentration is low which may be the case in polluted waters.

When using field collected animals an acclimation period is necessary for several reasons. Firstly, with temperatures in the field as low as 4 °C, any sudden increase will lead to a change in respiration and in turn feeding rate. For example a rise in temperature from 11°C to 18°C leads to a 65% increase in oxygen consumption, or respiration, by *L. peregra* as reported by Berg and Ockelmann (1959). Secondly, *L. peregra* must acclimate to the ionic composition of the artificial medium (Dussart and Kay, 1980).

The field collected animals were fed during acclimation to avoid starvation effects, which might include increased respiration rate (Berg and Ocklemann, 1959), during the feeding trials. In *Daphnia* for example, respiration rate is highly dependent on food level (Porter *et al.*, 1982). It is therefore necessary to ensure that all individuals to be used in experimentation are fully acclimated to laboratory conditions to eliminate possible effects associated with environmental variation.

In order to produce reliable and repeatable results, experimental parameters are manipulated to minimise variability in ingestion and egestion rates. In the *L. peregra* bioassay, it is difficult to standardise body size, instead it must be taken into account through the equation used to calculate ingestion and egestion rates (Equation 1).

The least destructive and disruptive measure of *L. peregra* body size proved to be shell length. Shell length is a suitable measure of body size to use for *L. peregra* as shell shape changes very little during development. Shell length has been used in several studies as a measure of growth (Skoog, 1978; Calow, 1981; Brendelberger, 1997a). A positive correlation between shell length and body wet weight (r^2=0.8) has been reported by Islam *et al.* (2001) for a laboratory reared population of *L. peregra.* Brendelberger (1997b) reported r^2 values of 0.87 when body dry mass (shell removed) and shell lengths were regressed. However, there is no mention in either of these papers of the specific shell dimension measured. The longest axis excluding the final two whorls, as illustrated in Figure 2.3, was the measure of shell size with the most significant relationship to both wet and dry weight (Figures 2.4a and 2.4b). Measuring shell length allows multiple measures of size to be taken throughout a long experiment with minimal distress to the animal.

Figure 2.5a shows the relationship between the amount of spinach eaten over a 24 hour period and shell length for 50 *L. peregra.* For snails with a shell size of less than 8mm the spread was considerable. This may be due to the higher metabolic rate of smaller animals per unit weight as compared with larger animals (Calow, 1977). Smaller animals would be expected to ingest comparatively more food but egest proportionally less as more energy is allocated to growth than in larger animals.

Figure 2.5b illustrates the relationship between the weight of faeces produced over a 24 hour period and shell size. There was a stronger relationship between the weight of faeces produced and shell length as opposed to the weight of spinach ingested

and shell length. However, this relationship appears to become more variable in snails with a shell length greater than 12 mm. Feeding rate is notoriously variable, but from this work, the relationship between egestion and shell length appears more significant.

Brendelberger (1997a) reported similar ingestion rates to those found in this study using *Radix peregra* (Gastropoda: Pulmonata: Lymnaeidae) fed on various foods (0.19 mg/mg/d to 0.34 mg/mg/d). However, the initial size of snails used in Brendelberger's study was from 2 to 3 mm indicating that they were juvenile animals. Lam (1996) also successfully employed gravimetric techniques to measure the feeding rate of *R. peregra* fed on lettuce. He found consumption rates a useful and rapid test for the effects of environmental toxicants.

A significant relationship exists between egestion and ingestion for *L. peregra* measured over a 24-hour period (Figure 2.6). Measuring faeces produced gives a lower coefficient of variation when compared with the amount of food eaten over time. As discussed by Calow (1970), measurement of total faecal production by *L. peregra* provides a direct indication of the amount of material ingested. Leppanen and Kukkonen (1998) also reported that the weight of faecal pellets effectively represented the feeding activity of *Lumbriculus variegatus* with reasonable accuracy. Faeces has also been used by other authors as a measure of ingestion rates (Calow, 1981; Brown *et al.*, 1994; Blandenier and Perrin, 1998). It is therefore possible to use the egestion rate of *L. peregra* as a surrogate measure for ingestion rate.

Figure 2.7 shows that under laboratory conditions, *L. peregra* exhibits a curvilinear increase in ingestion rate with increasing food concentration. Food effects may often go unnoticed in experiments with freshwater snails since it is widely assumed that such effects are eliminated whenever food is present in excess. It appears from Figure 2.7 that the feeding rate did not plateau out at the highest food concentration provided. The rate of increase in feeding rate did, however, decrease at the higher food concentrations. No individual consumed all food provided at the two highest concentrations. Van der Steen *et al.* (1973) reported finding no optimum range in food quantity with respect to food consumption in the snail *L. stagnalis*. Food may be consumed in excess, just not digested, or used for other functions. In this bioassay food was clearly supplied in excess. Additionally, each snail was fed independently so any density effects could be eliminated.

Table 2.4 shows changes in the powers of ANOVA performed with egestion rate data using 10, 12 or 15 individuals to obtain at least a 20 % difference between treatment means. Egestion rate was measured over a 24 hour period as this will be the end point used in the post-exposure tests. Despite power increasing with the number of individuals used, results obtained did not reach the desired power of 0.8. The number of individuals used in experiments will be dictated by practicability and the number which gives the highest power possible. Using more than 60 individuals in any one trial of this bioassay becomes unworkable in terms of time spent carrying out experimental procedures. For this reason 15 individuals were chosen which gives a power of 0.5.

Despite the length of time over which lymnaeids have been cultured in the laboratory no guidelines have been documented. It can therefore be problematic to set up and maintain successive laboratory generations while sustaining good general condition with minimal variability in performance.

Employing genetically uniform populations in toxicity tests offers a number of practical advantages in terms of test standardisation and repeatability (Jensen *et al.*, 2001). However, as many genotypes typically coexist, there is concern that tests performed on one or a few clones of a species may not adequately reflect the species' response in the field (Forbes and Depledge, 1993). Lymnaeids exhibit considerable intraspecific life-history variation, such as growth rate (Russell-Hunter, 1961) as well as age and size at reproduction (Brown, 1985). Generally these differences are considered to be phenotypic (Hunter, 1975; Brown, 1982). However, according to several authors some have a genetic basis (Calow, 1981). Lam and Calow (1989), studied three neighbouring populations of *L. peregra* and concluded that differences in reproductive timing and growth were attributable to proximate environmental factors such as water temperature, food availability and flow rate. If genetic factors have a large influence on tolerance, then differences between clones may be substantial, and the response of any single clone would provide a biased estimate of the specie's tolerance. This could lead to either over or underprotective estimates of toxicant effects, both of which are undesirable from an environmental perspective.

Organisms in a given population vary in their response to a given toxicant due to past and present biological and environmental influences (Forbes *et al.*, 1995). In a

study by Jensen *et al.* (2001), 4 clones of *Potamopyrgus antipodarium*, displayed interclonal differences in tolerance to sediment bound cadmium, with no consistent patterns among clones in terms of which individual life-history trait was most or least affected. Therefore it may be more environmentally relevant to use field collected animals as opposed to laboratory cultured clones when evaluating the possible effects of environmental pollutants on an organism.

There is extensive literature with regard to lymnaeid diet, growth rates, temperature, fecundity, oxygen requirement, water chemistry etc. The culture of *L. peregra* for use in the work outlined in this thesis was successful, producing 4 generations. Despite the apparently thriving culture of these snails, their general condition seemed to deteriorate through successive generations. The second generation had lost the characteristic brown shell colour and the foot appeared pale. Figure 2.9 shows the results of a comparison between the egestion rates of snails collected from the field and the third generation of laboratory bred animals. There is a significant difference between the two rates as shown by a one way ANOVA ($F=15.2$, $p<0.001$). With both the laboratory and field collected animals there were a series of peaks and troughs in egestion rates. The apparent anomaly on day three where the calculated egestion rate for laboratory cultured snails was higher than that of the field collected animals could be due to a cyclical change in feeding patterns. Despite the acclimation period, when the feeding trial was started the field-collected animals were on a peak whereas the laboratory bred animals were in a trough. As the field collected animals become acclimated to laboratory conditions the feeding cycle of peaks and troughs may become synchronised between the two groups, but it would be difficult to conclude this with any certainty.

The discrepancy in egestion rates was not due to genetic differences as the initial breeding population was collected from the same site as the field collected animals used in this trial. A more plausible explanation, in addition to the possible decrease in general condition, is the possibility that the laboratory bred snails had undergone conditioning in such a controlled environment. Conditioning of feeding in *L. stagnalis* through visual, chemical and tactile stimuli has been demonstrated (Andrew and Savage, 2000). Under field conditions, food availability for *L. peregra* is unpredictable, so feeding occupies a dominant place in the behavioural hierarchy. However when bred under laboratory conditions, the feeding experience is modified and consequently may decrease in importance. In addition, the amount of food in the gut also markedly affects the feeding response in most but not all gastropods (Kohn, 1983). For some gastropods the intake of food may decrease when they reach satiation (Susswein and Kupfermann, 1975) or when the crop or gut is distended (Reingold and Gelperin, 1980). However, satiation does not reduce feeding in the opisthobranch, *Haminoea zelandiae* which resulted in undigested material leaving the anus continuously (Rudman, 1971). Van der Steen (1972) reported finding no optimum food quantity with respect to food consumption for *L. stagnalis*; food consumed in excess was not digested or used for other functions. Whatever the explanation for a decrease in egestion rate in the laboratory bred population, from this point all experiments were carried out using field collected animals.

The preceding chapter has defined the following:

- Culturing and maintenance protocols for *L. peregra* under laboratory conditions
- Protocol for the feeding section of the laboratory bioassay, including amount of food required and size of animals to use
- Rationale for using egestion rate
- Rationale for using length as a measure of body size
- Rationale for using field collected animals

CHAPTER 3.

LYMNAEA PEREGRA BIOASSAY UNDER LABORATORY CONDITIONS: CADMIUM AND COPPER.

3.1 INTRODUCTION

Aquatic toxicity testing has traditionally relied on continuous exposure acute lethality tests to assess the risk associated with chemical substances. However, acute toxicity tests measuring mortality are not always adequately sensitive to assess the risk posed by chemicals released to aquatic systems (Rand *et al.*, 1995). In addition to lethality, toxicity tests have increasingly considered effects on various endpoints such as reproduction, growth and feeding as well as recovery processes, which include the observation of post-exposure behaviour. Brent and Herricks (1998) have suggested the incorporation of post-exposure observation into brief-exposure toxicity testing, due to the likely presence of delayed effects and organism recovery.

Negative effects on physiological and behavioural sublethal endpoints have been recorded during exposure to toxicants. For a post-exposure response to be useful as an endpoint, effects shown during exposure should be persistent in the period subsequent to exposure. Brent and Herricks (1998) found that following exposure to a range of cadmium concentrations, *Ceriodaphina dubia*, *Hyalella azteca* and *Pimephales promelas* exhibited delayed effects, resulting in increased immobility for up to 172 hours after exposure. In the same study, organisms regained mobility

following exposure to phenol indicating post-exposure recovery, which may otherwise have been missed. Taylor *et al.* (1998) indicated the potential for post-exposure feeding inhibition as an endpoint in their work with *Daphnia magna*. *D. magna* previously exposed to sublethal concentrations of cadmium exhibited persistent feeding depression following transfer to uncontaminated medium. McWilliam and Baird (2002a) have also recently demonstrated the use of feeding rate depression in a *D. magna* post-exposure bioassay to assess the effects of chemical contaminants in the laboratory and under field conditions.

Many substances have been shown to affect feeding behaviour in a wide range of organisms, as outlined in section 1.3.2. The following chapter will illustrate the use of the *L. peregra* bioassay under laboratory conditions to study the effects of metal exposure on the snail's ingestion and egestion rates.

Sublethal metal toxicity can induce changes in several aspects of animal behaviour. By inducing behavioural changes, metals could cause the death of individuals or populations by disrupting the normal function and life history of the exposed organism. As indicated by Rand *et al.* (1995) for behavioural approaches to be utilized successfully in contaminant evaluations, the behaviour studied should be: 1) easily observed in the laboratory or field, 2) sensitive to the chemicals of interest, 3) previously well-described, 4) ecologically relevant to species survival and 5) integrate several sensory and or mechanical modalities. Additionally the methods should be routinely available and simple to employ.

3.1.1. METAL CONTAMINATION IN THE AQUATIC ENVIRONMENT

Metal compounds are released into aquatic systems, where they can be distributed throughout the water phase, the suspended particulate matter, bottom sediments and biota. Five main sources of metal pollution to the environment can be identified 1) geological weathering, 2) mining effluents, 3) industrial effluents, 4) domestic effluents and urban storm water runoff and 5) atmospheric sources (Forstner and Wittmann, 1981).

Some metals are essential for normal growth and reproduction, but can become harmful when present in amounts exceeding dietary requirements. Metals such as mercury, lead and cadmium have no apparent biological function and may affect organisms by inducing deficiencies of essential elements through competition at active sites in biologically important molecules (Walker *et al.*, 1996). Heavy metals (those metallic elements with atomic weights greater than 40) of greatest concern in the aquatic environment are cadmium, copper, lead, mercury and zinc (Rand *et al.*, 1995).

Metals are persistent and as such do not biodegrade and may be present in various chemical forms, which may or may not be bioavailable to aquatic organisms. In aquatic systems the bioavailability of metals is governed by the amount and source of input, the physical and chemical properties of the metal as well as those of the system (Rand *et al.*, 1995).

Since this chapter concerns the heavy metals cadmium and copper, the properties of these metals will be considered more closely.

Cadmium

Cadmium is one of the most toxic metals and has been designated as one of the 100 most hazardous substances, with natural sources of cadmium including volcanic emissions, exudates from vegetation, forest fires and windblown material from leaching from rocks (Ayres and Hellier, 1998). Cadmium levels are naturally very low, with average freshwater concentrations of <0.01 to 0.06 μg/L and seawater concentrations of approximately 0.1μg/L (WHO, 1992). The current Environmental Quality Standard (EQS) set out by the European Commission is 5 μg/L for freshwater.

With such low concentrations existing under natural conditions, even a small addition of cadmium from an anthropogenic source can elevate local concentrations, and thus potentially impact receiving waters. The main anthropogenic sources are from metal ore smelting plants and coal combustion processes, as well as chemical manufacturing or textiles, plastics and paints (Nriagu, 1990). Additionally, historic and current mining activities are significant sources of cadmium (Clements *et al.*, 2000) and other sources include atmospheric deposition (Nriagu, 1990) and agricultural fertilisers (Gomot, 1998).

Although cadmium can also exist in colloidal, complexed and particulate forms, the free ionic species tends to predominate in most freshwaters and has generally been regarded as the most available cadmium species to aquatic organisms (Campbell,

1995). Since cadmium has a tendency to form electrochemical bonds (particularly with sulphur donors commonly found in cell surface groups), free cadmium ions readily adsorb to particulate and cell surfaces. Cadmium competes with zinc, displacing it in a number of enzymes and interferes with calcium metabolism by binding strongly to calcium receptors, decreasing the uptake of essential nutrients (Wittmann, 1981). Table 3.1 demonstrates that although invertebrates range in their sensitivity to cadmium, molluscs tolerate very high concentrations.

Copper

Copper is an essential metal and at least 12 major proteins include it as an integral part of their structure (Wittman, 1981). Natural sources of copper include volcanoes, decaying vegetation, forest fires and sea spray with copper levels in seawater of 0.15 µg/L and fresh water of 1 to 20 µg/L in uncontaminated areas (WHO, 1998). Copper usually has a valency of 2 but can exist in the metallic, +1 and +3 valence states and is sparingly soluble in water, salt or mildly acidic solutions (Campbell, 1995). Copper is found naturally in wide variety of mineral salts and organic compounds, and in the metallic form. Anthropogenic sources of copper include emissions from smelters, iron foundries, power stations, and municipal incinerators as well as mining activities (Aynes and Hellier, 1998). It is also used widely in industry, cooking utensils and distribution systems, fertilizers, bactericides, fungicides and algaecides (WHO, 1998).

Copper is released into water as a result of the natural weathering of soil and discharges from industries and sewage treatment plants. Much of the copper discharged to water is in particulate form and tends to settle out or be adsorbed by

organic matter. The bioavailability of copper in the aquatic environment depends on water hardness and alkalinity, ionic strength, pH and redox potential, complexing ligands, suspended particulate matter and carbon.

Copper concentrations as low as 1 to 2 μg/L have been shown to exert adverse reproductive, biochemical, physiological and behavioural effects on a variety of aquatic organisms (WHO, 1998). Table 3.2 demonstrates the wide range in concentrations of copper which show an effect on aquatic invertebrates.

Laboratory experiments have demonstrated the tolerance of gastropods to high concentrations of metals in their food and in the surrounding media. However a notable absence of molluscs in areas of high metal pollution has been reported (Laskowski and Hopkin, 1996). This suggests that mechanisms other than mortality resulting from direct poisoning may be responsible for the extinction of snails in highly polluted areas. Possibilities include reduced food consumption leading to starvation (Simkiss and Watkins, 1990) or to reduced fecundity (Russell *et al.*, 1981). High concentrations of metals might also be found in acidic waters which will also contribute to the decline of gastropod populations.

Cadmium and copper can elicit effects on organisms far below the lethal dose. This chapter will illustrate the sensitivity of *L. peregra* ingestion and egestion rates as sublethal endpoints of metal exposure.

Table 3.1: Examples of relative sensitivities of some invertebrates to cadmium.

Species	Effect	Duration (days)	Reported effect concentration (μg/L)	Reference
Physa gyrina (adult)	TL_{50}	2	4250	Weir and Walter 1976
		4	1370	
P. gyrina (immature)	TL_{50}	2	690	Weir and Walter 1976
		4	430	
P. gyrina	Hatching	2	1800-10,000	
Lymnaea luteola	LC_{50}	2	2100	Khangarot and Ray 1988
L. stagnalis	LC_{50}	4	2500	
	Egg laying inhibition	49	400	Gomot 1998
	Hatching inhibition	49	200	
Radix plicatulus	LC_{50}	4	2500	Lam 1996
	LOEC (feeding rate)	4	200	
Potamopyrgus antipodarum	LC_{50}	2	1000-4000	Moller *et al.*, 1994
Pisidium sp	LC_{50}	4	2000	Mackie 1986
Daphnia pulex	Reproduction inhibition		5	Bertram and Hart 1979
	LC_{50}	2	50	Hall *et al.*, 1986
Daphnia magna	LC_{50}	2	133	McWilliam and Baird 2000a
	IC_{50} (feeding rate)	2	1.31	
Daphnia magna	EC_{50} (feeding rate)	1		Barata *et al.*, 2002
Clone G-19			15.4	
Clone G-62			3.4	
Clone S-28			2.2	
Moina macropora	LT_{50}	2	100-1000	Wong and Wong, 1990

LC_{50}, median lethal concentration; TL_{50}, 50% tolerance limit; EC_{50}, median effective concentration; IC_{50}, median inhibition concentration; LOEC, lowest observable effect concentration.

Table 3.2: Examples of relative sensitivities of aquatic animals to copper.

Species	Effect	Duration (days)	Reported effetive concentration (μg/L)	Reference
Helix engaddensis	EC_{50} (feeding rate)	28	120	Swaileh and Ezzughayyar 2000
	EC_{50} (growth rate)	28	100	
Helix aspersa	EC_{50}(feeding rate)	120	3980	Swaileh and Ezzughayyar 2000
Lymnaea stagnalis	$NOEC_{(growth)}$	21	2.3	Girling *et al*, 2000
Daphnia sp.	LC_{50}	2	5	WHO, 1998
D. magna	LC_{50}	2	57	Naddy *et al*, 2002
	LC_{50}	2	58.77	McWilliam and Baird 2000a
	EC_{50} (feeding rate)		12.13	
	EC_{50} (feeding rate)	1	19.0	Barata *et al.*, 2000
Ceriodaphnia dubia	LC_{50}	2	16	Naddy *et al* 2002
Gammarus pulex	LC_{50}	4	133	Naddy *et al* 2002
Chironomus riparius	NOEC	10	17	Girling *et al*, 2000
Rainbow trout	LC_{50}	4	18	Naddy *et al.*, 2002

NOEC, no observable effect concentration.

AIMS

The aims of this chapter are as follows:

- To assess the effects of cadmium and copper on the ingestion and egestion rates of *L. peregra* during exposure and after exposure.
- Establish the EC_{50} values for ingestion and egestion rates of *L. peregra* exposed to cadmium and copper.

3.2. MATERIALS AND METHODS

3.2.1. ANIMALS AND ACCLIMATION

All animals used for the cadmium and copper experiments were collected from the River Devon site (see Figure 2.1). Animals collected were acclimated to standard laboratory conditions as outlined in section 2.3.2.2.

3.2.2 PREPARATION OF CADMIUM AND COPPER TEST SOLUTIONS AND ANALYSIS

Cadmium (1000ppm in solution with nitric acid): A 1 mg/L stock solution was prepared within 24 hours of experimentation and 4 concentrations of 10, 100, 250 and 500 μg/L were made. No lethality test was conducted for cadmium as the LC_{50} had been previously estimated for *L. stagnalis* at 2500 μg/L by Gomot (1989) and 2500 μg/L by Lam (1996) for *Radix plicatulus*. These two lymnaeids are closely related to *L. peregra* and exhibit a very high tolerance to cadmium. Due to the highly insensitive nature of lymnaeids to cadmium and the hazardous nature of using such high concentrations of the heavy metal, it was decided not to undertake lethality tests but instead to use these values as an estimate for the LC_{50} of *L. peregra*.

As previously stated by Barata (1998), the speciation of cadmium in the standard ASTM hard water medium which has the same range of pH but lower hardness (ASTM 180 mg/L $CaCO_3$, snail media 192 mg/L $CaCO_3$) as the snail media, has been shown to be predominantly (99%) in the free ion form. It was therefore deemed unnecessary to repeat the exercise as most of the total cadmium

concentration measured would be equivalent to the dissolved cadmium concentration.

Copper (1000 ppm in solution with nitric acid): A 1 mg/L stock solution was prepared within 24 hours of the start of the experiment and 5 concentrations of 5, 23, 107, 250 and 500 μg/L were prepared for the lethality test. For the ingestion and egestion test a log series of 5 concentrations of 5, 10, 22, 47 and 100 μg/L was prepared.

Three water samples were taken for analysis from each test solution at time 0 and time 48 and acidified to 1% with HNO_3 (Analar Grade, BDH) and stored at 4°C for later analysis. Three additional spinach mats were prepared for each test concentration to observe the amount of test substance taken up by the mat over the duration of the test. These were left in one of the 5 test vessels through the duration of the experiment. They were then collected, dried, weighed and stored at –4°C for later analysis.

Spinach mat digestion

The digestion technique used employed 10 ml, 15 ml and 20 ml Teflon chambers with tightly fitting screw top lids commonly known as 'bombs'. Each bomb was soaked in a 5% HCl (GLP grade) acid bath for 48 hours prior to use, bombs were then removed, rinsed 5 times with tap water and 3 times with distilled water. Each spinach mat was removed from its glass fibre filter paper backing and placed into one of the Teflon bombs containing 5 ml HNO_3 (Analar grade). For each trial, 3 blanks containing only nitric acid were included to ensure there was no

contamination from the bomb. Control bombs were rotated randomly throughout each of the digestions. Bombs were then securely sealed and placed in an oven set at 110°C for 2 hours. The bombs were cooled to room temperature before being transferred to a 15 ml Teflon sample tube and refrigerated for later analysis.

All metal analysis was carried out directly on a Unicam GF90 graphite-furnace atomic absorption spectrometer with an FS90 plus furnace auto sampler and a GF90 graphite furnace (detection limit 0.5 μg Cd/L) (Thermo Electron Corporation, UK).

3.2.3. LETHALITY TESTS - COPPER

Seventy two *L. peregra* were randomly assigned to individual 60 ml jars which were then placed in 6 treatment groups consisting of 12 animals. At each treatment level (0, 5, 23, 107, 250, 500 μg/L) 6 animals were fed spinach mats as outlined in section 2.3.2.3 and 6 were not. The number of individuals dead in each treatment was evaluated at 24, 48, 72 and 96 hours. Death was carefully assessed through several methods: a) discolouration of the soft tissue usually with white mucus covering the foot area, b) complete withdrawal into the shell, c) lack of movement or rigidity of the soft tissue when gently handled.

3.2.4. INGESTION AND EGESTION RATES DURING AND POST-EXPOSURE

Sixty snails were randomly assigned to 5 groups of 12 jars for cadmium and 90 snails were allocated to 6 groups of 15 jars for copper, each containing a pre-made spinach mat as outlined in section 2.3.2.3. Each jar was covered with <1 mm mesh

secured by an elastic band and placed into one of the 5 tanks containing culture medium made up to one of the test concentrations (cadmium 0, 10, 100, 250, 500 μg/L and copper 0, 5, 10, 22, 47, 100 μg/L).

Uneaten food and faecal pellets were collected following a 48 hour period as outlined in section 2.3.2.3. Ingestion and egestion rates were calculated as outlined in section 2.3.1.1. Water samples for analysis were taken at 0 hours and 48 hours from each tank and acidified with HNO_3 (1% final concentration). Water samples were stored at 4°C for up to 1 month before being analysed as outlined in section 3.2.2.

3.2.5. STATISTICAL ANALYSIS

From the copper lethality test, a 96 hour LC_{50} was calculated using probit analysis (SPSS, 2000).

Ingestion and egestion rate EC_{50} values were calculated from data generated during the 48 hour exposure period and the 24 hour post-exposure period. A one directional General Linear Model with a post hoc Bonferroni comparison (Minitab, 2000) was carried out to establish which concentrations produced rates that were significantly different from the control. Data were then fitted to an allosteric regression model using Sigma Plot version 7.0, to estimate the EC_{50} value for that metal. The allosteric model was fitted only to data which produced a significant change as defined by GLM analysis in either ingestion or egestion rates.

RESULTS

Actual concentrations of cadmium and copper measured differed by less than 10% from nominal concentrations. This difference was considered small enough to regard nominal and actual concentrations as equivalent. All concentrations used in the calculations and graphs were therefore nominal values.

3.2.6. CADMIUM

From Table 3.3 it can be seen that approximately half of the original cadmium concentration in solution was lost over the 48 hour exposure period. This may have been due to loss onto the spinach mats and to the sides of the test vessel. Figure 3.1 shows the uptake of cadmium by spinach mats over the 48 hour period in each of the 4 concentration as well as the control. This shows a linear increase in concentration of cadmium in the spinach mat with increasing cadmium concentrations in the solution. In a study of cadmium uptake by *Navicula peliculosa* mats Irving (2000) also found a linear uptake with increasing concentration. The regression equations from this study and from Irving (2000) were very similar and are given in Table 3.4.

Figure 3.2 shows ingestion and egestion rates during direct exposure to cadmium as well as the post-exposure feeding period for *L. peregra*. No mortalities were recorded during this experiment. Under direct exposure to cadmium no effects on either ingestion or egestion rates were observed (Figure 3.2 a and c). The control ingestion rate shown in Figure 3.2a was lower than those recorded in Chapter 2 and

in subsequent chapters. The post exposure control egestion rate (Figure 3.2b) increases to a level comparable to those recorded in subsequent chapters.

Both ingestion and egestion rates show effects which were significantly different from the control rates during the post-exposure period ($F_{4, 55} = 11.52$; $p<0.0001$ for ingestion rates and $F_{4, 55} = 14.65$; $p<0.0001$ for egestion rates). Figures 3.2 b and d (Table 3.5) show a greater than 50% reduction in both ingestion and egestion rates at 10 μg/L cadmium.

Figure 3.3 (Table 3.6) shows the fitted allosteric decay model for the effects of cadmium on ingestion and egestion rates. The variation in both cases was equal but high with coefficients of determination for the two curves at $r^2 = 0.45$ for ingestion and $r^2 = 0.46$ for egestion. From these models the EC_{50} values for post-exposure ingestion and egestion rates are 0.05 (SE 0.42) and 5.6 (SE 12.5) μg/L respectively.

Table 3.3. Percent decrease in cadmium concentration over 48 hour exposure from artificial media solutions.

Cadmium concentration (μg/L)	**Percent decrease over 48 hours**
10	61
100	61
250	56
500	42

Table 3.4: Linear regression equations calculated from uptake of cadmium onto spinach and *Navicula pelliculosa* mats (Irving, 2000). Values were calculated using dry weight in both studies.

Mat Material	Linear regression equation	r^2
Spinach	$y = 1.87 + 0.046x$	0.97
Navicula pelliculosa	$y = 2.03 + 0.078x$	0.92

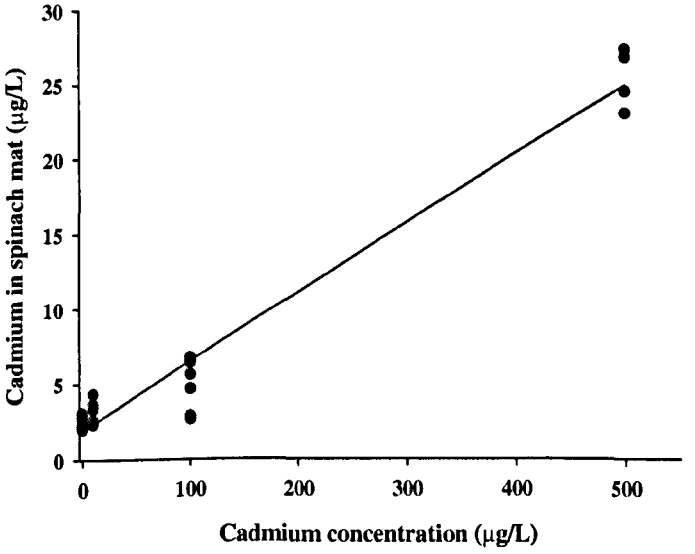


Figure 3.1: Cadmium concentration in spinach mats, calculated using dry weight, exposed to increasing aqueous cadmium concentrations over 48 hours ($r^2 = 0.97$; n = 16; $p<0.0001$).

EXPOSURE

POST-EXPOSURE

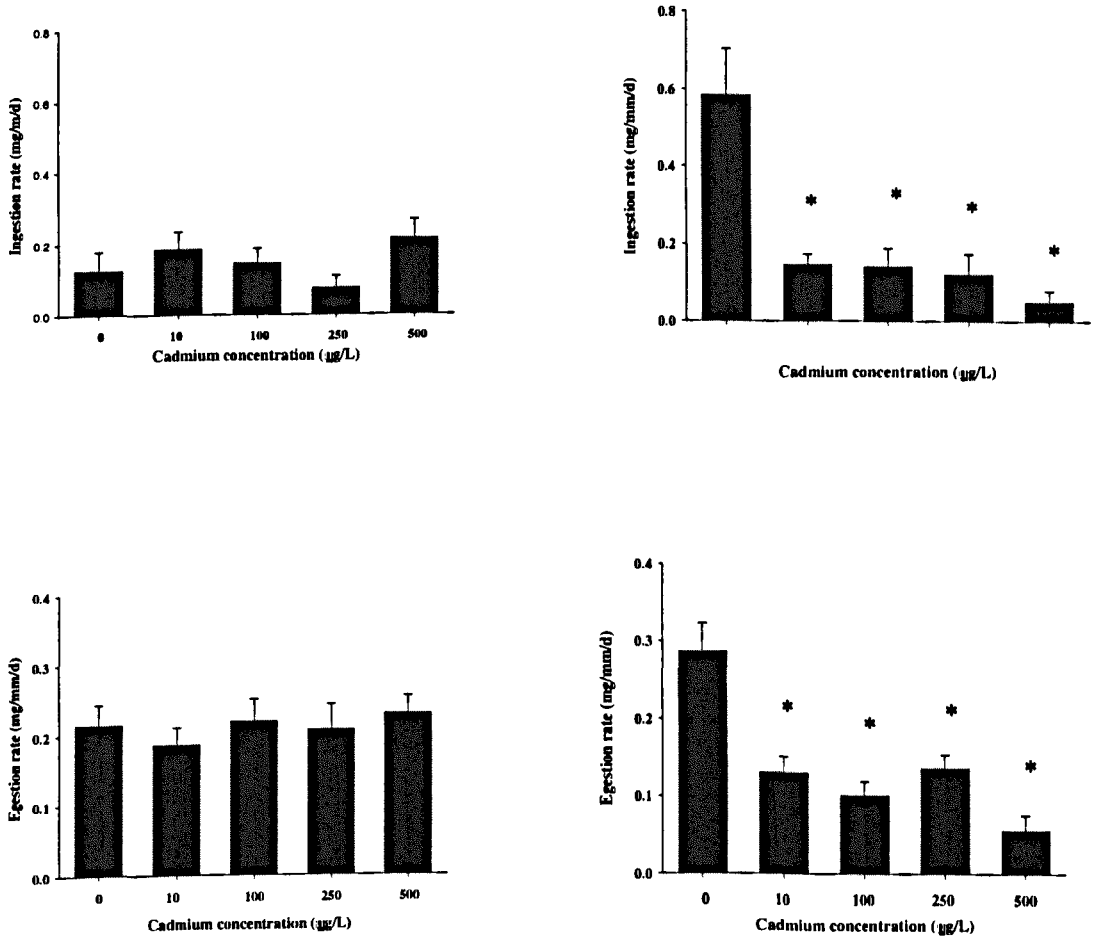
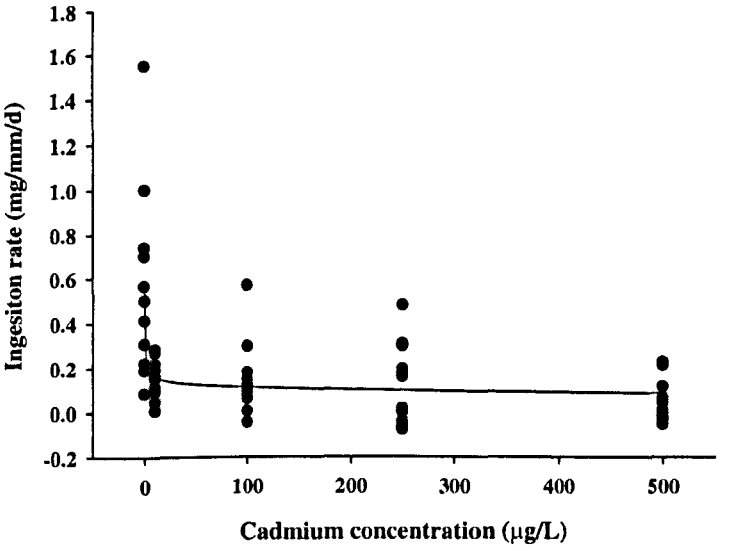


Figure 3.2: Ingestion and egestion rates during 48 hour exposure and 24 hour post-exposure periods following exposure to a range of aqueous cadmium concentrations. Asterisks denote rates that were significantly different from control calculated using the Bonferroni test ($n = 60$; $p<0.0001$). Bars indicate standard error.



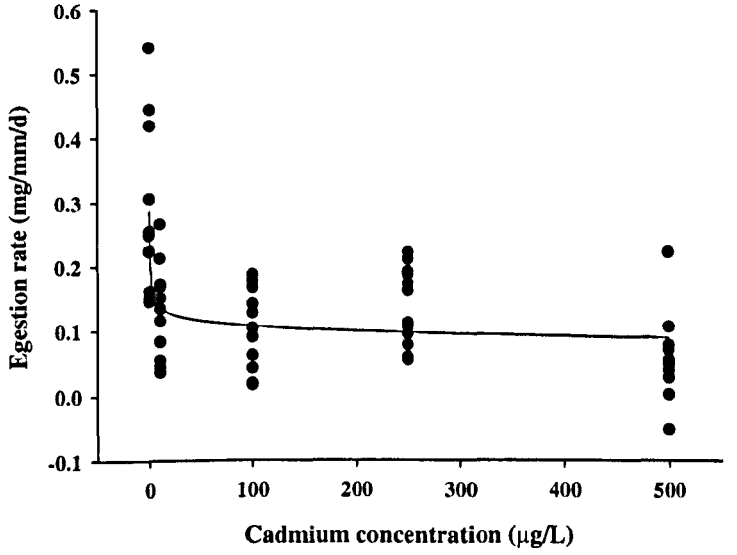


Figure 3.3: Allosteric decay curves for post-exposure a) ingestion (r^2 = 0.45; p<0.0001) and b) egestion rates (r^2 = 0.46; n = 60; p<0.0001) against aqueous cadmium concentration.

a). $y = \frac{0.59 \times 0.05^{0.2}}{0.05^{0.2}}$

b). $y = \frac{0.29 \times 5.6^{0.2}}{5.6^{0.2}}$

Table 3.5: Results of one way ANOVA to compare the effects of cadmium on *L. peregra* ingestion and egestion rate during and following a 48 hour exposure period.

Experiment	Source of error	Sum of squares	d.f.	F-value	p-value
During exposure ingestion	Treatment	0.14	4	2.06	0.099
	Error	0.96	55		
	Total	1.10	59		
During exposure egestion	Treatment	0.01	4	0.37	0.831
	Error	0.49	55		
	Total	0.50	59		
Post-exposure ingestion	Treatment	2.19	4	11.51	<0.001
	Error	2.62	55		
	Total	4.82	59		
Post- exposure egestion	Treatment	0.37	4	14.65	<0.001
	Error	0.35	55		
	Total	0.72	59		

Table 3.6: Fitted values of the allosteric decay equation for ingestion and egestion rates of *L. peregra* exposed to cadmium.

	k_t (=EC_{50}) (μg/L)	i	r^2	p
Ingestion	0.05	0.2	0.45	<0.0001
Egestion	5.6	0.2	0.46	<0.0001

3.2.7. COPPER

Over 50% of the copper was lost during the 48 hour exposure in the three lowest concentrations (5, 10, and 22 μg/L). In the 47 μg/L treatment loss was 16% and decreased to 2% at 100 μg/L. The linear relationship between copper concentration in spinach mats and the concentration of the exposure media over a 48 hour period is shown in Figure 3.4.

The lethal effect of copper on *L. peregra* over a 96 hour period was not affected by the presence or absence of food. Therefore the 96 hour LC_{50} values for animals exposed without and with food were the same. The calculated 96 hour LC_{50} value for *L. peregra* exposed to copper was 115 μg/L (95% C.I. 86.1, 170.9).

Figure 3.5 shows ingestion and egestion rates during direct exposure and post-exposure of *L. peregra* to copper. Table 3.7 shows the results from a one way ANOVA using the ingestion and egestion rates measured during exposure to copper. No mortalities were recorded in the control group during this experiment. Mortalities in the 5, 10, 22, 47 and 100 μg/L copper groups were 6%, 20%, 13%, 13%, and 20 % respectively.

Under direct exposure there was an average 31% decrease in ingestion rate at 22 μg/L over the 48 hour period when *L. peregra* were exposed to copper (Figure 3.5 a), but this difference was not statistically significant. The highest concentration of copper (100 μg/L) produced an ingestion rate that showed an average 42% decrease, which was significantly different from the control ($F_{5,\ 73} = 10.37$; $p<0.0001$).

Ingestion rates measured over the 24 hour post-exposure period (Figure 3.5 b) show a significant decrease in ingestion at the 3 highest copper concentrations ($F_{5, 73}$ = 9.71; $p<0.0001$). A very pronounced decrease in ingestion rate takes place when the percent difference from control increases from an average of 13% at the 10 µg/L treatment to 86% at 22 µg/L.

The post-exposure effects of copper on the egestion rate of *L. peregra* (Figure 3.5 c) shows a statistically insignificant 38% decrease at 10 µg/L copper, which drops to an 82% decrease at 22 µg/L copper which proves to be statistically significant. Again as with ingestion, the three highest concentrations of cadmium show a decrease in egestion rates when compared with the control ($F_{5, 73}$ = 13.07; $p<0.0001$).

Figure 3.6 (Table 3.8) shows the fitted allosteric decay model for the effects of copper on ingestion and egestion rates. The variation in both cases was high with coefficients of determination for the two curves at r^2 = 0.46 for ingestion and r^2 = 0.41 for egestion. From these models the EC_{50} values for post-exposure ingestion and egestion rates were 15 (SE 3.23) and 13 (SE 3.57) µg/L respectively.

Table 3.7: Results of one way ANOVA to compare the effects of copper on *L. peregra* ingestion and egestion rate during and following a 48 hour exposure period.

Experiment	Source of error	Sum of squares	d.f.	F-value	p-value
During exposure ingestion	Treatment	0.83	5	10.37	<0.0001
	Error	1.16	73		
	Total	1.99	78		
During exposure egestion	Treatment	0.001	5	0.11	0.990
	Error	0.165	73		
	Total	0.166	78		
Post-exposure ingestion	Treatment	3.01	5	9.71	<0.001
	Error	4.52	73		
	Total	7.54	78		
Post- exposure egestion	Treatment	0.96	5	13.07	<0.001
	Error	1.07	73		
	Total	2.03	78		

Table 3.8: Fitted values of the allosteric decay equation for ingestion and egestion rates of *L. peregra* exposed to copper.

	k_t (=EC_{50}) (μg/L)	i	r^2	p
Ingestion	15.14	0.45	0.39	<0.0001
Egestion	13.13	1.2	0.41	<0.0001

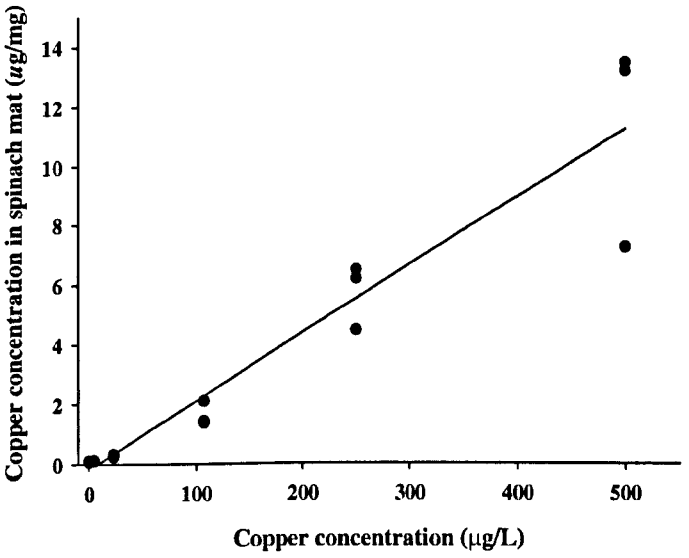


Figure 3.4: Copper concentration in spinach mats exposed to increasing aqueous copper concentrations over 48 hours ($r^2 = 0.93$; n = 15; $p<0.0001$).

$$y = -0.18 + 0.0236x$$

EXPOSURE

POST-EXPOSURE

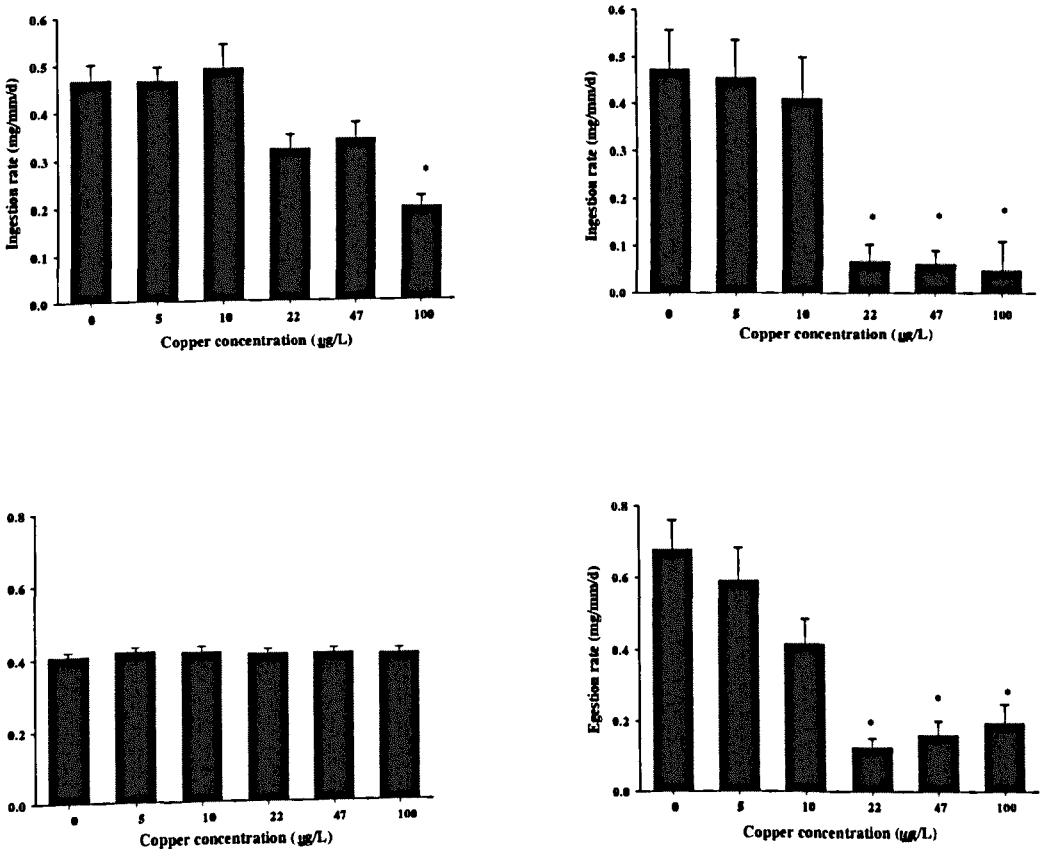


Figure 3.5: Ingestion and egestion rates during 48 hour exposure and 24 hour post-exposure periods following exposure to a range of aqueous copper concentrations. Asterisks denote rates that were significantly different from control using a Bonferroni test (n = 90; $p<0.0001$). Bars indicate standard error.

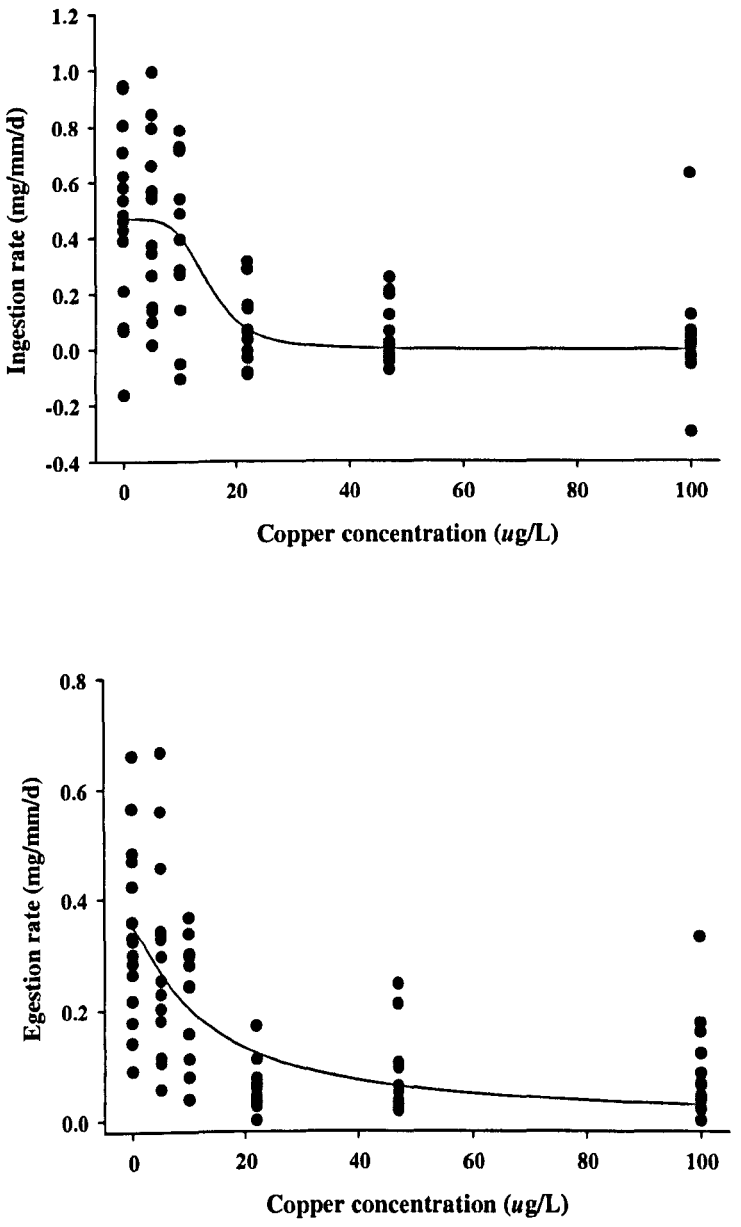


Figure 3.6: Allosteric decay curves for post-exposure a) ingestion (r^2 = 0.39; p<0.0001) and b) egestion rates (r^2 = 0.41; n = 90; p<0.0001) against aqueous copper concentration.

a). $y = \frac{0.46 \times 15^{4.5}}{15^{4.5}}$ b). $y = \frac{0.35 \times 13^{1.2}}{13^{1.2}}$

3.4 DISCUSSION

Both cadmium and copper uptake by spinach mats during a 48 hour exposure period were a linear function of the exposure concentration, as shown in Figures 3.1 and 3.4. Periphytic biofilms have been shown to bioconcentrate cadmium to more than 4 orders of magnitude above concentrations in the surrounding water (Hart and Scaife, 1977; Hill *et al.*, 1996). Cushing and Rose (1970) reported that the dominant mechanism acting with respect to uptake of zinc by periphyton mats was adsorption which occurs rapidly, usually within the first 10 minutes of exposure (Irving, 2000). This was proven through the exposure of live periphyton mats to no light and continuous light regime as well as by exposing dead mats to radiolabelled Zn^{65}. Irving (2000) exposed *Navicula pelliculosa* mats to between 0 and 2500 µg/L cadmium solutions for 15 minute time periods. The study concluded that cadmium uptake was a linear function of concentration and produced a regression equation with similar parameters to the one found in the present study (Table 4.3).

The food source suggested for use in this bioassay appears to act in the same manner with respect to metal uptake as a live biofilm or algal mat, making it a viable alternative to the relatively protracted task of producing diatom mats for food purposes. In addition, the uptake of metals by spinach mats may be acting as a sink for metals so may be responsible for the decrease in metal concentration in solution over the 48 hour period.

The high capacity of snails to accumulate metals has been attributed to the synthesis of metal-binding proteins (metallothioneins) and to the deposition of metals in

insoluble intracellular granules (Swaileh and Ezzughayyar, 2000). Xenobiotic metals such as cadmium may be accumulated more readily than nutritional metals such as copper. This, however, might not be applicable with respect to copper in animals containing haemocyanin, such as gastropods, which accumulate this essential metal over a wide range of environmental concentrations (Hopkin, 1990).

Despite the capacity of molluscs to take up high concentrations of heavy metals and survive, the possibility of sublethal effects remains. Both Taylor *et al.* (2001) and Laskowski and Hopkin (1996) reported a decrease in food consumption and aestivation in the terrestrial snail *H. aspersa* when fed a diet supplemented with metals. Swaileh and Ezzughayyar (2000) found that both feeding and growth were inhibited in *H. engaddensis* fed diets contaminated with either cadmium or copper.

The LC_{50} level for cadmium was not experimentally derived for *L. peregra.* Two authors have previously estimated this value for closely related species of lymnaeidae. Gomot (1998) estimated the 48 hour LC_{50} of adult *L. stagnalis* at 2500 µg/L, and Lam (1996) established a 96 hour LC_{50} for *Radix plicatulus* of 2500 µg/L. With these values estimated for lymnaeids in such close agreement there was no reason to suppose that *L. peregra* would be any more sensitive to cadmium toxicity than *L. stagnalis* or *R. pliculatus.* Additionally it was decided not to proceed with the lethality test due to the hazardous nature of working with the very high levels required to carry out this lethality test. Other aquatic gastropods exhibit equally high LC_{50} values; Cheung *et al.* (2002), reported a 96 hour LC_{50} value for *Nassarius festivus* (Powys) of 1520 µg/L (reported as nominal) and Devi (1997) for *Morula granulata* of 2060 µg/L (actual value).

Several studies indicate that cadmium is by far more toxic to gastropods than copper, with the 96 hr LC_{50} value of copper reported to be 20 fold that of cadmium. Devi (1997) estimated a Cu 96 hr LC_{50} value of 370µg/L (actual value) and Cheung *et al.*, 2002, reported a 96 hr LC_{50} of 360 µg/L (nominal value) for *N. festivus*. The LC_{50} value derived during this study for *L. peregra* exposed to copper was 115 µg/L over 96 hours of exposure.

Laboratory bioassays frequently equate chemical effects and body burden with the uptake of substances from water, deducing that this is only the important exposure route. However, dietary exposure can be important for substances that adsorb to material (Podemski and Culp, 2001). In a study by McWilliam (2001) it was found that the 48 hour LC_{50} values for *D. magna* exposed to cadmium, zinc, copper and lead were consistently higher in the presence of food. This indicated that the presence of food caused a reduction in the lethal toxicity of these metals and enhanced resistance of fed animals rather than differences in exposure route. The results from the copper lethality study presented in section 3.3.2, show that *L. peregra* was unaffected by the presence or absence of food. The subject of uptake route will be explored further in Chapter 5.

During the sublethal tests, no concentration of cadmium significantly altered the ingestion or egestion rates of *L. peregra* during the 48-hour exposure period, (Figure 3.2). The ingestion rates calculated during the 48 hour exposure period to cadmium were very low when compared to the control egestion rate under post-exposure conditions as well as egestion rates reported in subsequent chapters. The *L. peregra* egestion rates measured during direct exposure also showed no evidence

of cadmium effects, but the values obtained were normal when compared to those in subsequent chapters. Why ingestion rate values were so low during the exposure period is subject to conjecture. Perhaps the acclimation period was not sufficient and the control animals were beginning to acclimate to laboratory conditions following the 48 hours direct exposure period when the effects of cadmium toxicity began to become apparent in the exposed animals. The same could be said with respect to handling stress.

In the post-exposure period there was clear evidence of a significant reduction in both ingestion and egestion rates at all concentrations with a calculated EC_{50} value of 0.05 (SE 0.42) and 5.6 (SE 12.5) µg/L. These values were very low when compared to those found in the literature with high associated standard error. Lam (1996) reported a decrease in the rate of consumption and adsorption of *Radix plicatulus* with increasing cadmium concentration, calculating LOEC values of 200 µg Cd/L and 150 µg Cd/L respectively. The growth rate EC_{50} for *Potamopyrgus jenkinsi* exposed to cadmium was 16µg/L (measured value), as reported by Dorgelo *et al.* (1995).

The ingestion and egestion rates of *L. peregra* during the 48 hour exposure period to increasing concentrations of copper (Figure 3.5) do not mirror one another. Ingestion rates show a decrease of 31% at 22 µg/L. This decrease continues and becomes significant at 100 µg/L (42%). However, the egestion rate remains constant during the 48 hour exposure period. This could perhaps have been due to *L. peregra* avoiding the highly contaminated food, but still voiding any material remaining in their gut.

A significant decrease in the post-exposure ingestion and egestion rates as compared to control ($p<0.0001$) was observed for *L. peregra* exposed to copper at 22, 47 and 100 µg/L. There was some evidence of a decrease in egestion at 10 µg/L (38% reduction), however the difference was not statistically significant. The failure of this assay to detect small changes in ingestion and egestion rates can be attributed to the high variation seen in the data. Figure 3.3 and 3.6 show the fitted allosteric decay model for the effects of cadmium and copper on ingestion and egestion rates respectively. The variation in these 4 graphs was very high with r^2 values between 0.41 and 0.46.

The EC_{50} values estimated for *L. peregra* when exposed to copper were 15 µg/L (SE 3.23) and 13 (SE 3.57) µg/L for ingestion and egestion respectively. Cheung and Wong (1999) observed several behavioural changes in *Babylonia lutosa* when exposed to copper. At 0.06 µg/L individuals began to retract into the shell and over 50% of individuals exposed to 0.2 µg/L retracted their body within the first week. *Nassarius obsoletus* did not start retracting its body until 4 to 5 µg/L (MacInnes and Thurberg, 1973). There was a significant effect on burrowing at 0.06 µg/L. While most molluscs seem able to survive high concentrations of copper, sublethal effects appear at quite low concentrations.

Gastropods may use several strategies to reduce or avoid the toxic effects of metals. One widely reported strategy is the sequestering of metals into subcellular granules within soft tissues or even specific glands (Brown, 1984), which are regularly passed through the gut and voided with the faeces (Nott and Nicolaidou, 1996). It may be that these function as a route for the accumulation, storage and excretion of

metals (Simkiss, 1976). However, little is known about their capacities for loading and unloading metal ions (Pyatt *et al.*, 1997). As outlined by Cheung and Wong (1999), to avoid metal uptake, three strategies may be employed by an organism: limit uptake, increase excretion or immobilize the metal within the tissues. One of these strategies has been clearly demonstrated by the results presented in this chapter. Exposure of *L. peregra* to both cadmium and copper reduces ingestion therefore limiting uptake with a subsequent reduction in egestion rate, during the post exposure period.

With the exception of the ingestion rate of *L. peregra* exposed to copper, there were no mirroring of effects between the exposure ingestion and egestion rates and those observed during the post-exposure period. This may be due to the length of time required for the effects of metal toxicity to become apparent. There was also clearly no recovery observed in the post-exposure periods for either cadmium or copper.

Overall post-exposure ingestion and egestion rates showed significant depression when compared to control values ($p<0.0001$) indicating that the effects of metals are persistent in *L. peregra*. This also indicates that the post-exposure ingestion and egestion rate endpoint is sensitive to metal exposure. Additionally, post-exposure ingestion and egestion rates of *L. peregra* exposed to cadmium and copper have proved to be sensitive endpoints as the EC_{50} values were consistently lower than the LC_{50} values. Snails are known to be less sensitive to copper than cadmium and are able to accumulate large quantities of this metal (Hopkin, 1990; Swaileh and Ezzughayyar, 2000). Results from this study indicate copper is more acutely toxic to *L. peregra* than cadmium; and that *L. peregra* ingestion and egestion rates are

more sensitive to cadmium than copper as evidenced by the EC_{50} values obtained. However, there may be some difficulty with the elucidation of changes in the ingestion and egestion signal from the variability of the data presented. Despite the 31% decrease in ingestion rate during the 48 hour exposure of *L. peregra* to copper, the effect was not statistically significant. The failure of this test to pick up small changes in ingestion or egestion rates indicate low power and perhaps the need for increased number of replicates or further investigation into the feeding pattern of *L. peregra* and the associated variability.

This chapter has raised some questions with regard to uptake of metals by *L. peregra* over long and short time periods as well as prolonged effects on ingestion, egestion and growth. These issues will be explored further in Chapter 5. This chapter has illustrated the pertinence and sensitivity of the *L. peregra* laboratory based bioassay for metal toxicity. The following chapter will use the same procedures to look at the possible negative effects of selected organic substances on the ingestion and egestion rates of *L. peregra*.

CHAPTER 4.

LYMNAEA PEREGRA BIOASSAY UNDER LABORATORY CONDITIONS: PYRENE AND LAMBDA CYAHLOTHRIN.

4.1 INTRODUCTION.

Any chemical, which has carbon as the main structural element, is called an organic chemical and as a group they comprise a large variety of different compounds. Organic compounds are generally classified according to their structure (i.e. hydrocarbons, polychlorinated biphenols), but may also be classified according to their toxicological mode of action (i.e. narcotic, polar narcotic)

Organic chemicals have a range of water solubilities. Some groups of organic chemicals vary in their water solubilities such as carbamate insecticides, organophosphorus insecticides, and alkali salt formulations of phenoxy herbicides, detergents and chlorophenols. Others are persistent, pervasive and have a tendency to bioaccumulate such as polycyclic aromatic hydrocarbons (PAH's), halogenated dibenzo-p-dioxins, halogenated dibenzofurans, some polychlorinated biphenols (PCB's), several chlorinated pesticides, pyrethroid insecticides and organochlorine insecticides (Bartell *et al.*, 1998).

An organic chemical at low concentrations in the aquatic environment can exist in either or both of two forms – a dissolved phase and a sorbed phase (Rand *et al.*, 1995). Which form it will take will depend largely on its chemical properties. One

of the most important determinants of the environmental fate of organic chemicals is its polarity. In general, the more polar a compound is the higher its water solubility and the lower its octanol-water partition coefficient (K_{OW}). For example polar compounds with low K_{OW} values tend to remain in the water and not to move onto membranes (Walker *et al.*, 1996). Hydrophobic non-polar compounds tend to be excluded from the aqueous phase; instead they tend to become adsorbed to the surface of colloidal material. This results in compounds that are not very mobile in the environment and are often persistent. Other important properties are the chemical's vapour pressure, chemical stability and the presence of charged groups (Walker, 2001).

The extent to which a chemical will dissolve in water will also play a role in its fate within an organism. The fate of an organic chemical within an organism will depend upon several factors (Walker *et al.*, 1996):

1. Site of action – where the chemical acts upon the organism
2. Sites of metabolism – the organism acts upon the chemical
3. Sites of storage – the chemical is essentially inert within the organism
4. Sites of excretion – largely of biotransformed products

Hydrophobic organic chemicals (low polarity) pass readily across biological membranes and accumulate in the fatty tissues of organisms (Bartell *et al.*, 1998). Hydrophilic (polar) compounds will be dissolved in water, or associated with charged groups on proteins during transport through an organism (Walker, 2001).

The adsorption to organic material and the uptake of organic chemicals by *L. peregra* will not be under study in this chapter. However, it is worth pointing out

that hydrophobic chemicals such as those used in this study are likely to adsorb/bind strongly to organic material and move readily across membranes.

Modes of action may be divided into two main mechanisms: narcosis and specific action (Rand *et al.*, 1995). An individual chemical can be a specific toxicant in one way where the organism has a susceptible system, yet be a narcotic in another where the test organism does not. Specifically acting chemicals work by binding to a particular molecule and modifying or inhibiting some biological process (Rand *et al.*, 1995). In contrast, narcosis can be described as a non-specific reversible disturbance of the functioning of the cell membrane resulting in decreased activity which can ultimately lead to death (Van Wezel and Opperhuizen, 1995). The exact mechanisms and sites of action of narcosis are still unclear (Franks and Lieb, 1997).

Compared to the literature covering the toxic effects of metals on gastropods, work regarding the effects of organic chemicals is much smaller. In a study by Cuppen *et al.* (2000) application of the fungicide carbendazim to freshwater microcosms elicited a decrease in the population of the snail *Bithynia* sp. but an increase in numbers of all other gastropods including *Lymnaea* sp. In an earlier study by Cuppen *et al.* (1997) the effects of the herbicide linuron on *L. stagnalis*, again applied to a freshwater microcosm, were shown to be secondary in nature. The decrease of available food through death of the resident algal population, resulted in the complete eradication of *L. stagnalis* from the microcosms. Scorgie (1980) also demonstrated the secondary effects of the herbicide cyanatryn. In this study *L. peregra,* initially the single most abundant species was completely eradicated, where previous toxicity studies had shown no detectable effects of long term

exposure of the pond snail to cyanatryn (Scorgie and Cooke, 1979). Bennett *et al.* (1999) also found secondary effects of PAH exposure on gastropods. Initially, there was no difference in the feeding rate of the periwinkle snail *Littorina irrorata* in high and low PAH treatments, but snails exposed to high concentrations eventually became resource limited due to the reduction in microphytobenthic biomass.

Recently interest has risen in a number of organic compounds that are reportedly capable of interfering with the normal endocrine function of animals and humans. One of the most widely studied chemicals, Tributyltin (TBT) has masculinising effects on approximately 150 species of prosobranch molluscs (Oehlemann *et al.*, 2000). However, the majority of studies regarding the effects of endocrine disrupting substances have been carried out in vertebrates due to the comparatively poor understanding of invertebrate hormonal systems.

Oehlemann *et al.* (2000) have recently published a series of papers looking at the effects of bisphenol A, octylphenol, triphenyltin and vinclozolin on the freshwater prosobranch snail *Marisa cornuarietis*. Their findings indicate that some of these chemicals affect the hormonal system of this snail at levels far below those measured in the environment. This series of papers has sparked interest in the use of snails as indicators of environmental contamination throughout Europe and America. Conversely, in an experiment by Czech *et al.* (2001) no adverse effects on the fertility or fecundity of *L. stagnalis* were observed when exposed to the endocrine disrupting chemicals TBT, β-sitosterol, 4-nonlyphenol and t-methyltestosterone.

The two organic chemicals chosen for study in this work were from the PAH and pyrethroid groups. These two classes of chemicals will be considered further.

Polycyclic aromatic hydrocarbons

Hydrocarbons are compounds comprised of the elements hydrogen and carbon only. Polycyclic aromatic hydrocarbons (PAHs) fall within this group and consist of three or more six-membered rings directly linked together (Walker *et al.*, 1996). Major sources include the incomplete combustion of organic materials during industrial processes as well as domestic activities, forest fires and crude oil. Globally, the largest emissions of PAHs are into the atmosphere, where they can be adsorbed onto airborne particles which eventually enter surface water through precipitation or diffusion (Walker, 2001). Once in water, they tend to become adsorbed to the organic material of sediments and taken up by aquatic organisms (Bennett *et al.*, 1999).

PAHs are unreactive and their toxicity is as a consequence of their oxidative transformation which is usually catalyzed by cytochrome P450, O-diphenol oxidase and peroxidase (Walker, 2001). Evidence of some biotransfomation enzymes have been found in lymnaeids such as benzo[a]pyrene hydroxylase (phase I) (Baturo and Lagadic, 1996) and glutathione S-transferase (phase II) (Wilbrink *et al.*, 1991; Baturo and Lagadic, 1996). These two enzymes commonly take part in biotransformation reactions. Some evidence of cytochrome P450 has been reported in the pond snail *L. stagnalis* (Wilbrink *et al.*, 1991). Despite the presence and activation of phase I and II enzymes in lymnaeids, their precise action has not been defined (Baturo and Lagadic, 1996).

PAH's can also become considerably more toxic in the presence of UV radiation which can also transform them into their oxidative products (Walker *et al.*, 1996).

Pyrene was the PAH chosen for this study as a representative and widely studied member of this group of organic compounds.

Pyrethroids

Pyrethroids were created as a more stable, synthetic compound copy of the naturally occurring pyrethrin insecticides found in the flowering heads of *Chrysanthemum* sp (Walker *et al.*, 1996). Pyrethroids are used extensively in agriculture. Spray drift, run off from fields into surface waters and the use of sheep dips can all be sources of pyrethroid contamination to water courses. Pyrethroids have very low water solubility and may become strongly adsorbed to particulate matter. They are reasonably persistent in sediments, but as they are readily biodegradable by many species of microbes, they do not tend to undergo biomagnification (Walker, 2001). However, due to their persistence in sediments there is concern that many benthic invertebrates lacking detoxifying enzymes may bioconcentrate them (Walker, 2001).

The primary mode of action is through interference with ion channels in the nerve axon, resulting in hyperactivity of the nervous system with subsequent lack of control of normal function. In addition to their action on the nervous system, pyrethroids have been reported to interfere with certain ATPase enzymes associated with maintaining ionic concentration gradients across membranes (Solomon *et al.*, 2001). They are highly toxic to terrestrial and aquatic arthropods and somewhat

less to fish. Pyrethroids are metabolised through two pathways, oxidation by cytochrome P450 and esteractic hydroxylation (cleavage of the ester bond) (Walker, 2001). In some cases the metabolites of some pyrethroids may be more toxic than the parent compound (Lutnicka *et al.*, 1996).

In the aquatic environment, pyrethroids are subject to degradation and metabolic processes. The main route of their decomposition comes in the water column through hydrolysis of the ester bond and oxidation. Half-lives vary considerably between pyrethroids, e.g. for cypermethrin in water it ranges from 0.3 to 3 days, and for deltamethrin up to 300 days (Lutnicka *et al.*, 1996). Due to their lipophilic nature, pyrethroids bind strongly to substrates containing organic matter, which results in rapid dissipation from the water column with a reported DT_{50} for lambda cyhalothrin (i.e., time for initial concentration to decline by 50%) of 5 to 11 hours (Hand *et al.*, 2001).

Despite the apparently limited capacity of lymnaeids to detoxify organic compounds, possible toxic effects are not precluded. Lutnicka *et al.* (1999) reported no appreciable accumulation of a variety of pyrethroids in the snail *Physa fontinalis*, but all animals died following a 15 day exposure to each of the chemicals individually at concentrations apparently found in natural waters. Tripathi and Agarwal (1997) reported that a mixture of three pesticides was more effective when compared to a mixture of two or a single pesticide to *L. acuminata*. These authors hypothesized that the increased toxicity of a mixture was due to the inhibition of some enzyme systems by two of the pesticides which increased the toxicity of the third.

Lambda cyhalothrin was chosen as a member of the pyrethroid group for this study. Lambda is a type II cyano group containing synthetic pyrethroid, used mainly as an agricultural pesticide on a wide variety of crops (WHO, 1990).

AIMS

The aims of this chapter were as follows:

- Measure the effects of pyrene and lambda cyhalothrin on the ingestion and egestion rates of *L. peregra* during exposure and post-exposure.
- Establish the ingestion and egestion EC_{50} values of pyrene and lambda cyhalothrin to *L. peregra.*

4.2 MATERIALS AND METHODS.

4.2.1 ANIMALS AND ACCLIMATION.

All animals used for the cadmium and copper experiments were collected from the River Devon site (see Figure 2.1). Animals collected were acclimated to standard laboratory conditions as outlined in section 2.3.2.2.

4.2.2 PREPARATION OF PYRENE AND LAMBDA CYHALOTHRIN TEST SOLUTIONS AND ANALYSIS.

Stock solutions for pyrene and lambda cyhalorthrin were prepared using a thin-layer evaporation technique (Barata and Baird, 2000). Initial stock solutions of concentrated test substance were made in acetone. Aliquots of these stock solutions

were pipetted into 2 L conical flasks, which produced a thin layer of the acetone/test substance over the base of the flask. The acetone was then evaporated off leaving a layer of test substance on the base of the flask. Following the addition of one litre of snail media to the flask, the solution was shaken for 2 days on an orbital shaker to allow the test substance to dissolve into the media.

Pyrene (Aldrich, 98% purity): A 200 µg/L stock solution in acetone was prepared and 5 concentrations of 10, 20, 40, 75 and 150 µg/L were made for both the lethality and post-exposure response tests.

Lambda cyhalothrin (Reidel-de-Haen, 98% purity): A 3 µg/L stock solution in acetone was prepared and 5 concentrations of 0.08, 0.2, 0.5, 1.2 and 3.0 µg/L were made for both the lethality and post-exposure response tests.

Analysis of the stock solutions was done using a fluorescence detector HPLC (Spectra system FL3000 fluorescence detector), by injecting 100 µl of sample into a Phensophere next 5 µm 250x4.6 mm C18 column. Flow rates for both substances. The wavelength used for pyrene was, mobile phase, 14.75 minutes retention time. Only stock solutions were analysed for both pyrene and lambda cyhalothrin.

4.2.3 LETHALITY TESTS.

For each of 5 concentrations of test substance, plus control, 5 x 12 individual *L. peregra* were randomly assigned to 60 ml jars. At each concentration, 6 animals were fed spinach mats as outlined in section 2.3.2.3 and 6 were not. The number of

individuals dead in each treatment was evaluated at 24, 48, 72 and 96 hours. Death was carefully assessed through several methods: a) discolouration of the soft tissue usually with white mucus covering the foot area, b) complete withdrawal into the shell, c) lack of movement or rigidity of the soft tissue when gently handled.

4.2.4 INGESTION AND EGESTION RATES DURING AND POST-EXPOSURE.

Seventy two snails were randomly assigned to 6 groups of 12 jars for both pyrene and lamda cyhalothrin exposure each contained a pre-made spinach mat as outlined in section 2.3.2.3. Each jar was covered with <1 mm mesh secured by an elastic band and placed into one of the 5 tanks containing culture medium made up to one of the test concentrations.

Uneaten food and faecal pellets were collected following a 48 hour period as outlined in section 2.3.2.3. Ingestion and egestion rates were calculated as outlined in section 2.3.1.1. Water samples for analysis were taken at 0 hours and 48 hours from each tank and were stored at 4°C for up to 1 month before being analyzed as outlined in section 4.2.2.

4.2.5 STATISTICAL ANALYSIS.

Ingestion and egestion rate EC_{50} values were calculated from data generated during the 48 hour exposure period and the 24 hour post-exposure period. A one directional General Linear Model with a *post hoc* Bonferroni comparison (Minitab, 2000) was carried out to establish which concentrations produced rates that were

significantly different from the control. Data were then fitted to an allosteric regression model using Sigma Plot version 7.0, to estimate the EC_{50} value for that metal. The allosteric model was fitted only to data which produced a significant change as defined by GLM analysis in either ingestion or egestion rates.

4.3 RESULTS.

The pyrene stock solution was initially made to 10 μg/ml. The actual concentration of pyrene stock analysed using HPLC was 13.75 μg/ml.

Lambda cyhalothrin was not analysed at the time of printing, so all concentrations will be presented as nominals.

LC_{50} values for pyrene (highest exposure concentration was at 75% solubility limit in water) and lambda cyhalothrin (highest exposure concentration was at solubility limit in water) could not be calculated for *L. peregra* as no lethality was observed for either chemical over a 96 hour period.

Mortalities were very low during the pyrene experiment with one dead in the 20 μg/L treatment over the 48 hour exposure period and one in each of the 75 and 150 μg/L treatments during the post exposure period.

Figure 4.1a shows the ingestion rates for *L. peregra* exposed to pyrene over 48 hours. Statistical analysis (Table 4.1) showed no significant difference between the control and exposed treatments. Although Figure 4.1a shows a decrease between

the control ingestion rate and those presented between 10 and 75 µg/L, the raw data shows that three high values increased the control level and in fact most of the data values were very low. Again the same was true for the data collected from the 150 µg/L treatment. The ingestion rates shown in Figure 4.1a were very low. The post exposure ingestion rates (Figure 4.1b) are again very low and unsurprisingly show no significant difference between the control and treatment rates.

Figure 4.1c shows the egestion rates calculated from the 48 hour exposure of *L. peregra* to pyrene. Again there was not a significant statistical difference from the control (Table 4.1). During the post exposure period all egestion rates decreased showing no statistically significant difference between the control and exposed treatments.

In the lambda-cyhalothrin experiment, two mortalities were recorded during the 48 hour exposure period, one each in the 0.2 and 3.0 µg/L treatments. Subsequently in the post-exposure period one mortality in each of the 0, 0.08 and 0.5 treatments was recorded.

Figure 4.2a shows the ingestion rates of *L. peregra* exposed to lambda cyhalothrin over a 48 hour period. No treatment showed a significantly lower value when compared to control (Table 4.2). The ingestion rates (including control) measured during the exposure and post exposure periods were high when compared to previous chapters.

There was no statistically significant difference between control and treated rates either during exposure or post exposure period for egestion (Figures 4.2 c and d). As with ingestion rates, overall the egestion rates were higher than those measured in previous chapters.

Table 4.1: Results of one way ANOVA to compare the effects of pyrene on *L. peregra* ingestion and egestion rate during and following a 48 hour exposure period.

Experiment	Source of error	Sum of squares	d.f.	F-value	p-value
During exposure ingestion	Treatment	0.682	5	1.39	0.237
	Error	8.157	83		
	Total	8.83	88		
During exposure egestion	Treatment	0.119	5	1.17	0.333
	Error	1.456	83		
	Total	1.576	88		
Post-exposure ingestion	Treatment	0.143	5	0.51	0.770
	Error	4.614	82		
	Total	4.757	87		
Post- exposure egestion	Treatment	0.539	5	2.05	0.08
	Error	4.307	82		
	Total	4.846	87		

Table 4.2: Results of one way ANOVA to compare the effects of lambda-cyhalothrin on *L. peregra* ingestion and egestion rate during and following a 48 hour exposure period.

Experiment	Source of error	Sum of squares	d.f.	F-value	p-value
During exposure ingestion	Treatment	0.604	5	0.95	0.453
	Error	10.420	82		
	Total	11.024	87		
During exposure egestion	Treatment	0.523	5	1.19	0.319
	Error	7.186	82		
	Total	7.709	87		
Post-exposure ingestion	Treatment	0.735	5	1.08	0.375
	Error	10.704	79		
	Total	11.438	84		
Post- exposure egestion	Treatment	0.102	5	0.45	0.811
	Error	3.565	79		
	Total	3.666	84		

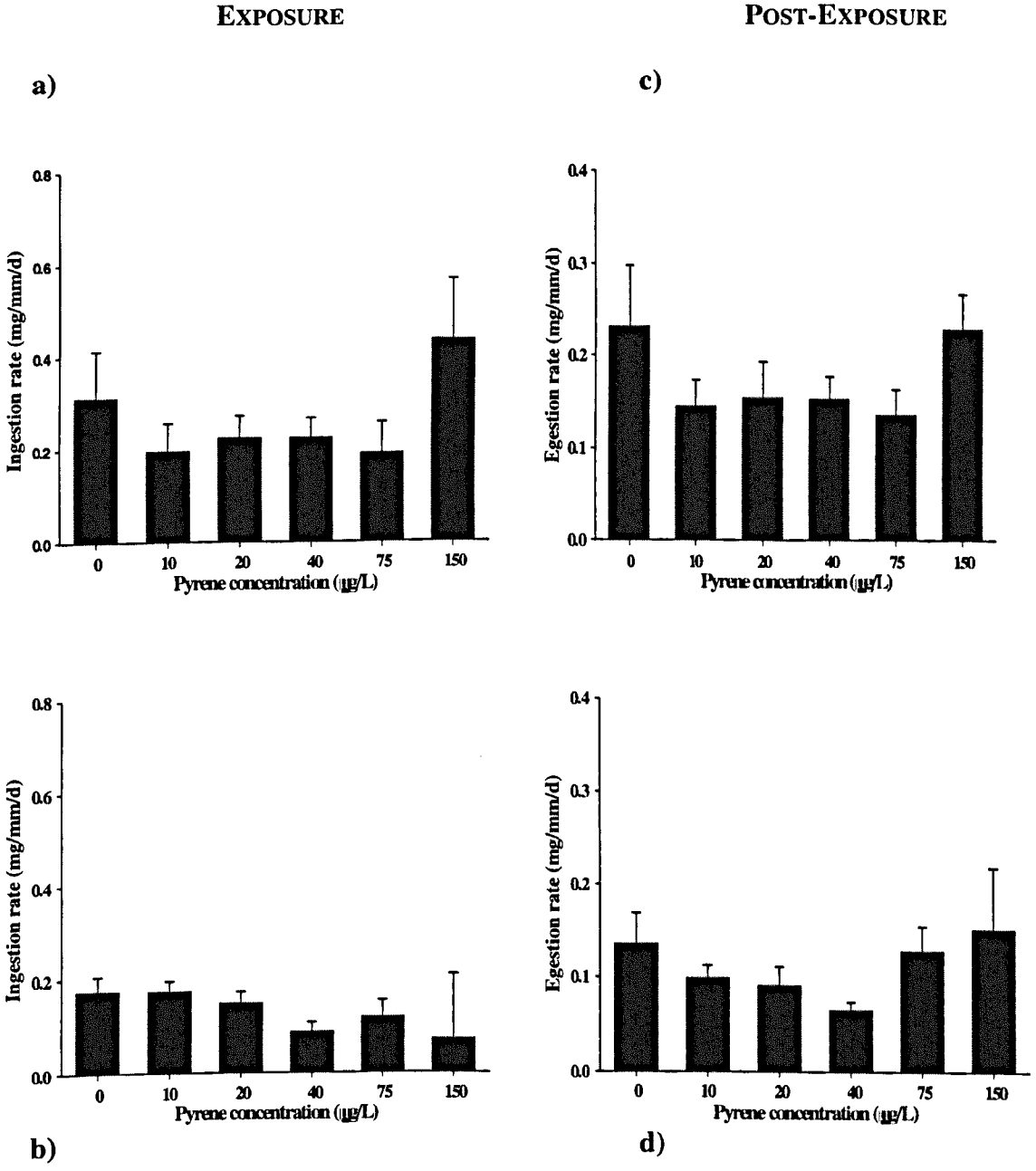
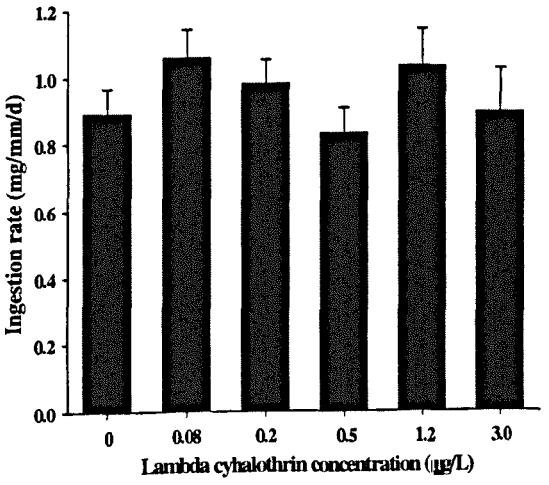


Figure 4.1: Ingestion and egestion rates during 48 hour exposure and 24 hour post-exposure periods following exposure to a range of pyrene concentrations. Error bars indicate standard error.

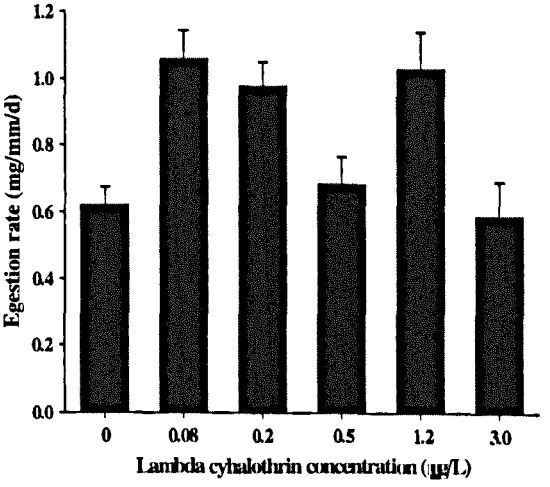
EXPOSURE

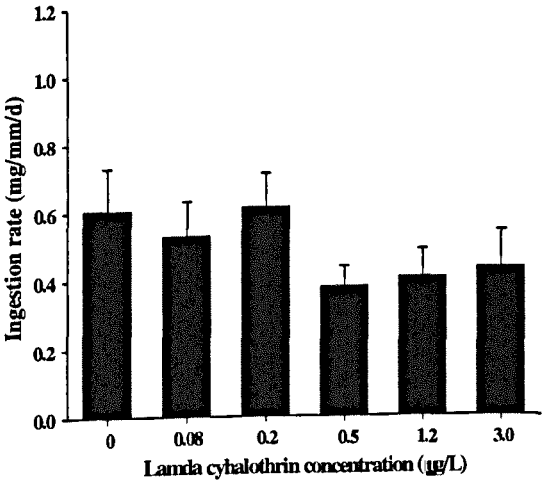
POST-EXPOSURE

a)

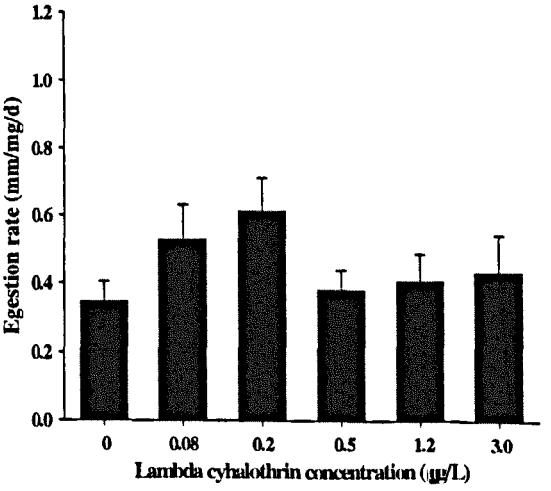


c)





b)



d)

Figure 4.2: Ingestion and egestion rates during 48 hour exposure and 24 hour post-exposure periods following exposure to a range of lambda cyhalothrin concentrations. Error bars indicate standard error.

4.4 DISCUSSION.

Exposure to pyrene and lambda cyhalothrin had no lethal effect at or below 75% and 100% respectively, of the experimental solubility limit in freshwater. Low acute toxicity at high PAH concentrations of *Lumbriculus variegatus* has been rationalized by Kukkonen and Landrum (1994), as a failure to bioaccumulate the contaminant to levels expected to cause mortality by non-polar narcosis. As reported by Lotufo (1998), lethality occurs at tissue concentrations of organic contaminants active via non-polar narcosis at about 2 to 8 μmol (g wet wt) for most invertebrates. In this study by Lotufo (1998), *Schizopera kanabeni* and *Coullana* sp. reached this concentration in less then 12 hours, however mortality did not occur until days 4 to 10 of the exposure period. Despite the differences between copepods and lymnaeids, during the lethality experiment *L. peregra* was exposed to very high concentrations of pyrene, apparently feeding at normal levels for 96 hours yet ostensibly not reaching this critical level. The lymnaeids must have been either excreting efficiently, storing large quantities of pyrene, not ingesting at a normal rate or may require a longer exposure period before mortality is observed.

Despite the general lack of effects on ingestion or egestion rates of pyrene on *L. peregra*, sublethal effects on feeding have been reported in PAH exposed animals. In a study by Lotufo and Fleeger (1996), low mortality of *Limnodrilus hoffmeisteri* was reported when exposed to high concentrations of pyrene in sediment. However, there were significant sublethal toxic effects which were detected as a decrease in sediment ingestion rates. Feeding rate of various species of aquatic invertebrates has reportedly decreased as a result of exposure to PAHs and is likely to be as a

direct consequence of the bioaccumulation of organic contaminants with a narcotic mode of action (Van Wezel and Opperhuizen, 1995). Decreased grazing rates with increasing body burden of fluoranthene were found for both *S. knabeni* and *Coullana* sp. (Lotufo, 1998).

Differences in the patterns of bioaccumulation of PAHs are likely related to an organism's ability to biotransform these chemicals (Lotufo, 1998), which will affect their rate of elimination. Invertebrate species that biotransform PAHs efficiently will also eliminate them rapidly (Lotufo, 1998). However, several animals with poor ability to biotransform, such as *S. knabeni* and *Collana* sp., are capable of eliminating PAHs quickly (Lotufo, 1998). In a study by Legierse *et al.* (1998) the uptake and elimination rates of chlorthion in *L. stagnalis* were found to be higher than those in the guppy indicating that the pond snail does not significantly biotransform this organic chemical. Additionally the location of only limited biotransformation enzymes and an inability to define their action in lymnaeids may mean that they do not metabolise organic pollutants to any extent.

The relatively low fat content of lymnaeids (0.5%) (Legierse *et al.*, 1998) indicates that they are unlikely to store any substantial amount of PAH in these reserves. Proteins, however, also play an important role in the accumulation of hydrophobic organics in some aquatic species (Seydel and Schaper, 1982). With the haemolymph contributing one third to one half of the total body weight in lymnaeids containing 2 to 6% protein (McMahon, 1983), this might represent a considerable store for hydrophobic organic chemicals.

During exposure to pyrene no statistically significant differences were present between the control and exposure ingestion rates despite the ingestion rates for the control and 150 µg/L treatments appear elevated compared to the 10, 20, 40 and 75 µg/L treatments. However, the raw data shows that the ingestion rates measured for two snails in the control group and three snails in the 150 µg/L group were comparatively high and increased the means associated with these treatments. In actual fact the overall ingestion rates for animals during the 48 hour exposure period were very low compared to those measured in past chapters. The post exposure ingestion rate again shows no significant difference between the control ingestion and egestion rates and again the ingestion rates measured were very low. Possible reasons for this as discussed in Chapter 3 were an insufficient acclimation period, handling stress or poor overall condition of animals. Seasonality may have also been a factor in this experiment as these animals were collected in the late fall when the breeding season has concluded and animals are on the decline. However, as will be discussed in the following section, low ingestion rates were not observed for lambda cyhalothrin exposed animals despite collection from the same site at the same time of the year.

Due to their lipophilic nature, pyrethroid insecticides bind strongly to substrates containing organic matter, resulting in a very rapid rate of dissipation from the water column (Hand *et al.*, 2001). It is likely that most of the lambda cyhalothrin in solution would have become adsorbed to the food source and animals within the first 24 hours of exposure. In this study by Hand *et al.* (2001), the adsorption of lambda cyhalothrin to macrophytes was rapid with a DT_{50} in a semi realistic system of 3 hours.

Singh and Agarwal (1991) reported the sublethal effects of cypermethrin (cyano group containing pyrethroid) on enzyme activity in the nervous tissue of *L. accuminata* by exposing them to 40 and 80% of the previously estimated 24 hour LC_{50} concentration. The exposure concentrations were high at 320 µg/L and 640 µg/L when compared with effective concentrations: 24 hour cypermethrin LC_{50} for *Daphnia magna* of 2µg/L (Stephenson, 1982) and 24 hour LC_{50} for *Gammarus pluex* of 0.09 µg/L (Shires, 1983). Lymnaeids are more resistant to pyrethroids than most aquatic invertebrates and do not respond to concentrations of these chemicals in natural waters.

When exposed to a range of lambda cyhalothrin concentrations no statistically significant effects were observed. The ingestion rates measured for snails during this experiment were very high compared to those in past chapters. However the egestion rates for both during and post exposure periods fall within the observed range. Again the reasons for an elevated ingestion rate can only be speculated upon. During the acclimation period animals were fed, all animals were of a similar length (between 8 and 12.5 mm) and were collected from the same site at the same time. Had the ingestion rates during exposure shown a significant increase from the control, some excitatory effect of lambda cyhalothrin on feeding might have been conjectured. However, as stated previously there was no statistically significant difference from the control and while Figures 4.1a and b show a small increase in ingestion rates it is not consistent across all treatment levels.

No lethal effects of pyrene or lambda cyhalothrin were observed for *L. peregra*. Subsequently, no consistent sublethal effects on either ingestion or egestion rates

were observed during a 48 hour exposure or post exposure periods to very high concentrations. From the limited literature it seems that lymnaeids do not transform hydrophobic organic chemicals to any extent, despite the presence of phase I and II enzymes and may either store them in the haemolymph or excrete them rapidly. Whatever the case, while lymneaids are subject to secondary effects of some organic chemicals such as herbicides under semi-natural conditions, their use in a laboratory based bioassay to assess the toxicity of PAHs or pyrethroids is limited.

A more comprehensive study of the effects of these two chemicals on *L. peregra* would have been undertaken had the initial results shown some toxic effects. Further work with *L. peregra* and both PAHs and pyrethroids should include measurement of body burden as well as uptake and elimination studies to look more closely at the possible biotransformation of these organics by this snail.

CHAPTER 5

ROUTE OF CADMIUM UPTAKE, BODY BURDEN AND ASSOCIATED FEEDING AND GROWTH EFFECTS.

5.1 INTRODUCTION

Aquatic animals are potentially exposed to metals from both the surrounding water and through their diet. The relative contribution of these exposure routes to metal uptake is fundamental in predicting bioavailability, which can be defined as the proportion of the total quantity of contaminant that is potentially available for biological action (Spacie *et al.*, 1995).

The primary purpose of the work in this chapter was to carry out an investigation into the predominant route of cadmium uptake in *L. peregra* (food versus water). In addition, to correlate the possible effects of cadmium on ingestion and egestion rates on growth and body burden over a 4-week period.

Aquatic invertebrates are exposed to metals from both the particulate and dissolved phases. The transfer of metals from the surrounding medium is a result of the actions and interactions between a large set of factors controlling the partitioning of the elements within the dissolved and particulate phases, their chemical speciation and bioavailability (Plette *et al.*, 1999). The accumulation and toxicity of a metal depends largely on its bioavailability, which is determined by its rate of uptake (Gomot de Vaufleury and Phian, 2002), and is greatly influenced by the exposure

routes and characteristics of the organism. Total metal concentrations in solution alone do not predict bioavailabilities so are inadequate predictors of metal toxicity.

In the environment, under natural conditions, the bioavailability of a metal is influenced by many factors including speciation, pH, reactions with other chemicals, complexation with organic matter and water hardness (Walker *et al.*, 1996; Plette *et al.*, 1999), all of which influence the degree or potential to which the metal is available for biological action on the organism. Following uptake metal bioavailability is influenced further by nutritional status, size, age, feeding strategy, trophic level and gut pH (Sabalinunas *et al.*, 1998). It is important to consider the reactions that take place following uptake, as the presence of a metal in an organism is not an adverse biological effect in itself. Only the biological responses induced by the presence of the metal constitute potential adverse effects (Spacie *et al.*, 1995).

In general it is assumed that metal uptake depends almost exclusively on the concentration of the element in the water phase, without considering the possible influence of food, particulate matter and sediments and other compounds present (Guerrero *et al.*, 2000). The importance of uptake route depends on the physiology of the organism, the geochemistry of the metal and the physicochemical parameters of the environments (Blackmore and Morton, 2002). The accumulation of a chemical from the water alone is called bioconcentration. Under natural conditions bioconcentration must be interpreted with caution as it is difficult to exclude the possibility that some uptake may be associated with dissolved, colloidal and particulate organic carbon phases (Spacie *et al.*, 1995). In addition to uptake

through water and sediments, food may also represent an important source of metals to aquatic invertebrates. The uptake of chemical(s) from any source (water, sediment, and other organisms) or possible routes (i.e. diet, respiration, dermal) is called bioaccumulation. Proper distinction between bioaccumulation and bioconcentration is relevant because it is increasingly recognised that, besides uptake from water, uptake from food, sediment, and suspended particles may also be relevant, depending again on the physicochemical characteristics of the studied compound, the biology of the organism and the exposure scenario (de-Maagd, 2000).

Initially upon intake, cells in the digestive tract take up elements from ingested food and from here they may be distributed to other cells in the body (Blackmore and Morton, 2002). It is therefore expected that a metal taken up mainly from food should become concentrated in the body tissues. Metals taken up from solution are either adsorbed onto exoskeletons or pass across permeable tissue surfaces where they may be distributed to the rest of the animal (Miramand and Bentley, 1992). Evidence that food is a significant source of some metals in some organisms can be gleaned from comparisons of metal uptake from food and solution, mass balance models of the biological fate of metals and indirect observations upon tissue distributions (Blackmore and Morton, 2002). It is, however, difficult to quantify metal bioavailability from food (Wand and Fisher, 1999). Additionally routes of metal uptake may vary between organisms and between metals for the same organism.

No consensus exists in the literature on the relative importance of trace metal uptake from dietary or aqueous sources in aquatic organisms (Timmermans *et al.*, 1992). Depending on whether the uptake route is from water or via ingested food, the properties of the biological barriers that separate the organisms from their surrounding medium e.g. gills, mantle, gut wall, lead to varying degrees of metal distribution in different organs (Baudrimont *et al.*, 1997). Several studies have investigated the influence of ingestion on uptake and accumulation of contaminants (e.g. Timmermans *et al.*, 1992; Warren *et al.*, 1998; Roy and Hare, 1999; Guerrero, 2001). Metal accumulation from water only generally occurs across gills, the outer epithelium or the exoskeleton. However, *L. peregra* do not possess gills, so for these animals, the digestive tract should be the principal tissue involved in metal uptake (Guerrero *et al.*, 2000). In some studies it has been reported that that presence of food increases the uptake of contaminants by increasing the rate of feeding or offering additional surfaces for the adsorption of metals. On the contrary, other studies have shown a decrease in feeding rate in animals exposed to metals (Guerrero *et al.*, 2000).

The route of uptake can modify exposure to a contaminant, as absorption efficiencies from food are usually lower than absorption efficiencies associated with bioconcentration. While exposure via ingestion may contribute only a small amount to body burden, the effect is additive (Fisher *et al.*, 1986) and may have a significant impact on the effects of exposure. Laskowski and Hopkin (1996) found that the assimilation efficiency of *H. aspersa* for cadmium at 68-72%, was approximately equal to the value for food. Despite the similarity of assimilation efficiency between cadmium and food, the metal concentrations in snail tissues after a four-

month exposure were higher than those in the food. This was however, only the case for cadmium. Laskowski and Hopkin (1996) also found that zinc was not accumulated to levels exceeding that in food and that lead was regulated efficiently.

Kungolos and Aoyama (1993) reported that the addition of food significantly increased the toxicity of chromium and cadmium to *Daphnia*, even though bioconcentration was by far the most important uptake route. Dietary exposure may also be particularly important for toxicants that produce effects by inhibiting feeding or digestion. As found by Taylor *et al.* (1998), cell ingestion was inhibited by the presence of cadmium contaminated algae leading to reduced growth and reproduction in *Daphnia magna.*

Negative effects on various physiological and behavioural sublethal endpoints have been recorded during exposure to metals. Previous chapters have illustrated the sublethal effects of metals on behavioural endpoints such as feeding. Another sublethal endpoint related to feeding behaviour is growth. Several studies have demonstrated a decrease in growth of *H. aspersa* when fed a diet contaminated with cadmium (Russell *et al.*, 1981; Simkiss and Watkins, 1990; Gomot, 1997; Gomot de Vaufleury, 2000). Results from these studies appear contradictory, as some report a decrease in food intake with no related decrease in growth. The question posed by Gomot de Vaufleury (2000) following a thorough review of available literature with regard to snails was, 'Is the reduction in food intake the cause or the consequence of the reduction in growth? '

Despite the apparent contradictions in the literature with regard to the effects of cadmium on feeding and growth, all authors concur that in general gastropods accumulate metals primarily in their tissue (as opposed to the shell). Aquatic invertebrates accumulate metals in three phases: 1) metal uptake, 2) metal transport, distribution and sequestration and 3) metal excretion (Rainbow and Dallinger, 1993). As discussed in Chapter 3, snails possess the capacity to take up large quantities of metals and sequester them with little or no apparent consequences for their survival.

AIMS

The aims of this chapter are as follows:

- To investigate the major routes of cadmium exposure to *L. peregra* (food or water?)
- To investigate whether or not the uptake from water vs. food corresponds with the short term sublethal response on ingestion and egestion rates as discussed in previous chapters.
- To look at the possible effects of 26 day cadmium exposure on growth (measured as shell length).

5.2 MATERIALS AND METHODS

5.2.1 CADMIUM CONTENT OF UNCONTAMINATED SPINACH.

The use of fertilisers can elevate the cadmium content of commercially produced spinach. The level of cadmium in spinach used as a food source during experiments was therefore established. Spinach mats were made as outlined in section 2.3.2.3, dried and weighed. As a comparison small pieces of lettuce were washed, placed in a metal weigh boat and dried at 60°C for 24 hours, then weighed. Additionally, 3 controls containing 1ml of media and 5 ml of nitric acid were also analysed as outlined in section 3.2.4.

5.2.2 SHORT TERM EXPERIMENT

All animals used in the short and long experiments were collected from the Howietoun site and acclimated to laboratory conditions as outlined in section 2.3.2.2.

Sixty snails were randomly assigned to 5 groups of 12 jars; any food given during this experiment was in the form of a spinach mat prepared as outlined in section 2.2.3.2. The 5 treatments were as follows:

1. 0 µg/L in water with no food given
2. 0 µg/L in water with food
3. 0 µg/L in water with contaminated food as outlined in section 5.2.2.1
4. 150 µg/L in water with no food
5. 150 µg/L in water with food

For further clarification of the experimental set-up, see Figure 5.1.

Each tank contained 5 L of culture media at the beginning of the experiment. Each jar was covered with <1 mm mesh secured by an elastic band and placed into one of the 5 tanks containing culture medium made up as outlined above.

Uneaten food and faecal pellets were collected following a 48 hour period as outlined in section 2.3.2.3. Ingestion and egestion rates were calculated as outlined in section 2.3.1.1. Water samples for analysis were taken at 0 hours and 48 hours from each tank and fixed with HNO_3 (1% final concentration). Water samples were stored at 4°C for up to 1 month before being analysed as outlined in section 3.2.2. Three further spinach mats were placed in each of the three 'with food' treatments for the duration of the 48 hour exposure period and analysed as outlined in section 3.2.4.

Following the 48 hour feeding period, animals were placed in a 74 µg/L solution of EDTA for 24 hours without food, measured for length and frozen at -4°C for future analysis.

5.2.2.1 CONTAMINATION OF SPINACH MATS

A quantity of boiled and macerated spinach was prepared as outlined in section 2.1.2. Cadmium stock was added to this to make the solution up to 100 µg Cd/L, to ensure contamination of the spinach mats. Spinach mats were then prepared as outlined in section 2.3.2.3. Three spinach mats were placed in

artificial snail media for 48 hours and subsequently analysed for cadmium content as outlined in section 3.2.4.

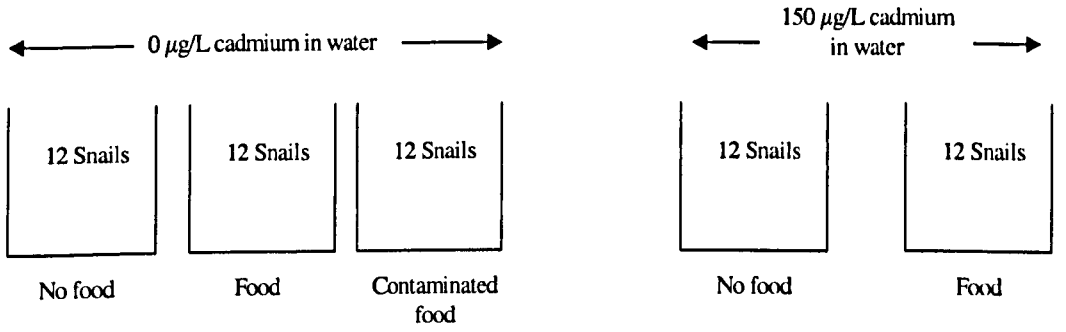


Figure 5.1: Diagram of experimental set up for short term experiment.

5.2.2.2 TISSUE DIGESTION.

Soft tissue removed from the snail shell was dried at 100°C and weighed. The dried tissue was then placed in one of 10 ml, 15 ml and 20 ml Teflon bombs that had been acid washed as outlined in section 3.2.4. Each Teflon bomb contained 5 ml of HNO_3 (Analar grade). For each trial, 3 blanks containing only nitric acid were included to ensure there was no contamination from the bomb. Control bombs were rotated randomly throughout each of the digestions. Bombs were then securely sealed and placed an oven set at 110°C for 2 hours. The bombs were cooled to room temperature before being transferred to a 15 ml Teflon sample tube and refrigerated for later analysis.

All metal analysis was carried out directly on a Unicam GF90 graphite-furnace atomic absorption spectrometer (detection limit 0.5 μg Cd/L).

5.2.3 LONG TERM EXPERIMENT

Thirty snails were randomly assigned to 3 groups of 3 tanks containing 5 L of culture medium made up to 0, 50 or 100 μg Cd/L. Culture medium was exchanged every 4 to 5 days when 3 ml of boiled and macerated spinach was given with approximately 0.5 g of Tetramin fish food. Water samples for analysis were taken before the medium was exchanged and immediately after from each tank and fixed with HNO_3 (1% final concentration). Water samples were stored at 4°C for up to 1 month before being analysed as outlined in section 3.2.2.

Feeding trials were carried out at the beginning, middle and end of the 27 day period over which this experiment was run. During the feeding trials snails were measured for length and then placed individually into a glass jar containing a pre-made spinach mat. Snails were allowed to feed for 48 hours at which time uneaten food and faecal pellets were collected as outlined in section 2.3.2.3. Ingestion and egestion rates were calculated as outlined in section 2.3.1.1.

Snails were measured for length on day 27 of the trial and placed in a 74 μg/L solution of EDTA without food. This depuration period in the presence of EDTA was to ensure that no excess metal adhered to the foot tissue and to void the gut contents which may have biased the final body cadmium concentration. Snails were then frozen for future analysis. Tissue analysis was carried out as outlined in section 5.2.2.2.

5.2.4 STATISTICAL ANALYSIS

A one directional General Linear Model (Minitab, 2000) was carried out to establish which concentrations produced rates that were significantly different from one another.

5.3 RESULTS

5.3.1 CADMIUM CONTENT OF UNCONTAMINATED SPINACH

The mean concentration of cadmium in uncontaminated spinach was 0.45 μg/mg (sd 0.18, n = 10) as compared to that in lettuce at 0.0084 μg/mg (sd 0.013, n = 8). Commercially bought spinach contains 50 times the amount of cadmium when compared to lettuce. Some uptake of cadmium will occur through feeding, but uptake into the tissues will be accounted for in the measurement of final body burden.

5.3.2 SHORT TERM EXPERIMENT

There were two mortalities during the course of this experiment. Both were in the treatment including cadmium in the media with food provided.

Cadmium concentration in media for all treatments is given in Table 5.1. Both food and media in the two control treatments were below the limit of detection for cadmium. The treatment in which snails were exposed to cadmium through the

food only showed some leaching of the metal from the spinach mat, however the concentration in the media remained below 1 μg/L. Cadmium concentrations in the treatments with and without food decreased by and average of 37% and 31% respectively. The spinach mats in the treatment with cadmium in the media contained 9.56 (± 1.5 sd) μg/L of the metal at the end of 48 hours exposure. This value concurs with those found in Chapter 3, for the uptake of cadmium onto spinach mats. The initial cadmium concentrations in the two treatments with contaminated media were approximately 10 to 12 % higher than nominal and decreased predictably (as outlined in Chapter 3) by the end of the 48 hour period.

Cadmium concentration in the soft tissue only of *L. peregra* is illustrated in Figure 5.2. Cadmium uptake from food only did not differ significantly from either of the two treatments containing no cadmium in the media ($p<0.05$). However, the metal uptake from the two treatments with cadmium contaminated media were significantly different from the treatments with no cadmium in the media, but not significantly different from each other ($p<0.05$). Snails in the two treatments with cadmium in the media showed an approximately 16 fold increase in concentration from the food only contamination source.

Figure 5.3 a and b show the ingestion and egestion rates of *L. peregra* from the 3 water and food combination treatments over a 24 hour period. Ingestion and egestion rates were not significantly different between the any of the treatments.

Table 5.1: Average cadmium concentrations in the media and spinach mats for short experiment. Spinach mats are given ± standard deviation.

	0 Cd no food	0 Cd food	Cd in food	Cd in media no food	Cd in media with food
Conc. time 0 (μg/L)	0.03	-0.002	0.062	170.4	166.2
Conc time 48 (μg/L)	0.097	0.56	0.586	117	104.4
Cd in spinach mats (mg/kg)		<0.05	98.06 ± 5.2		9.56 ± 1.5

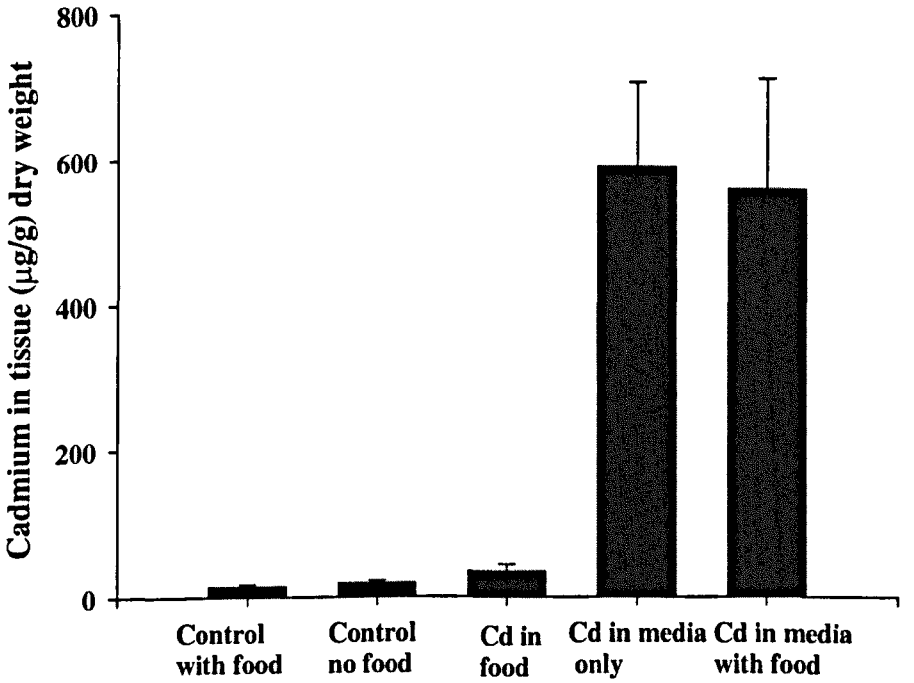
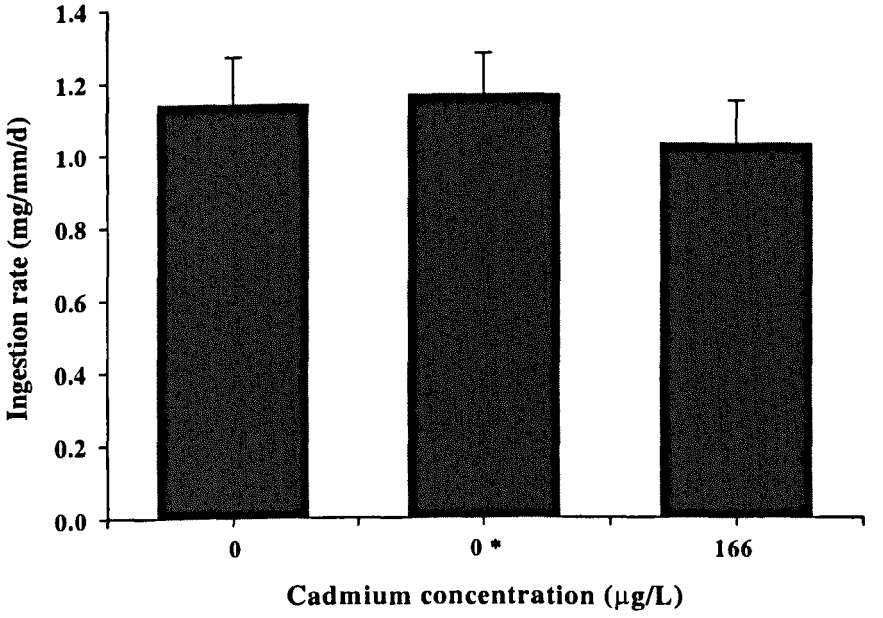


Figure 5.2: Cadmium concentration in whole body soft tissue (dry weight) of *L. peregra*, following 72 hours total exposure to different combinations of Cd contaminated food and water. Bars denote standard error.

a)



b)

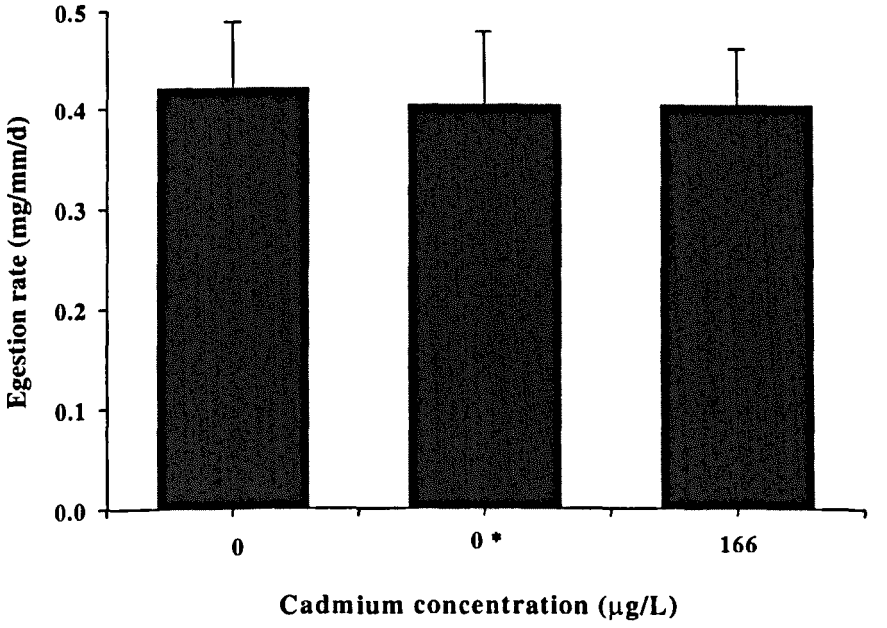


Figure 5.3: Ingestion (a) and egestion (b) rates for *L. peregra* exposed to different combinations of Cd contaminated food and water. Asterix denotes treatment with no Cd in medium but in food. Bars denote standard error.

5.3.3 LONG TERM EXPERIMENT

Mortality rates for the long term experiment were high at 30% in each treatment by the end of the 26 days.

Table 5.2 shows the cadmium concentrations in the media before and after exchange. As shown in Table 5.2a, there was some increase in the cadmium concentration of the media in the 0 µg Cd/L treatment over the 4 to 5 day period between exchanges with levels in the media remaining below 0.3 µg/L. As observed during the short term experiment, this was likely due to the presence of some cadmium in the food source. Cadmium concentrations in the 50 µg/L treatment are shown in Table 5.2b. Again as seen in Chapter 3 a high percentage of cadmium was lost over the 4 to 5 days. The highest cadmium concentration used was 100 µg/L (Table 5.2b). On days 5 and 10 the starting concentrations were quite low compared to nominal, but still high enough to produce effects on ingestion and egestion rates as maintained by the results obtained in Chapter 3.

Cadmium concentrations measured in the soft tissue of *L. peregra* at the end of the 26 day experiment are shown in Figure 5.4. Snails exposed to 0 µg Cd/L had an average concentration of 9.8 (±12.7, sd) µg Cd/g. Those exposed to 50 and 100 µg Cd/L contained 250.6 (±83.1, sd) µg Cd/L and 1066.9 (553.4, sd) µg Cd/L, respectively.

Table 5.2: Cadmium concentrations taken before and after media exchange every 4 to 5 days for the duration of the experiment at nominal concentrations of a) 0 µg/L b), 50 µg/L c), and c)100 µg/L.

a)

	DAY						
	0	5	10	14	19	21	26
Before		0.077	0.028	0.049	0.043	0.025	0.034
After	0.257	0.099	0.106	0.051	0.047	0.064	0.058

b)

	DAY						
	0	5	10	14	19	21	26
Before		12.55	24.05	9.615	5.595	36.97	26.583
After	48.1	46.14	45.65	43.97	55.44	57.45	53.25
Percent decrease over 4 to 5 days	74	48	79	87	33	54	

c)

	DAY						
	0	5	10	14	19	21	26
Before		32.14	57.03	33.29	36.58	79.53	58.29
After	107.1	62.1	65.89	100.8	89.5	122.4	105.6
Percent decrease over 4 to 5 days		70	8	49	64	11	52

Ingestion rates fell over the 26 day period as illustrated in Figure 5.5a. Control ingestion rates fell successively from the initial feeding trial to the third. Ingestion rates measured at the end of the 26 day period decreased in all treatments. It can also be noted from Figure 5.5a that the ingestion rates measured at the beginning of the 26 day period were slightly below those found in previous chapters.

The control egestion rates measured at the beginning and end of the 26 day period were consistent and fell within the range seen in previous chapters (Figure 5.5). However, the egestion rates measured in the second feeding trial during the 26 day period were consistently lower for all treatments which was mirrored by a drop in ingestion rate for the same feeding trial. Egestion rates remained the same or increased during the third and final feeding trial which did not correspond with the very low ingestion rates seen during the same period. Overall the egestion rates measured for animals exposed to 50 and 100 μg/L showed a decrease when compared to the control animals. The egestion results from this experiment indicate a decrease within increasing cadmium concentration. However, the ingestion rates measured for the control animals over the same three time periods indicate that the animals may have been suffering from handling stress and were voiding the contents of their guts during this period.

Snails from all treatments grew over the 26 day period (Figure 5.6) with average growth per week of 0.24 mm (control), 0.55 mm (50 μg/L treatment) and 0.4 mm (100 μg/L treatment). The controls grew the least showing that there was no apparent effect of cadmium on the growth of *L. peregra*.

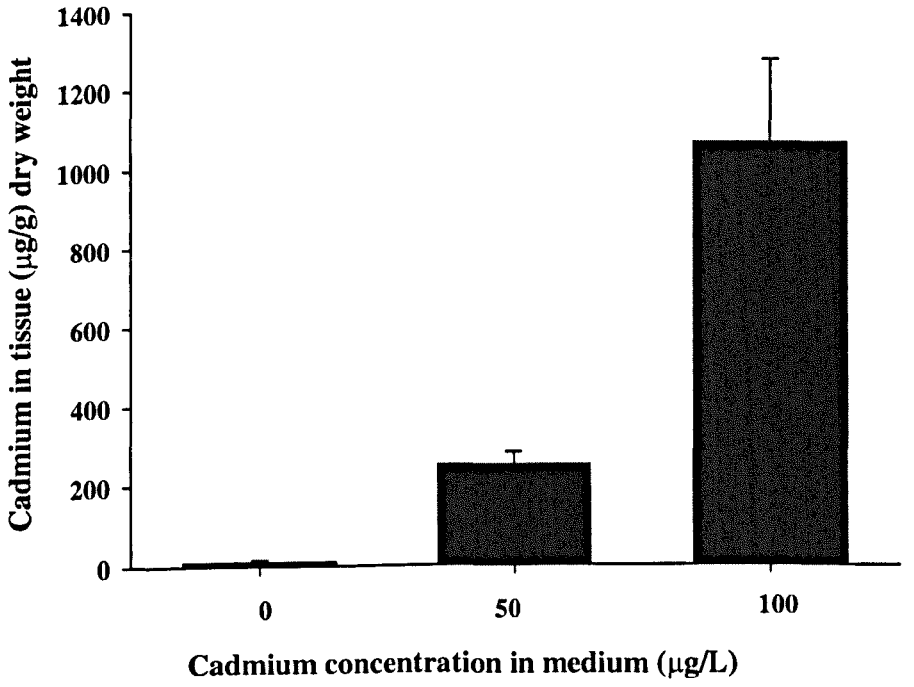
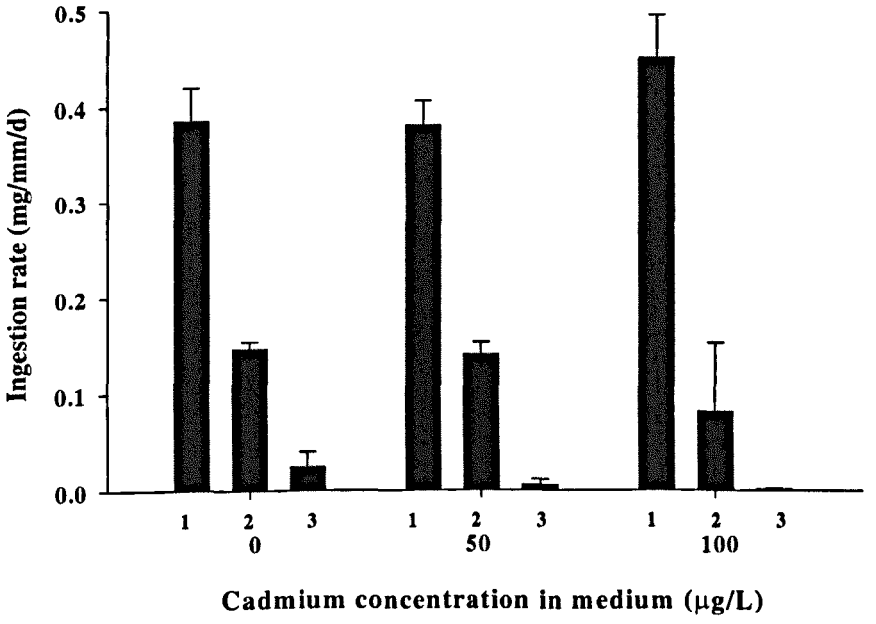


Figure 5.4: Cadmium concentration in whole body soft tissue (dry weight) of *L. peregra*, following 26 days exposure to each cadmium concentration. Bars denote standard error.

a)



b)

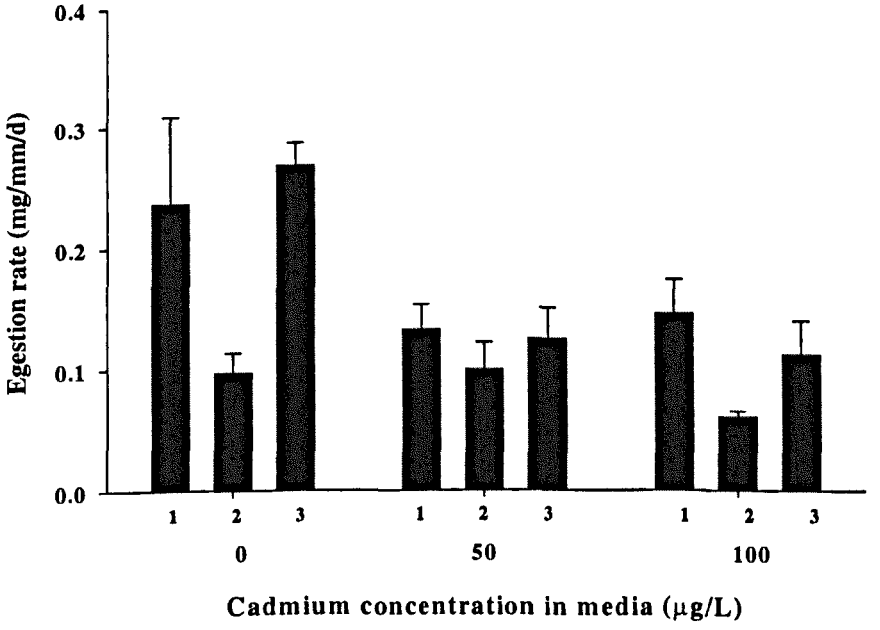


***Figure 5.5*:** Ingestion (a) and egestion (b) rates at beginning (1), middle (2) and end (3) of long term experiment

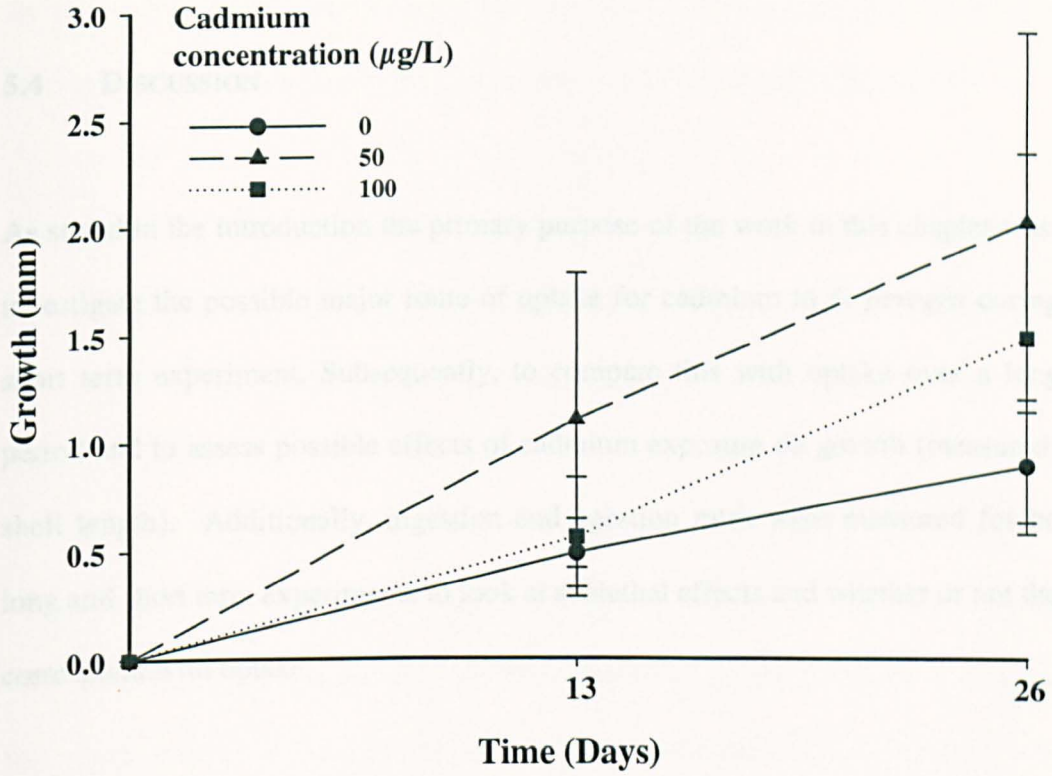


Figure 5.6: Average shell length measured for each individual at the beginning, middle and end of the 26day trial. (n=21)

5.4 DISCUSSION

As stated in the introduction the primary purpose of the work in this chapter was to investigate the possible major route of uptake for cadmium in *L. peregra* during a short term experiment. Subsequently, to compare this with uptake over a longer period and to assess possible effects of cadmium exposure on growth (measured as shell length). Additionally, ingestion and egestion rates were measured for both long and short term experiments to look at sublethal effects and whether or not these correspond with uptake.

Due to intensive cultivation in hothouses or greenhouses, and the use of cadmium containing phosphorous fertilizers (Stenstrom and Vahter, 1974), the concentration of cadmium in spinach was measured to establish if this might affect levels in *L. peregra* tissue. The results in section 5.3.1, show a mean cadmium concentration in spinach of 0.045 μg/mg and lettuce of 0.0084 μg/mg. Presing *et al.* (1993) reported a mean cadmium concentration of 14.3 (± 5.14) μg/g in hothouse grown lettuce used as a food source during experiments. This resulted in a 0.37 ug/g increase in *L. stagnalis* soft tissue concentration of cadmium per month. The cadmium concentration measured in the spinach used as a food source in this experiment was higher than that found in lettuce by Presing *et al.* (1993). Over a long time period this may lead to elevated concentrations of cadmium in the soft tissues and possible desensitization to the effects of the metal.

Mortality rates were low in all 5 treatments used during the short-term experiment. In the treatment with food provided in cadmium contaminated medium there was 20% mortality. These deaths may have been due to direct cadmium toxicity.

Soft tissue concentrations of controls snails without and with food contained 14.40 µg Cd/g (SD = 8.79) and 18.45 µg Cd/g (SD = 12.40) respectively (Figure 5.2). The snails in the control treatment without food had not been exposed to any cadmium source in the laboratory and had been fed with Tetramin fish food once upon arrival. The snails used in this trial were collected from the Howietoun site (see Chapter 2) and analysis of water from this site showed a cadmium concentration of 1.65 µg/L. It is therefore feasible that the cadmium measured in the tissue of the control snails was accumulated naturally before collection. Timmermans *et al.* (1992) maintains that the majority of invertebrates collected in unpolluted ecosystems have cadmium concentrations ranging from 0.01 to 1 µg/g dry weight. However, given the capacity for snails to accumulate metals in their tissues it is not surprising that the value for control snails seems quite high. Desy *et al.* (2002) reported tissue cadmium concentrations of 0.71 µg/g, 1.51 µg/g and 0.43 µg/g for *Bithynia tentaculata*, *Physa gyrina* and *Gammarus fasciatus* respectively collected from an area with apparently moderate cadmium contamination. The cadmium tissue concentrations for *P. gyrina* and *B. tentaculata* are quite different again showing the capacity of pulmonates to take up and sequester heavy metals. Presing *et al.* (1993) reported a whole soft body cadmium concentration of 2.33 µg/L in field collected animals and 9.02 µg/L in *L. stagnalis* following 18 months of laboratory culture before experimentation. Cadmium levels in the soft tissues were attributed to the use of intensively cultivated lettuce used as a food source.

Although the level of cadmium in the soft tissue of the snails collected from the Howietoun site was high, this could only be attributed to the metal concentration in the water collected from the field, which was below the EQS of 5 μg/L.

The soft tissue cadmium concentrations in the snails exposed to uncontaminated medium were 14.40 (SD = 8.79) μg/mg without food and 18.45 (SD = 12.42) μg/mg with food. The snails in the treatment without food had not been exposed to cadmium in the laboratory prior to or during experimentation. So the metal concentration measured in their soft tissue was considered to be indicative of levels occurring in animals collected directly from this site. As the standard deviation was high in both the snails with and without spinach it could not be concluded that the increase in cadmium concentration in the soft tissues was from a contaminated food source.

The concentration of cadmium in the soft tissues of snails exposed to cadmium through food only was 34.27 (± 31.15) μg/g (see Figure 5.2). Despite a cadmium concentration in the food source of 98.06 μg/g the soft tissue cadmium concentration of these snails did not differ significantly ($p<0.05$) from those measured in the two treatments with 0 μg/L metal in the media. Looking more closely at the data, the standard deviations are very large so preventing any significant difference being revealed. So again it would not be prudent to say that this soft tissue concentration of those snails given a highly contaminated food source was definitely the same as those snails in the control treatments and subsequently those collected from the field. However, this increase in cadmium

concentration was likely a consequence of the uptake of a contaminated food source.

The soft tissue concentration of cadmium in snails exposed to a contaminated food source (34.27 µg/mg as discussed above) was low compared to those measured in animals exposed to metal contaminated media with (166 µg/L) and without (170 µg/L) food. As outlined in Figure 5.2 the tissue concentrations of cadmium were 589.95 (±396.37) µg/g and 559.88 (±474.02) µg/g for snails without and with food respectively. These results along with the results from ingestion rate, which show no difference between any treatments, indicate that the major source of uptake of cadmium in *L. peregra* was most likely from the media.

As discussed in Chapter 1 many studies have been undertaken attempting to deduce the major source of metal uptake for invertebrates. However, due to the absence of gills in *L. peregra*, the route of metal uptake would be via the digestive tract (i.e. from food). As reported by De With (1996) water taken in orally by *L. stagnalis* contributes 20 to 30 % of urine production with an uptake rate calculated at 12 µl/h/d. De With (1996) concluded that water ingestion was mainly a consequence of taking bites and swallowing as the ingestion rate increased almost 6 fold in the presence of food. During observation carried out in the course of this study, the biting action of *L. peregra* was induced in the presence of food at a rate of between 6 and 18 bites per minute. However with no food present the bite rate dropped to between 0 and 2 bites per min. During this experiment, bites were defined as a protrusion of the radula. However, the author did note many of the snails opening and closing their mouths without taking a 'bite'. The increase in bites taken may

therefore explain the high concentration of cadmium in the tissues of *L. peregra* in the presence of food. However, when food is not present, water may still be ingested through the opening and closing of the mouth, but this requires further investigation.

The absence of any difference between ingestion and egestion rates in control and cadmium contaminated treatments (see Figure 5.3) conflicts with the findings of Chapter 3. The ingestion rates measured during the short term experiment were quite high when compared to those in previous chapters, at between 1.0 and 1.2 mg/mm/d. This may indicate that the snails used in this experiment were healthy and perhaps preconditioned to the presence of cadmium. Despite this the level of cadmium used in this experiment was very high, and as seen in Chapter 3 the ingestion and egestion rates of *L. peregra* should have shown significant effects at 50 μg/L. The source population used for experiments in Chapter 3 was different from those used in the present chapter, so it can be assumed that this was the source of the disparity in effective cadmium exposure concentrations.

Mortality rates during the long term experiment were high at 30% in each treatment. Deaths were spaced at regular intervals throughout the duration of the experiment in each treatment. This may indicate that the snails chosen for use were in sub standard condition.

Figure 5.4 clearly demonstrates the difference in accumulation in the soft tissue of *L. peregra* at increasing concentrations of cadmium in the media. At 0 μg Cd/L the average soft tissue concentration was 9.81 (±12.73, sd) μg/g. This was 32% lower

than the value reported for control animals in the short term experiment, but in line with the values reported by Presing *et al.* (1993) (see above discussion). Additionally the spread of data around the mean given for these control snails was very wide lowering the confidence placed in this value as a true representative of cadmium concentration in the tissues of these animals. The tissue concentrations measured for the snails exposed to 50 and 100 µg Cd/L were 250.61 (±83.12, sd) µg/g and 1066.89 (±553.54, sd) µg/g. Presing *et al.* (1993) reported a linear uptake of cadmium by *L. stagnalis* exposed to 100 µg/L from day 6 to 16, followed by an intensive increase until day 21 at which the apparent saturation level occurred at 200 µg/g in the soft tissue (measured as dry weight). Clearly for the animals used in this study no such saturation point appears to be present.

The pattern of ingestion rates measured during the 3 feeding trials (Figure 5.5a) shows a clear decreasing trend over time. This may have indicated a decrease in overall condition of the snails over the 26 days. As reported in Chapter 2, egestion rates for *L. peregra* maintained under laboratory conditions for extended periods of time tend to decrease notably. The ingestion rates measured for all three treatments were also quite low compared to those measured in previous chapters. Despite this, snails from all three treatments grew over the 26 day period. There was no effect of cadmium on growth rate as the smallest increase in shell size was observed for snails in the control treatment (Figure 5.6). The values for shell growth recorded in this study do concur with Brendelberger (1997) who reported shell growth rate of *Radix peregra* of 0.36 mm per week under uncontaminated conditions. The unusually low ingestion rates measured may have been due to handling stress and not to poor condition.

Figure 5.5b shows evidence of a decrease in egestion rate at increasing cadmium concentrations. Rates measured at interval 2 were low in all treatments and may have been as a consequence of satiation effects or stress from handling. The egestion rates measured were low when compared to those from previous chapters, again perhaps indicating the poor overall condition of the animals. Considering egestion rate only measured over the 3 time intervals, might lead to the conclusion that a dose dependent decrease in egestion rate was seen over time. However, this conclusion does not seem to hold true when control ingestion rates are also considered. Alternatively, the unusually low ingestion rates measured over the length of the long term experiment may have been due to excessive handling and the egestion rates were in fact indicative of exposure to cadmium.

The extremely low ingestion rates measured again provide evidence for the uptake route of cadmium by *L. peregra.* If the animals were not ingesting food and contaminated media along with it as postulated by De With (1996), then how were such high levels of cadmium accumulated in the tissue? The only possible explanation was the oral uptake of water without food (drinking) or cutaneous uptake. This point continues to be debated in the literature.

A future long term experiment should include the sequential removal of animals over the duration of the experiment to look at cadmium uptake over time. Subsequently these animals would undergo a 24 hour post-exposure feeding period to observe whether or not the exposure to uncontaminated media alters ingestion or egestion rates. Additionally the measurement of the rate of water uptake and how

this relates to accumulation or excretion of cadmium would be beneficial to calculate what proportion of the metal is sequestered and how much is excreted.

The results from this preliminary study indicate that bioconcentration appears to be more important to the uptake of cadmium in *L. peregra* than bioaccumulation. This point has long been debated in the literature for many animals and equally as many chemicals. Clearly cadmium was available to *L. peregra* for uptake from the surrounding media. It appears from the results of this study that while *L. peregra* may accumulate cadmium from food, this route of uptake did not make a significant contribution when compared to the amount taken in from surrounding media. It may be that the cadmium bound to food particles was not available to the snail and was excreted.

Some important points concerning the ingestion/egestion *L. peregra* bioassay were raised by this work. As shown in Figures 5.3 and 5.5 the ingestion and egestion rates measured for *L. peregra* exposed to high concentrations of cadmium in the media (with the exception of egestion rate in the long term study), were seemingly not dose related. In both the short and the long term experiment this may have largely been due to the source population. Snails used for both the long and short term experiments were collected from the Howietoun site, whereas animals used in Chapter 3, in which significant effects were observed, were collected from the Devon site (Figure 2.1). However, considering only the egestion rates measured during the long term experiment, an effect over time can be distinguished. Could it be that egestion rate is a more reliable measure of effects than ingestion?

CHAPTER 6.

MICROCOSM EXPERIMENTS:

ZINC, LAMBDA-CYHALOTHRIN AND LINURON.

6.1 INTRODUCTION.

Previous chapters have described the use of the *L. peregra* bioassay to measure both during and post-exposure ingestion and egestion rates under strictly controlled laboratory conditions. The present and subsequent chapters consider the response of the post-exposure egestion rate of *L. peregra* to various contaminants under semi-field and field conditions.

The functional response (for example changes in feeding rate, reproduction or growth) of an organism to contaminants or pollutants may provide a warning of the deterioration in other species at higher trophic levels or changes in the community structure. For example, a change in the feeding rate of one organism can be associated to effects on organisms in the same and other trophic levels as well as changes in community structure. As well as providing an early warning signal, the correlation of a functional response to effects at higher trophic levels indicates that the endpoint under study (i.e. feeding rate) is ecologically relevant. The purpose of this chapter was to assess the performance of the *L. peregra* post-exposure egestion rate when the bioassay was deployed in model systems contaminated with single pollutants, and to assess its performance in relation to the response of various simultaneously deployed bioassays.

Microcosms or mesocosms provide simplified models of natural ecosystem behaviour, a more realistic exposure regime as well as an intermediate between field ecosystems and single-species toxicity tests (Rand *et al.*, 1995). The definition of a microcosm or a mesocosm can vary between authors. Rand *et al.* (1995) defines mesocosms as artificially constructed earthen ponds, with volume ranging from 100 to 1000 m^3 which are allowed to colonize before use. Neither of the systems in this study fall under this category so both shall be defined as microcosms for the purposes of this thesis.

Many types of artificial test system are used to simulate natural field conditions. These include outdoor meso and microcosms as defined by their size, construction and use as well as indoor lotic or lentic microcosms. Other types include artificial enclosures such as limnocorrals, which enclose the pelagic regions of ponds, lakes or marine systems and can vary widely in size; and littoral enclosures comprising plastic dividers used to isolate the littoral regions of ponds and which are usually between 1000 to 50000 L with a maximum depth of 2 m (Rand *et al.*, 1995).

Many authors consider the use of results from single-species laboratory tests alone inadequate to assess the potential hazards of contaminants in aquatic environments (Cairns, 1984; Kostel, 1999). Simple laboratory tests generally overestimate the magnitude and duration of exposure relative to that observed in a more natural field system (Pratt *et al.*, 1990). Artificial systems facilitate the assessment of chemical transformation, partitioning and biological effects of a toxic chemical on organisms under conditions of realistic exposure (Kostel, 1999). Under these more realistic circumstances, toxic effects might occur at concentrations different from those in

the laboratory, since a complex range of abiotic and biotic factors will influence reactions (Juttner, 1995).

An important reason for conducting mesocosm studies is also to investigate higher order structural and functional properties of ecosystems (Rand *et al.*, 1995). In comparison to laboratory bioassays, artificial systems allow the assessment of a range of species and biological effects, as well as the evaluation of interactions between organisms in a controlled environment (Kostel, 1999). Artificial systems can provide predictable variation or control of nutrients, temperature, chemical composition, flow regimes, illumination intensity and periodicity allowing the clarification of cause and effect relationships and interactions (Crossland *et al.*, 1991). Furthermore, these systems allow the replication of treatments and therefore the statistical analyses of results (Kostel, 1999).

The use of artificial systems allows the detection of ecotoxicological effects on complex and interacting communities (Juttner, 1995). Endpoints measured using these systems can include structural aspects such as species composition, succession and diversity, and also functional changes through competition and predation.

6.1.1 MICROCOSM EXPERIMENTS

All three of the microcosm studies described below were carried out as part of the training and validation section of the TARGET project. The aims of each were to test the usefulness of all bioassays and endpoints when exposed to single chemical stressors under semi-field conditions. Additionally these experiments aimed to

establish links between contaminant effects on endpoints of individual bioassays and community responses.

6.1.2 SHEFFIELD UNIVERSITY STREAM MICROCOSM.

The artificial stream microcosms used in the first two studies were located at the University of Sheffield, Sheffield, England. The stream system consisted of six independent stainless steel channels (3 m long, 20 cm wide, 30 cm deep), with a light bank 1.5 m long by 1 m wide positioned 70 cm above the water surface at the top end of the channels and operated on a 12 h light: 12 h dark photoperiod. Each channel had a 5 cm pea gravel substrate covered by 15 cm of artificial pond water, supplemented with 10 μg P/L and 100 μg N/L to facilitate algae growth. Stream water was recirculated at a rate of 4000 L/min, giving a mean flow rate of approximately 0.02 m/s.

Each channel was stocked with invertebrate communities comprised of locally sourced crustraceans (*Gammarus pulex*, *Asellus aquaticus*), stonefly larvae (Leuctridae, Nemouridae), case-bearing caddis larvae (Sericostomatida, Limnephilidae) and snails (Planorbidae, Lymnaeidae). Naturally-inoculated leaf material, also collected from local streams was added to each channel 20 days prior to dosing to act as inoculum for aquatic hyphomycetes, and left in place for the duration of the experiment.

6.1.3 OUTDOOR POND MICROCOSMS – TNO, DEN HELDER.

The microcosms used for the third study were located at the TNO Institute of Environmental Sciences, Energy Research and Process Innovation, Den Helder, The Netherlands. Each of the 9 microcosms used comprised circular glass-fibre tanks 195 cm high, with an internal diameter of 225 cm at the top and 170 cm at the bottom, with a maximum capacity of 3 m^3 of water. Each tank was partially buried in the ground to ensure some degree of diurnal temperature buffering.

A 9 cm layer of sediment collected from the relatively unpolluted shallow Lake Markermeer was placed into each microcosm. Water was also collected from Lake Markermeer with a volume of 2.5m^3 added to each microcosm. Each tank had an overflow situated approximately 15 cm below the rim of the microcosm to allow run-off following rainfall. A small pump was placed in each microcosm to prevent stagnation and promote mixing.

Each microcosm was stocked with 168 *Gammarus* spp. and 69 *Asellus aquaticus* collected from local streams prior to bioassay deployment. Approximately 60 g of reed were also added to each microcosm to introduce leaf associated fungi as outlined in the set-up of the Sheffield stream system. Phytoplankton and zooplankton communities resident in the lake water were counted and identified at the beginning and end of the trial.

6.1.4 ENDPOINTS MEASURED.

The general purposes of all three microcosm studies were to determine the individual impacts of the metal zinc, the pyrethroid lambda-cyhalothrin and the herbicide linuron on the structural and functional parameters of the study system including primary production, detritus processing, algal grazing and biodiversity of zooplankton and phytoplankton populations. These parameters were determined through the series of bioassays outlined in the TARGET project. These bioassays included:

1) The survival and post-exposure feeding rate of *L. peregra*
2) The survival and post-exposure feeding rate of the algal consumer *Daphnia magna*
3) The survival and feeding rate of the detritivore shredders *Gammarus pulex*, *Calamoceras marsupus* and *Sericostoma personatum.*
4) The growth of the freshwater microalga *Chlorella vulgaris*
5) The extent of *Alnus glutinosa* and *Phragmites australis* decomposition
6) The survival, feeding rate and growth of the detrivore shredder *Atyaephyra desma*
7) The survival, feeding rate and growth of the collector *Chironomus riparius*

AIMS

The aim of this chapter was to study the performance of the *L. peregra in situ* bioassay in two types of artificial systems (as described above) exposed to three contaminants to assess its response outside the laboratory under more natural

conditions. It was expected that the *L. peregra* bioassay would perform predictably under a defined set of conditions and contaminants. It was envisioned that together with a suite of bioassays, the *L. peregra* test might potentially be used to assess ecological quality in rivers.

6.2 MATERIALS AND METHODS.

6.2.1 ANIMALS AND ACCLIMATION.

Animals used in the Sheffield stream microcosm trials were collected from the River Devon site. *L. peregra* for use in the experiments were transported in 10 L plastic buckets aerated with a battery operated pump. Upon arrival the animals were fed (section 2.1.3) and allowed to acclimate to test conditions for at least 48 hours prior to use.

Animals used in the Den Helder trial were *L. peregra* collected from a pond close to TNO. Animals were acclimated to microcosm water for 48 hours prior to deployment in a constant temperature room at between 15 and 18°C with a photoperiod regime of 12 hours light: 12 hours dark.

6.2.2 DEPLOYMENT PROCEDURE.

Individual *L. peregra* were transferred from the acclimation media to a PVC cylinder with mesh caps on each end (Figure 6.1). Ten of these cages each containing one *L. peregra* were placed within a wire mesh basket and secured with

plastic ties. The baskets were made using wire meshing cut to an appropriate size and rolled into a cylindrical shape held together using plastic ties. One wire basket containing the *L. peregra* cages was then placed with the mesh ends of the cages perpendicular with the direction of water flow (Figure 6.1) and secured to the sides of the microcosm with string. The baskets were removed from each of the microcosms following a 48 hour exposure period and the animals allowed to feed for 24 hours. Egestion rate was then measured as outlined in sections 2.3.1.1 and 2.3.2.3.

Zinc

Three groups of 2 stream channels were randomly assigned one of the following zinc concentrations: control at 0 mg/L, low zinc at 0.5mg/L nominal concentration and high zinc at 2 mg/L nominal concentration. Zinc solutions were prepared using $ZnSO_4$ $7H_2O$ dissolved in deionised water and zinc additions were make at least daily to maintain aqueous zinc concentrations. Water samples were analysed by atomic absorption spectrophotometry.

Sixteen *L. peregra* were deployed at each treatment level (i.e. 8 per stream) in January 2001.

Lambda-cyhalothrin.

Three concentrations (control, low and high) were randomly assigned to 2 of each of the six stream channels. Stock solutions were prepared using C_{14} labelled lambda-cyhalothrin (416 µg a.i./mL acetone). Stream channels were dosed immediately before bioassays were deployed and water samples were taken from

each stream after 24 and 48 hours. Nominal concentration was taken to be the concentration measured in the media at time 0. Thirty *L. peregra* were deployed at each treatment level (i.e. 15 per stream) in February 2002.

Water quality parameters measured for both the zinc and lambda-cyhalothrin studies included dissolved oxygen, temperature, conductivity and pH.

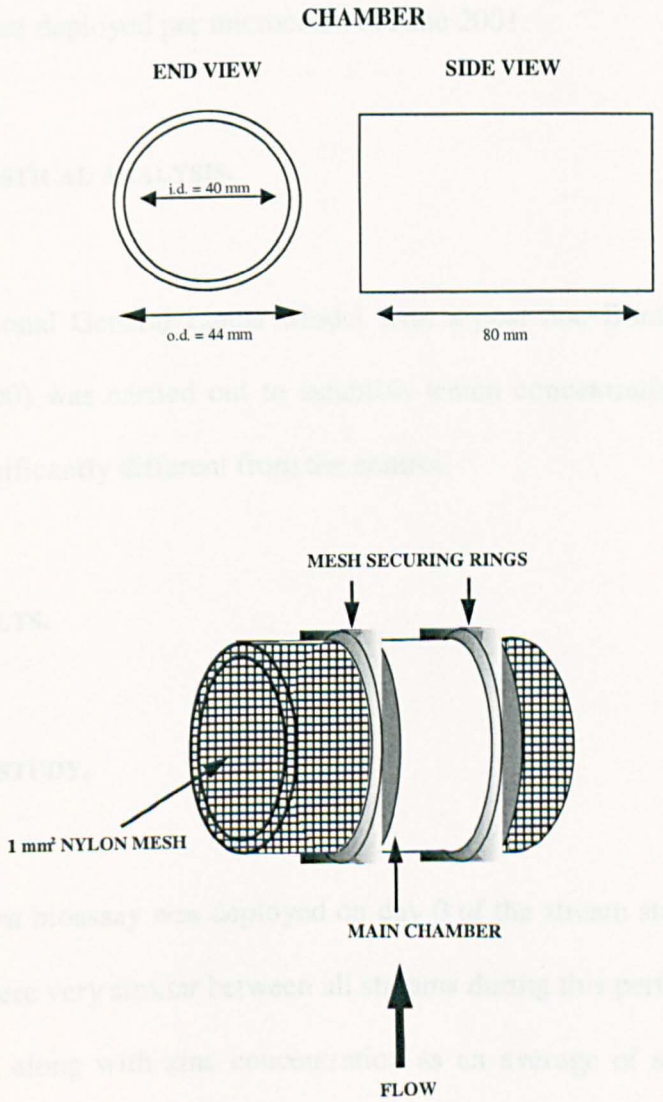


Figure 6.1: Diagrammatic representation of *L. peregra* deployment cages.

Linuron

Due to the low nutrient content of the lake water used, the microcosms were spiked with 1.26 g K_2HPO_4 and 13.65 g $NaNO_3$. Three of the 9 microcosms were randomly assigned as the control group. The remaining 6 micrcososms were randomly assigned to 3 groups of 2 and each group was dosed with one of 20, 60 and 180 µg Linuron/L. Water samples were taken and analysed on day 0 (at 4 hours) and on day 6. Water samples were analysed using HPLC. Water quality measurements taken included dissolved oxygen, conductivity, and temperature. Ten *L. peregra* were deployed per microcosm in June 2001.

6.2.3 STATISTICAL ANALYSIS.

A one directional General Linear Model with a *post hoc* Bonferroni comparison (Minitab, 2000) was carried out to establish which concentrations produced rates that were significantly different from the control.

6.3 RESULTS.

6.3.1 ZINC STUDY.

The *L. peregra* bioassay was deployed on day 0 of the stream study. Water quality parameters were very similar between all streams during this period and are outlined in Table 6.1, along with zinc concentration as an average of samples taken from time 0 to 48 hours.

L. peregra survival was greater than 90% for all zinc treatments. Post-exposure egestion rates of *L. peregra* exposed in the low and high concentration streams dosed with zinc, were significantly lower ($F_{2, 43}$; $p<0.05$; Table 6.2) than in the control (Figure 6.2). The post-exposure egestion rate calculated for snails exposed to 0.5 mg zinc/L treatment was slightly lower than the 2.0 mg zinc/L treatment, but the difference was not statistically significant.

Table 6.1: Average water quality parameters and zinc concentrations taken on days 0 to 2 of the zinc study. Each treatment level consisted of 2 separate stream channels. Values are given as means with standard error in parentheses.

Parameter	**Stream treatment**					
	Control		Low		High	
	1	2	1	2	1	2
Dissolved oxygen (mg/L)	9.5 (0.10)	9.57 (0.09)	9.47 (0.07)	9.37 (0.09)	9.40 (0.12)	9.23 (0.07)
Temperature (°C)	14.97 (0.12)	14.83 (0.13)	15.67 (0.18)	14.90 (0.21)	14.93 (0.19)	16.17 (0.17)
Conductivity (µS/cm)	597.67 (14.24)	634.00 (12.86)	586.00 (11.55)	613.00 (8.50)	613.67 (8.01)	599.00 (8.14)
pH	7.57 (0.07)	7.57 (0.07)	7.57 (0.03)	7.60 (0.00)	7.53 (0.03)	7.57 (0.07)
Zinc concentration (mg/L)	0.02 (0.00)	0.02 (0.00)	0.38 (0.02)	0.35 (0.03)	1.48 (0.10)	1.48 (0.10)

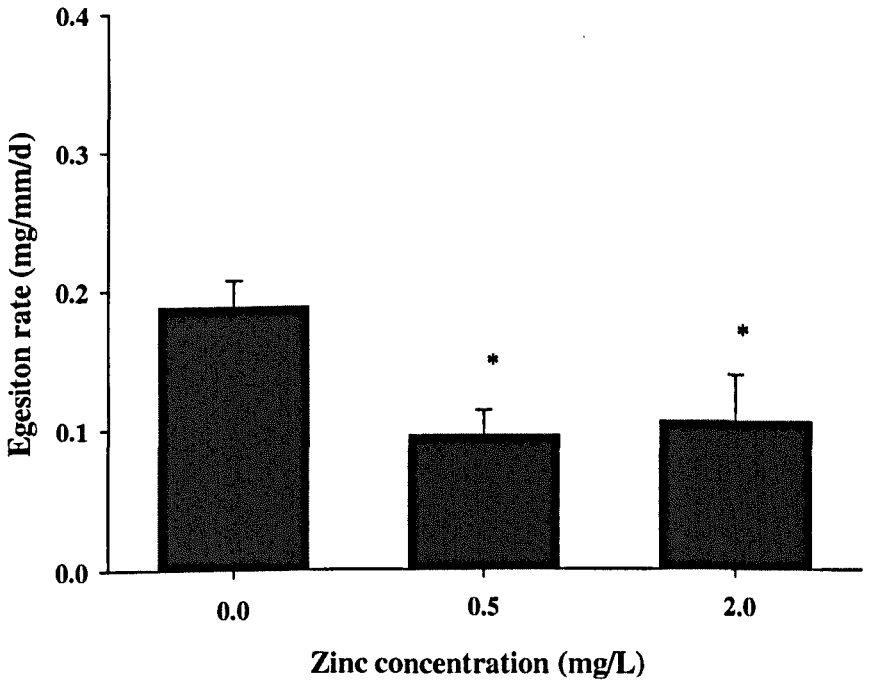


Figure 6.2: 24 hour post-exposure egestion rate of *L. peregra* following 48 hour exposure to zinc in laboratory streams. Asterisks denote egestion rates that were significantly different from control ($p<0.05$; $n = 46$). Bars indicate standard error.

Table 6.2: Results of one way ANOVA to compare the effects of zinc on *L. peregra* egestion rate following a 48 hour exposure period.

Source of error	Sum of squares	d.f.	F	p
Treatment	0.080	2	4.61	0.015
Error	0.377	43		
Total Error	0.458	45		

6.3.2 LAMBDA-CYHALOTHRIN STUDY.

The *L. peregra* bioassay was deployed on day 0 of the stream study. Water quality parameters were very similar between streams during this period and are outlined in Table 6.3 together with lambda-cyhalothrin concentrations.

Survival was greater than 80% for all treatments. There was no significant effect of lambda-cyahalothrin on post-exposure egestion rates of *L. peregra* in this study (Figure 6.3).

Table 6.3: Water quality parameters and lambda-cyhalothrin concentrations for days 0 to 2 of the study. Lambda-cyhalothrin concentrations were measured 48 hours after the start of the experiment. Values are given as means with standard error in parentheses.

Parameter	**Stream treatment**					
	Control		6.3		24.9	
	1	2	1	2	1	2
Dissolved oxygen (mgO_2/L)	8.58 (0.08)	8.42 (0.07	8.72 (0.08)	8.80 (0.09)	8.66 (0.09)	8.56 (0.12)
Temperature (°C)	16.16 (0.09)	16.22 (0.15)	14.78 (0.11)	14.5 (0.10)	14.7 (0.10)	14.9 (0.11)
Conductivity (µS/cm)	650 (3.66)	591.4 (4.59)	610.4 (3.68)	648.6 (3.29)	549.2 (3.91)	643.2 (2.18)
pH	7.94 (0.05)	7.88 (0.03)	7.94 (0.04)	7.96 (0.02)	7.96 (0.02)	7.9 (0.00)
Lambda-cyhalothrin concentration (ng/L)	<0.01	<0.01	2.66	3.36	9.49	10.85

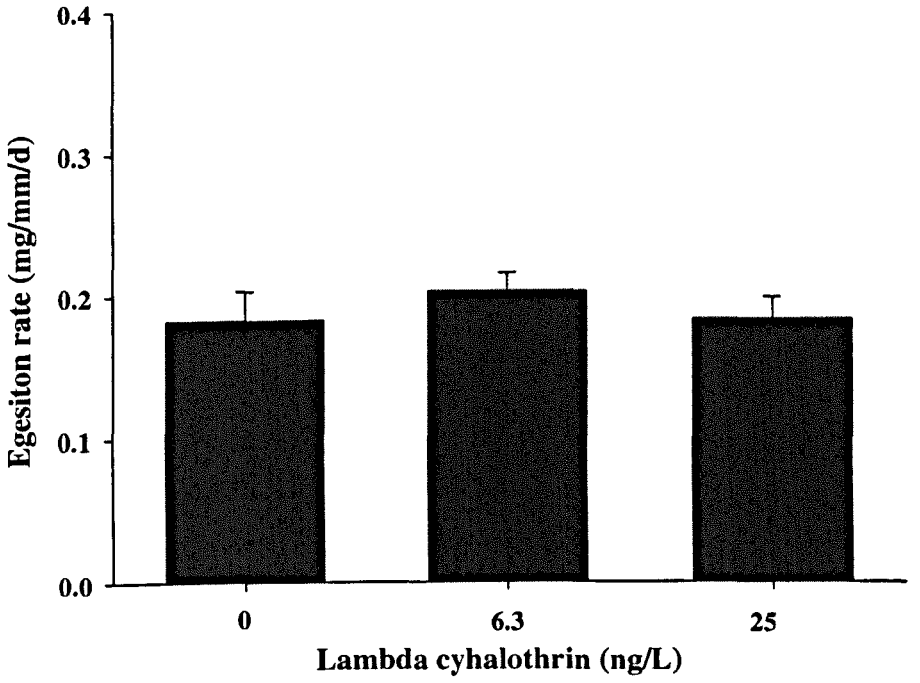


Figure 6.3: 24 hour post-exposure egestion rate of *L. peregra* following 48 hour exposure to lambda-cyhalothrin in laboratory streams (n = 80).

6.3.3 LINURON STUDY.

The *L. peregra* bioassay was deployed on day 0 of the outdoor microcosm study. Water quality parameters were taken at hour 4 on day 0 and were very similar among treatments as outlined in Table 6.4, linuron concentrations are also shown. Some variability of linuron concentration was observed within treatments.

L. peregra survival was greater than 90% for all treatments. There was no significant effect of linuron on post-exposure egestion rates of *L. peregra* in this study (Figure 6.4).

Table 6.4: Water quality parameters and linuron concentrations taken on day 0 of the study.

Parameter	**Linuron concentration (µg/L)**								
	Control			20		60		80	
	1	2	3	1	2	1	2	1	2
Dissolved oxygen (mgO_2/L)	7.0	6.9	7.1	6.9	6.9	6.3	6.4	6.3	6.3
Temperature (°C)	15.8	15.6	15.7	15.6	15.7	15.8	15.8	15.7	15.7
Conductivity (µS/cm)	722	720	719	717	715	720	715	716	715
pH	8.14	8.15	8.15	8.15	8.16	8.14	8.15	8.08	8.08
Linuron concentration (µg/L)	0.0	3.0	0.0	12	14	30	43	53	115

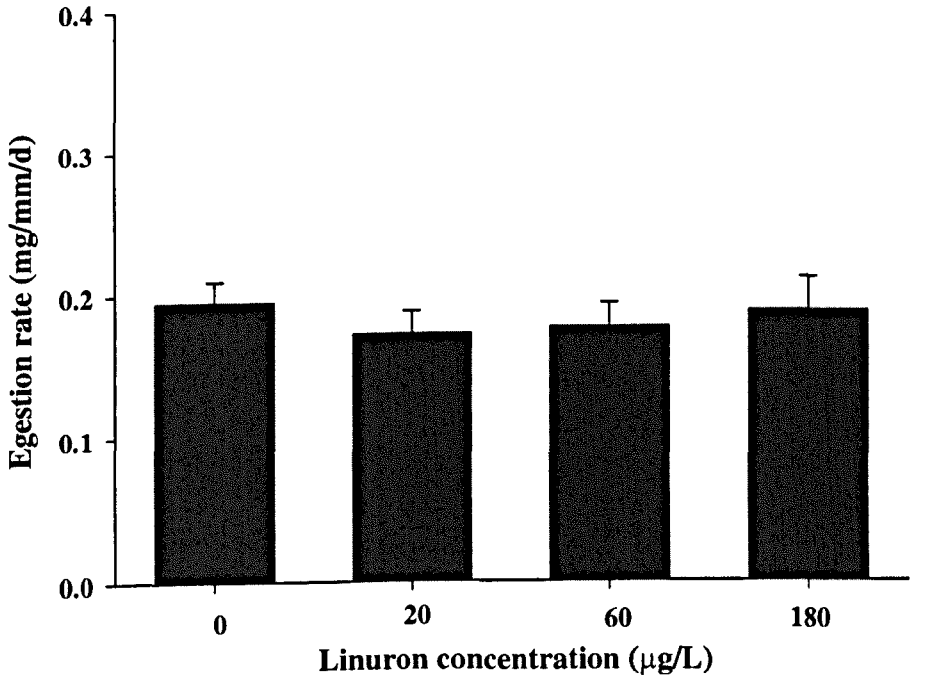


Figure 6.4: 24 hour post-exposure egestion rate of *L. peregra* following 48 hour exposure to linuron in laboratory streams (n = 85).

6.4 DISCUSSION.

Zinc

Measured concentrations of zinc in the control, low and high concentrations were identical between the two control (0.02 mg/L) and high treatments (1.48 mg/L) with only a slight difference between the two low treatments (0.38 and 0.35 mg/L) as averaged over the 48 hour exposure period. The survival rate of *L. peregra* used in the zinc stream study was greater than 90% for all treatments with only two mortalities, one in the control group and one at treatment level 2.0 µg/L. The development of the laboratory *L. peregra* bioassay for use in microcosm studies was therefore successful in terms of survival and recovery of individuals for use in the post-exposure portion of the test.

Egestion rates calculated for the three treatments show a significant difference between the control and dosed streams (Figure 6.2). The post-exposure egestion rates of snails exposed to 0.5 mg/L and 2.0 mg/L zinc were 51% and 56% lower than the control rate respectively. Although the 0.5 mg/L treatment showed a lower egestion rate than the 2.0 mg/L stream, the difference was not significant. Overall the control egestion rate was slightly lower than in previous studies, which may have been due to poor conditions under which the animals were transported to the study site.

LC_{50} values for a variety of molluscs as cited in the literature are presented in Table 6.5. The 96 hour LC_{50} value for *L. luteola* and *L. acuminata* (at pH 7.0 and 7.8) were 15 mg/L and 10.5 mg/L respectively (Hunt and Hedgecott, 1996). In the

present study the egestion rate of *L. peregra* decreased by 56% at a zinc concentration of 0.5 μg/L (pH 7.6). This value is in line with those given in Table 6.4 and is lower than the LC_{50} values presented for the other two lymnaeids. Without lethality data for the effects of zinc on *L. peregra* no conclusions about the sublethal dose can be drawn. However, the EQS value for zinc at water hardness (as $CaCO_3$) above 200 mg/L is between 125 and 500 μg/L. If the egestion rate were used as a surrogate for ingestion, a decrease of 56% would be seen in polluted areas (as defined by the EQS value), which could be detrimental to a natural population. The results from this experiment also concur with the effects of metals on *L. peregra* found in Chapter 3.

Table 6.5: 96 hour LC_{50} values for several freshwater snails from Hunt and Hedgecott (1996).

Snail	LC_{50} (mg/L)
L. luteola	15
L. acuminata	10.5
Physa gyrina	1.3
Ancylus fluviatilis	3.2

In the zinc stream study algal growth was influenced by the difference in light intensity, water temperature and nutrient concentrations between treatments. A multiple regression model, incorporating temperature, light intensity, nitrate concentration and zinc concentration, explained 98% of the variation in algal growth rate; algal growth decreased as zinc concentration increased. There was also no significant effect of zinc on leaf decomposition.

Other results from this microcosm study included some evidence of depressed feeding rates of *D. magna*, but with no significant effect of zinc on feeding rate. Mc

William (2001) found post-exposure effects of zinc on the feeding rate of *D. magna* at concentrations starting at 100 µg/L, with some evidence of post-exposure recovery up to a concentration of 1350 µg/L, but not enough to mask the effects of the metal on feeding rates. With this in mind, some effects should have been seen at both treatment levels used in this stream experiment. There was a significant decrease in feeding rate of *G. pulex* at low zinc concentrations and decreased survival and feeding rates at both treatment levels. However, there was no evidence of feeding rate inhibition in either of the other two shredders.

Lambda-cyhalothrin

Concentrations of lambda-cyhalothrin measured in the low and high dosed streams at the end of the 48 hour period were 50% and 41 % of nominal. As discussed in Chapter 4, lambda-cyhalothin has low water solubility and may become strongly adsorbed to surfaces and organic matter as a consequence the concentration in the water column will decrease over time.

Despite the greater than 80% survival of individuals during this study, this value is lower than recorded in previous experiments. The control treatments lost 20% of individuals over the 48 hour exposure period as compared with 6% in both of the other treatments. The only environmental difference between the treated and control streams was an approximately 1.5°C increase in water temperature, which would not have significantly influenced the stress response of *L. peregra*. However as in the zinc experiment animals may have been damaged or severely stressed during transport to the site.

No significant statistical difference was observed between the post-exposure egestion rates calculated for the control animals and those exposed to lambda-cyhalothrin. This agrees with findings from Chapter 4. Again, as seen in the zinc study, the egestion rates of control and exposed animals were below the control rate seen in previous chapters.

Other endpoints considered during this study showed significant effects including a decrease in the decomposition of *Alnus glutinosa* leaves exposed to lambda-cyhalothrin. Survival of *D. magna* deployed was 100% with there was no significant effect of lambda-cyhalothrin on post-exposure feeding rate. Mc William (2001) found effects of lambda-cyhalothrin of the feeding rate of *D. magna* at a concentration as low as 0.008 µg/L, with evidence of some post-exposure recovery, but not enough to mask the effects of the chemical. With this in mind, a decrease in the feeding rate of *D. magna* would have been expected at the highest concentration used in this study. A significant reduction in the *in situ* feeding rate of the crustacean shredder *G. pulex* was seen in both treatments. The insect shredders *S. personatum* and *C. marsupus* also showed a significant reduction in feeding rate in treated streams. Again the feeding rate of the collector *Chironomus riparius* showed a significant reduction in the high and low treatments. Results for most invertebrates deployed in the lambda-cyhalothrin study were as expected bar the *D. magna* bioassay.

Linuron

Linuron concentrations measured in the outdoor microcosms showed some disagreement between nominal and actual concentrations as well as between microcosms within a treatment over the 48 hour exposure period. The highly lipophilic nature of linuron means that it may adsorb easily to surfaces, which accounts for some of the observed variability. However, as there were no effects of linuron on the post-exposure egestion rate of *L. peregra*, differences within treatments are not considered further here.

As outlined in section 4.1, the effects of some herbicides on resident snail populations are secondary in nature as in the case of cyanatryn (Scorgie, 1980) rather than due to direct toxic effects. In a microcosm study by Cuppen *et al*, 1997, the chronic application of 50 and 150 µg/L linuron negatively affected the snails *Physa acuta* and *Lymnaea* due to the decrease in biomass of *Elodea nuttallii* and its associated periphyton. Islam *et a.l* (2001) reported no significant mortality of *L. peregra* exposed to concentrations of the herbicide simazine up to 5000 µg/L. In the same study growth was affected in a dose dependent manner (from 10 µg/L) and egg production ceased at the highest concentration (5000 µg/L). Had the microcosms been stocked with molluscs throughout the duration of the study, some secondary effects may have been observed.

Within the linuron microcosm study other endpoints used included various measures of primary production. During a microcosm study by Cuppen *et al* (1997) the first observed response to linuron was the rapid inhibition of the photosynthetic

efficiency of primary producers at the 5 µg/L and higher concentrations. However in the microcosm study at Den Helder, there was only a slight decrease in chlorophyll a concentration over time, but no concentration dependent effect was observed. Additionally, no effect of linuron on periphyton growth was observed. The inhibition of photosynthesis of algae and macrophytes observed at low concentrations (0.5 µg/L) of linuron by Cuppen *et al.* (1997) is in disagreement with the results of the linuron study at Den Helder.

In line with the findings of Cuppen *et al.* (1997), there was no effect of linuron on leaf decomposition. There was a decrease in copepod, rotifer and cladoceran numbers at the 60 and 180 µg/L concentrations in the present study. This is in partial disagreement with Cuppen *et al.* (1997) who found a decrease in rotifers but no change or an increase in cladocerans and ostracods.

The EQS for linuron currently set by the Scottish Environmental Protection Agency (SEPA) is between 2 µg/L to 20 µg/L. At these concentrations impairment of phytosynthetic activity would be expected with subsequent secondary effects on the grazing macroinvertebrate population.

The comparison of field and laboratory data is a critical issue in ecotoxicology as most regulatory decisions are based upon the latter. Without reference points closer to the real world, judging the relevance of bioassay results or the reliability or inferences drawn from them is difficult (Giddings, 2001). The results obtained from some of the bioassays deployed during these experiments were not consistent with past studies. So as evidenced by data set out in this chapter, the results obtained

from experiments carried out under semi-field conditions should be interpreted with some caution.

Results from the microcosm deployments demonstrated that the *L. peregra* bioassay could be successfully adapted for use under semi-field conditions. Very low mortality rates allowed for the successful completion of the post-exposure section of the test which demonstrates robustness. Post-exposure egestion rate was inhibited at environmentally relevant concentrations (i.e. levels found in the environment) of zinc which confirms the sensitivity of the bioassay to metal contamination, as well as the effectiveness of using post-exposure egestion rate as an endpoint. Ecological relevance (as defined in section 1.6) was also demonstrated in the zinc experiment as the response of the *L. peregra* bioassay agreed with that of the *G. pulex* test.

As expected no decrease in post-exposure egestion rate was elicited when *L. peregra* was exposed to either linuron or lambda-cyhalothrin. The *L. peregra* bioassay has been shown to respond predictably and sensitively to cadmium, zinc and copper under laboratory and semi-field conditions. The use of a bioassay, which ostensibly responds to only one group of compounds, as a stand-alone measure must be questioned. However, as one of a suite of bioassays used to estimate freshwater quality in a river it may prove to be a useful tool.

CHAPTER 7.

FIELD DEPLOYMENT OF THE *L. PEREGRA* POST-EXPOSURE EGESTION RATE BIOASSAY.

7.1 INTRODUCTION.

Laboratory toxicity tests have been used for many years to estimate the potential hazard of chemicals. However, the extrapolation of hazard evaluated under laboratory conditions to the risk of damage to aquatic systems is often problematic (Crane, 1995). A commonly cited source of uncertainty when extrapolating lab-based test results to natural environments is that they are done under strictly controlled conditions (Sibley *et al.*, 1999). The toxicity of chemicals to organisms will be modified under natural conditions through physical, chemical and biological processes (Cairns, 1984), which cannot adequately be reproduced under laboratory conditions.

In situ bioassays provide ecologically realistic assessments of in-place pollutants by placing selected organisms in the field in order to expose them to contamination occurring under natural conditions (Schulz, 1999), and therefore integrating the physical, chemical and ecological processes that are not represented by simpler laboratory tests (Giddings *et al.*, 2001). The results obtained are considered to be more relevant to the natural situation than those achieved through laboratory experiments (Chappie and Burton, 1997).

In situ bioassays represent a link between laboratory experiments and field studies. If meaningful conclusions are to be drawn, they must fulfill two requirements in particular (Schulz, 1999):

1. There must be a clear relationship between the environmental stress of interest and the response measured in the bioassay and;
2. The response of the bioassay must directly or indirectly reflect responses of the same species (or the whole community) in the field.

For the purposes of this chapter, the laboratory bioassay developed in chapters 2 and 3 was adapted for deployment in the field. The exposure period remains 48 hours (now in the field) with the post-exposure feeding period, upon return to the laboratory, at 24 hours. However, the animals were no longer fed during exposure, as this was a logistically difficult and problematic task.

A number of recent studies have reported the results of *in situ* deployments using a variety of test organisms including *Daphnia magna* (Pereira *et al.*, 1999; McWilliam and Baird 2002b), *Ceriodaphnia dubia* (Sasson-Brickson and Burton, 1991; Ireland *et al.*, 1996; Pereira *et al.*, 1999), *Gammarus pulex* (Maltby *et al.*, 1990; Crane *et al.*, 1995; Schulz and Leiss, 1999), *Anodonta cygnaea* (Crane *et al.*, 1995), *Chironomus tentans* (Chappie and Burton, 1997; Sibley *et al.*, 1999), *Pimephales promelas* (Wilde and Parrott, 1984), *Salvelinus fontinalis* (Simonin *et al.*, 1993), *Rhinichthys atratulus* (Simonin *et al.*, 1993), *Lumbriculus variegatus* (Monson *et al.*, 1995; Sibley *et al.*, 1999), *Hyalella azteca* (Chappie and Burton, 1997), *Scenedesmus subspicatus* (Twist *et al.*, 1998), *Corbicula fluminea* (Soucek, *et al.*, 2000), *Limnephilus lunatus* (Schulz and Liess, 1999) and *Mercenaria*

mercenaria (Ringwood and Keppler, 2002). Most of these studies have found that *in situ* toxicity responses provide an effective means of assessing water and sediment quality.

The majority of these *in situ* bioassay use death as an endpoint, which, in nearly all cases is not the most sensitive indicator of toxicity. As discussed in section 1.3.1, sublethal endpoints are acknowledged as a more sensitive endpoint than death, as often the first reaction of an organism to stress is a physiological one (Gerhardt, 1996). Methods based on sublethal responses of single-species include the use of chemical biomarkers to indicate exposure to specific stressors (e.g. glutathione-S-transferase induction) or physiological assays to indicate impact (e.g. Scope for Growth).

Currently the assessment of water quality/ecological status is carried out through regular monitoring. Monitoring is an important component of environmental management as discussed in section 1.2. In the U.K. biological monitoring relies on detecting variations in macroinvertebrate community structure, these alterations can then be associated with changes in water and sediment quality (Maltby, 1995). However, community level effect measures are insensitive to sublethal levels of stress (Chappie and Burton 1997); therefore, *in situ* bioassays that employ more sensitive, sublethal endpoints offer the potential for measuring impacts at an earlier stage. Regular monitoring of sites goes some way to keeping long-term damage to a minimum, but an early warning system would be more protective of aquatic systems.

In situ bioassays are proactive in the sense that they present the possibility of documenting harm before it has been done (Cairns and Pratt, 1989). The ability of *in situ* bioassays to provide a rapid indication of water quality may well provide an early warning system as effects measured at the individual level are often manifested more rapidly than resulting changes in community structure. Maltby *et al.*, (2000) demonstrated the possibility of relating endpoints measured at the individual level to effects measured at the community level, in theory providing a rapid assessment of water quality. Short term results gained from *in situ* deployments therefore could be useful in determining the initial causes of long-term alterations in macroinvertebrate community structure (McWilliam and Baird, 2002b). Additionally results from *in situ* bioassays could potentially help to identify causal mechanisms between environmental stressors and both population and community level effects (Maltby, 1999).

AIMS

The aims of this chapter were:

- To adapt the *L. peregra* laboratory bioassay for deployment under field conditions.
- Establish a background egestion level at control sites.
- Measure the magnitude of response at sites upstream and downstream of a discharge point. Post-exposure egestion rates at downstream sites should be reduced compared to upstream.

- Sample the benthic macroinvertebrate community in order to link post-exposure egestion rate inhibition in *L. peregra* to community structure based measures of water quality.

7.2 MATERIALS AND METHODS.

Deployment sites were chosen in consultation with SEPA and the Environment Agency of England and Wales. Deployment of the *L. peregra* bioassay took place during the summer of 2002 initially at 6 control sites (Table 7.1) and subsequently at 6 upstream (US) or reference sites and 6 downstream (DS) or contaminated sites (Table 7.2).

Table 7.1: Deployment date and national grid references for control sites in Scotland.

Site	Date	NGR
Allt Breaclaich	22/05	NN615342
Achmore Burn	22/05	NN584318
Auchlyne Burn	22/05	NN513295
Innerhadden Burn	29/05	NN673574
Allt a Chreagain Odhair	29/05	NN607617
Aulich Burn	28/05	NN605605

Table 7.2: Deployment date and national grid references for upstream/downstream sites in Scotland and Northern England.

Site	Date	NGR
Carron ValleyUS	16/07	NS689824
Carron ValleyDS	16/07	NS688825
Howietoun US	25/07	NS776877
Howietoun DS	25/07	NS784885
Leadhills US	9/07	NS838098
Leadhills DS	9.07	NS887177
Langholm US	9/07	NY365840
Langholm DS	9/07	NY370837
New Mill Dyke US	18/06	SK004919
New Mill Dyke DS	18/06	SK003922
Coombes BrookUS	17/07	SK164072
Coombes Brook DS	17/07	SK164076
Habergham Clough US	25/06	SD813312
Habergham Clough DS	25/06	SD810318
River Tame US	24/07	SD989042
Rive Tame DS	24/07	SD988042
River Drone US	17/07	SK362781
River Drone DS	17/07	SK368777

7.2.1 PHYSICO-CHEMICAL PARAMETERS.

The following physico-chemical parameters were measured at each site on the first day of deployment: depth, flow rate, water temperature, pH, dissolved oxygen, conductivity, hardness, alkalinity, and suspended solids (Tables 7.3 and 7.4).

Portable meters were used to measure pH and temperature (Jenway 3150, Dunmow, Essex, U.K.) and conductivity (HI9033, Hanna, Ronchi de Villafranca, Italy); dissolved oxygen (Hanna HI9142). Flow rates were measured using an Ott C2 flow meter (A. Ott, Kempten, Germany). Analysis of alkalinity used a Palintest photometer 5000 and accompanying reagents (Palintest Ltd., Tyne & Wear, U.K.).

Suspended solids were measured by filtering 1 L of sample water onto a pre-washed, dried and weighed GF/C filter paper (Whatman). Following filtration the filter + solids was dried overnight at 100°C and weighed.

Water samples for nutrient analysis were collected by immersing 250 mL opaque plastic bottles in running water until all air was expelled. Whenever possible, samples were analysed immediately on return to the laboratory. When this was not possible, samples were refrigerated (<5°C) and analysed within 24 hours.

Water samples for metal analysis were taken from the Leadhills, New Mill Dyke and Coombes Brook sites. These samples were acidified to 1% with HNO_3 (Aristar Grade, BDH), and refrigerated upon return to the laboratory for later analysis. Zinc, lead, aluminium, copper, cadmium, iron, magnesium and nickel were analysed using atomic adsorption spectrometry.

Table 7.3: Environmental measurements taken at the control deployment sites.

Site	Depth (cm)	Flow (m/s)	Temp (°C)	pH	DO (mg/L)	Cond (µs/cm)	Hard* (mg/L)	SS (mg/L)
Breaclaich	30	2.03	11.3	7.5	10.7	40	10.2	1.3
Achmore	90	0.06	10.5	7.8	12.0	92	28.4	2.0
Auchlyne	35	0.04	10.0	7.3	11.2	34	9.9	0.7
Innerhadden	30	<0.025	9.9	7.2	10.4	25	6.8	1.5
Chreagain	8.5	<0.025	10.3	7.3	11.2	23	4.9	0.3
Odhair Aulich	40	<0.025	10.7	7.2	10.6	25	9.9	1.3

*Hardness was measured in $CaCO_3$ mg/L.

Table 7.4: Environmental measurements taken at the upstream and downstream deployment sites.

Site	Depth (cm)	Flow (m/s)	Temp (°C)	pH	DO (mg/L)	Cond (µs/cm)	Hard (mg/L)	SS (mg/L)
Carron US	25	0.023	10.7	7.1	8.9	109	37.5	2.4
Carron DS	27	0.001	11.0	8.1	9.2	88	35.4	1.5
Howietoun US	13	0.033	13.6	6.9	11.9	90	36.4	3.4
Howietoun DS	15	0.102	14.8	6.9	9.5	125	44.7	8.5
Leadhills US	18	0.171	11.9	7.8	10.1	99	23.7	1.2
Leadhills DS	10	0.271	10.7	7.6	9.4	156	39.1	5.6
Langholm US	30	0.040	13.0	7.7	8.6	110	179.3	4.6
Langholm DS	15	0.277	12.0	8.1	8.4	174	135.0	4.2
New Mill US	20	0.036	11.7	7.4	8.3	163	25.5	6.7
New Mill DS	15	0.085	12.0	7.4	8.0	3420	54.4	2.9
Coombes US	20	0.001	13.2	7.3	9.8	290	50.6	3.2
Coombes DS	50	0.001	13.2	7.1	8.0	704	269.3	20.2
Habergham US	33	0.021	13.5	7.5	8.4	227	67.5	1.1
Habergham DS	28	0.065	12.9	8.1	9.2	524	67.5	7.7
Tame US	16	0.053	13.2	7.1	10.2	147	62.9	6.8
Tame DS	12.5	0.227	14.6	7.0	8.7	263	62.9	6.8
Drone US	10.0	0.045	14.0	9.5	6.8	521	227.0	9.1
Drone DS	22.0	0.030	16.8	7.1	8.7	620	355.1	6.4

7.2.2 BIOASSAY DEPLOYMENT.

All animals used in these studies were collected from the Howietoun site. Following collection animals were kept under standard laboratory conditions until used (see section 2.1.3).

The *in situ* chambers used in these studies were of the same construction as those used for the microcosm studies. For details of deployment cages and material used to house cages see section 6.2.2. Groups of 7 and 8 cages were housed as outlined in section 6.2.2., and weighted to the stream bed using bricks and string. Following a 48 hour exposure period, cages were collected and transported back to the laboratory in stream water. Upon return the post-exposure part of the test was

carried out as outlined in section 2.3.2.3 and egestion rates were calculated as outlined in section 2.3.2.4.

7.2.3 STUDY SITES.

All field sites used in this study were classed as siliceous with respect to their geology. Control and contaminated sites were chosen with the assistance of expert opinion as outlined in section 7.2.

7.2.3.1 Control Sites

a) Loch Tay Catchment

Two sites were chosen on the Loch Tay catchment in Perthshire. This area has historically supported a high level of sport fishing which is still thriving today.

Allt Braeclaich

The study site along this burn was 4.9 km from the source at an altitude of 107 m and a channel width of 2.3 m. The Allt Breachlaich drains northwards from the reservoir, through grassland and trees, emptying into Loch Tay near Fiddlers Bay with an average discharge of $<10 m^3/s$.

Auchmore Burn

The study site chosen along this burn was 4.5 km from the source with a very rocky substrate at an altitude of 168 m and a 4 m channel width. Again this burn drains into Loch Tay near Fiddlers Bay through grassland and trees at an average discharge of >10 to 25 m^3/s.

b) Loch Rannoch Catchment

Two sites were chosen on the Loch Rannoch catchment in Perthshire. This is a popular tourist, walking and sport fishing area.

Allt a Chreagain Odhair

The study site along this burn was 4.8 km from the source with a rocky substrate at an altitude of 300 m and a channel width of 4 m. The Allt a Chreagain Odhair drains into Loch Rannoch at an average discharge of >10 to 25 m^3/s through heather and grassland populated with deer.

Aulich Burn

The study site along this burn was 47 km from the source with a substrate of rocks and pebbles at an altitude of 290 m and a channel width of 2.5 m. The Aulich Burn drains into Loch Rannoch through woodland at an average discharge of >10 to 25 m^3/s.

c) Auchlyne East Burn

The Auchlyne East Burn is on an estate in Perthshire and runs into the popular sport fishing River Dochart. The study site chosen along this burn was 4.1 km from the source with a mostly rocky/pebble substrate with some areas of gravel and an altitude of 152 m and a channel width of 6 m. This section of the burn runs through a wooded area with an average discharge of <10 m^3/s.

d) Innerhadden Burn.

The Innerhadden Burn is located in Perthshire near Loch Rannoch. The study site was 6.1 km from the source with a rock and gravel substrate at an altitude of 220 m and a width of 8 m. This section of the river runs through woodland and shrubs with an average discharge of <10 m^3/s.

7.2.3.2. Upstream and Downstream sites

Deployment took place at the following sites during the spring/summer season of 2002. Each deployment location consisted of two sites situated near to a point of pollution discharge. Each location consisted of two sites one upstream of the discharge (control) and one downstream (contaminated). Environmental measurements for these sites can be found in Table 7.4.

a) Carron Valley

The Carron Valley is situated in Stirlingshire. Areas of the Carron Valley have historically been the sites of forestry operations. The main water quality issues are: the erosion and siltation resulting from cultivation, drainage, road construction and harvesting; nutrient enrichment of run-off following aerial fertiliser applications; and the contribution of forests to surface water acidification, due to their ability to scavenge acid pollutants from the atmosphere (Harriman and Morrison, 1982; Ormerod *et al.*, 1993). Water quantity issues are also becoming increasingly important, including the effects of forestry on groundwater recharge and the generation of summer low flows.

Burnhouse Burn (upstream)

The uncontaminated site chosen along this burn was 2.5 km from the source at an altitude of 245m, and a channel width of 2 m. This burn runs through grassland with some shubs and trees at an average discharge of <10 m^3/s.

Bin Burn (downstream)

The contaminated site chosen was 3 km from the source of the Bin Burn at an altitude of 240 m and a channel width of 1.5 m. A tributary of the Carron River, it flows north-east through the valley to the reservoir through grassland with some shrubs. The average discharge is >10 – 25 m^3/s.

b) Howietoun

Howietoun is a freshwater fish farm located in Stirlingshire. Effluent from fish farms contain organic material which exerts a biochemical oxygen demand (BOD) on the river water. Additionally, it will contain ammonia and suspended solids (Jones, 1990) and chemicals and drugs used to treat parasites and bacterial infections (Boaventura *et al.*, 1997).

Loch Coulter Burn (upstream)

The uncontaminated site was chosen upstream of the fish farm and runs through grassland as well as woodland. The burn has steep banks through this section at 2.3 km from its source. The channel width was 3 m at an altitude of 110 m and an average discharge of 25 – 50 m^3/s.

Loch Coulter Burn (downstream)

The contaminated site chosen was downstream of the fish farm, running through woodland at this point. The burn was 4.2 km from its source with a channel width of 1.2 m and an altitude of 80 m. The average discharge was >10- 25 m^3/s

c) Leadhills

The Leadhills area is located in Lanarkshire and has been extensively mined for lead since Roman times up until 1930 (Clyde River Purification Board). Historically the major metal contaminants of concern have been lead and zinc.

Mennock Water (upstream)

The uncontaminated site chosen was 6.6 km from the source of the Mennock at an altitude of 184 m. At this point the river has grassy banks with a channel width of 2.5 m and a average discharge of >10 – 25 m^3/s. The Mennock Water rises in the Lowther Hills and flows 10 km south westwards through the Mennock Pass to join the River Nith at Mennock.

Glengonnar (downstream)

The site chosen for study was 3.3 km from the source of the Glengonnar which has historically been contaminated up to its headwaters. The study site was at 322 m altitude with a channel width of 3m and an average discharge of >25 – 50 m^3/s. The Glengonnar rises in the Lowther hills east of the Leadhills, flowing north for 6 km to merge with the Glancaple Burn.

The area the Glengonnar water flows though contains numerous old mine shafts and smelter sites.

d) Langholm

The Langholm study site was located in the Dumfries and Galloway area of Scotland. The main contaminant at the downstream site was thought to be sewage treatment plant effluent containing the pyrethroid permethrin.

River Esk (upsteam)

The study site chosen was along the river Esk 45.5 km from the source at an altitude of 70 m and a channel width of 20 m. Here the river has steep banks with mostly small shrubs and scrub. The average discharge is >50 – 100 m^3/s.

River Esk (downstream)

The contaminated study site was 46.2 km from the source, just downstream of the sewage treatment plant. The channel width was 25 m at an altitude of 67 m and a discharge of >10 – 25 m^3/s. The banks were steep sided with grass and shrubs covering them.

e) Jackson Bridge

Jackson Bridge is a small town in West Yorkshire. Historically this area has been heavily mined for metals. Many abandoned mine sites in this area flood periodically, discharging various contaminants such as iron, ammonia, aluminium or sulphate to local river systems.

New Mill Dyke (upstream)

The uncontaminated site was 2.4 km from the source at an altitude of 195 m and a channel width of 4.3 m. The average discharge was <10 m^3/s.

New Mill Dyke (downstream)

The contaminated site was 2.6 km from the source. One side of the stream consisted of a high brick wall with grass on the opposite bank. The channel width was 4.7 m at an altitude of 195 m and an average discharge of 10 – 25 m^3/s.

f) Coombes Brook.

Coombes Brook is located in Derbyshire. Situated along the river is a textile dye manufacturer with the major contaminants of concern from this industry being cadmium, iron and aluminium.

Coombes Brook (upstream)

The upstream site was 1 km from the source with a channel width of 2 m and at an altitude of 180 m. The brook ran through woodland and grassy fields with an average discharge of <10 m^3/s.

Coombes Brook (downstream)

The contaminated site was 1.2 km from the source at an altitude of 170 m and a channel width of 2 m. The average discharge again was <10 m^3/s at this point.

g) Hapton Colliery

Hapton Colliery is located in Lancashire in an area which has been extensively mined for coal for hundreds of years. The effects of drainage from abandoned mines on riverine systems include a decrease in pH, increase in dissolved iron and siltation (Kimmel *et al.*, 1981).

Habergham Clough (upstream)

The upstream site chosen was 2.4 km from the source at an altitude of 170 m with a channel width of 2.5 m. The banks were grassy with some shrubs and the average discharge was <10 m^3/s.

Habergham Clough (downstream)

The downstream site was 3 km from the source just downstream of a gravel works at an altitude of 160 m and a channel width of 3.7 m. Here the banks were wooded with some shrubs and the average discharge was >10 m^3/s.

h) River Tame

The River Tame, located in South Yorkshire, has been degraded in past years through discharge of sewage treatment works effluent. The major pollutants affecting this stream are increased ammonia concentrations, metals and insecticides.

River Tame (upstream)

The upstream site was located 8.5 km from the source at an altitude of 150 m and a channel width of 10 m. The site was located approximately 10 m

from the base of a weir with fields and some trees on the banks. The average discharge was >10 – 25 m^3/s.

River Tame (downstream)

The contaminated site was located just downstream of a sewage treatment effluent outfall, at 8.6 km from the source. The channel width at the study site was 9m at an altitude of 150 m and an average discharge of >25 – 50 m^3/s.

i) River Drone

The River Drone, in Derbyshire, is a tributary of the River Rother running through a busy industrial district in which there are numerous coal mines and iron foundries. The river is also abstracted for use by industry.

River Drone (upstream)

The upstream site was 3 km from the source at an altitude of 105 m and a channel width of 5 m. The average discharge was <10 m^3/s.

River Drone (downstream)

The downstream site was 4.1 km from the source with pool and riffle areas and a channel width of 4 m. The altitude was 105 m with an average discharge of >10 – 25m^3/s.

7.2.4 BENTHIC MACROINVERTEBRATE COMMUNITY SAMPLING

The benthic macroinvertebrate community was sampled once at each site after deployment. A standard pond net, with mesh size 0.5 mm^2 was used to take a 3-minute kick sample over the width of the river. Each sample was preserved using 70% ethanol for analysis at a later time. Invertebrates were enumerated at x40 under an Olympus dissecting microscope and identified to family level using freshwater identification keys (Fitter and Manuel, 1986). The number of families and individuals were then used to calculate Biological Monitoring Working Party (BMWP) and Average Score Per Taxon (ASPT) scores. The BMWP system assigns each family present in the sample with a score in the range of 1-10 according to its perceived tolerance to organic pollution (10 = least tolerant). The result for each site is then the sum of the family score. The BMWP score can then be divided by the number of families (or taxa) present to give the ASPT score. Sites are then given an ecological quality grade from A (best quality) to D (worst quality). Invertebrate family identification lists are given in Appendix 2.

7.2.5 STATISTICAL ANALYSIS

Upstream and downstream post-exposure egestion rates were tested for significant difference using a Student's one way 2 sample t-test.

7.3 RESULTS

Survival of animals deployed at control sites during the spring/summer season of 2001, was 100% following the 48 hour exposure period, with the exception of Innerhadden at which mortality was 6.7% (one animal).

Figure 7.1 shows the post-exposure egestion rates calculated for *L. peregra* over the 24 hour feeding period. Rates shown for Allt a Chreagain Odhair and Aulich Burn were lower than those recorded at other control sites. However, egestion rates at Allt a Chreagain Odhair and Aulich Burn were within control rates measured in past chapters (0.2 to 0.7 mg/mm/d).

The mortality rates for animals used in the upstream/downstream trials are given in Table 7.5. The number of animals dead at upstream sites was between 0 and 2 individuals for all sites. The mortality rate for animals deployed at downstream sites was again low with between 0 and 1 individual dying at each site with the exception of Coombes Brook where 5 animals died during the exposure period.

Table 7.5: Mortality rates for *L. peregra* bioassay deployed at upstream and downstream sites (n=15).

Site	Upstream mortality %	Downstream mortality %
Carron Valley	0	6.7
Howietoun	13	0
Leadhills	0	6.7
Langholm	0	6.7
New Mill Dyke	6.7	6.7
Coombes Brook	13	33
Habergham Clough	6.7	6.7
River Tame	13	6.7
River Drone	0	0

Five of the 9 upstream/downstream sites show a decrease in post-exposure rates (Figure 7.2). The Carron valley downstream site showed a 13% decrease in egestion rate compared to the upstream site. The Leadhills a 23% decrease, Coombes Brooke a 65 %, Habergham Clough 31% and the River Tame a 22% decrease. However, only the downstream sites at Coombes Brook and Habergham Clough showed a statistically significant difference ($p<0.05$) in post-exposure egestion rate from the upstream sites.

The remaining upstream/downstream sites fall into 2 categories: those showing equal egestion rates between the two sites and those showing a higher rate at down stream sites. The post-exposure egestion rates at the Langholm and New Mill Dyke sites show no apparent difference between upstream and downstream sites. The remaining two sites, Howietoun and River Drone, show a greater post-exposure egestion rate at the downstream sites when compared to the upstream sites.

Table 7.6: Metal concentrations measured at potentially contaminated sites.

Site	Cd µg/L	Cu mg/L	Zn mg/L	Pb mg/L	Fe mg/L	Al µg/L	Mn mg/L	Ni mg/L
Leadhills us	5.1	0.02	0.016	<0.01	0.15	75.3	0.1	<0.02
Leadhills ds	12.3	0.01	0.096	0.05	0.16	41.3	0.1	<0.02
New Mill us	7.2	0.02	0.129	<0.1	1.91	3027	3.2	0.04
New Mill ds	2.8	0.01	0.017	<0.1	11.2	131	2.3	<0.02
Coombes us	11.2	0.01	0.003	0.1	0.18	30	<0.05	<0.02
Coombes ds	13.1	0.01	0.108	<0.1	0.39	174	0.07	<0.02
EQS	5	0.006*	0.175*	0.1*	1	NA	NA	0.1

* At hardness 50 – 100 mg $CaCO_3$/L.

* NA – not available

Table 7.6 shows metal concentrations measured at sites historically contaminated with mining effluent and the EQS for each of the metals as outlined by SEPA. All sites measured were contaminated to some extent with cadmium with the exception

of the downstream site at New Mill. All sites were contaminated with copper. All sites were below the EQS for zinc and at or below the EQS for lead. Leadhills and Coombes were not contaminated with iron, but both upstream and down stream sites at New Mill were highly contaminated. A survey of British rivers by Dixon and Garner (1998) reported concentrations of aluminium in rivers between 8.9 to 458 μg/L at pHs between 7.3 and 8.7. The only site contaminated with aluminium would therefore be New Mill upstream. There was no information available on manganese concentrations in freshwater rivers in the U.K. Nickel concentrations at all sites were below the EQS.

Table 7.7 shows the BMWP and ASTP scores for all sites where the *L. peregra* bioassay was deployed. All control sites scored within the highest water quality classification (A1) with the exception of the Auchlyne Burn. All sites used for deployment scored within the top two water quality classifications with the exception of the Coombes Brook downstream site and both upstream and downstream River Drone sites.

Table 7.7: Biological monitoring working party score (BMWP), average score per taxon (ASPT) and corresponding SEPA water quality classification.

Site	BMWP score	ASPT score	Class
Allt Breaclaich	146	6.95	A1
Achmore Burn	141	7.05	A1
Auchlyne Burn	80	5.71	A2
Innerhaddon Burn	85	6.54	A1
Allt a Chregian Odhair	80	7.27	A1
Aulich Burn	101	6.31	A1
Carron US	75	6.25	A1
Carron DS	67	6.7	A1
Howietoun US	124	6.53	A1
Howietoun DS	71	4.18	A2
Leadhills US	128	6.4	A1
Leadhills DS	98	6.53	A1
Langholm US	152	6.61	A1
Langholm DS	101	6.73	A1
New Mill US	86	5.38	A1
New Mill DS	87	5.12	A1
Coombes US	112	6.59	A1
Coombes DS	42	4.67	B
Habergham US	94	5.88	A1
Habergham DS	94	5.88	A2
Tame US	67	6.09	A1
Tame DS	70	5.38	A2
Drone US	50	4.55	B
Drone DS	50	5	B

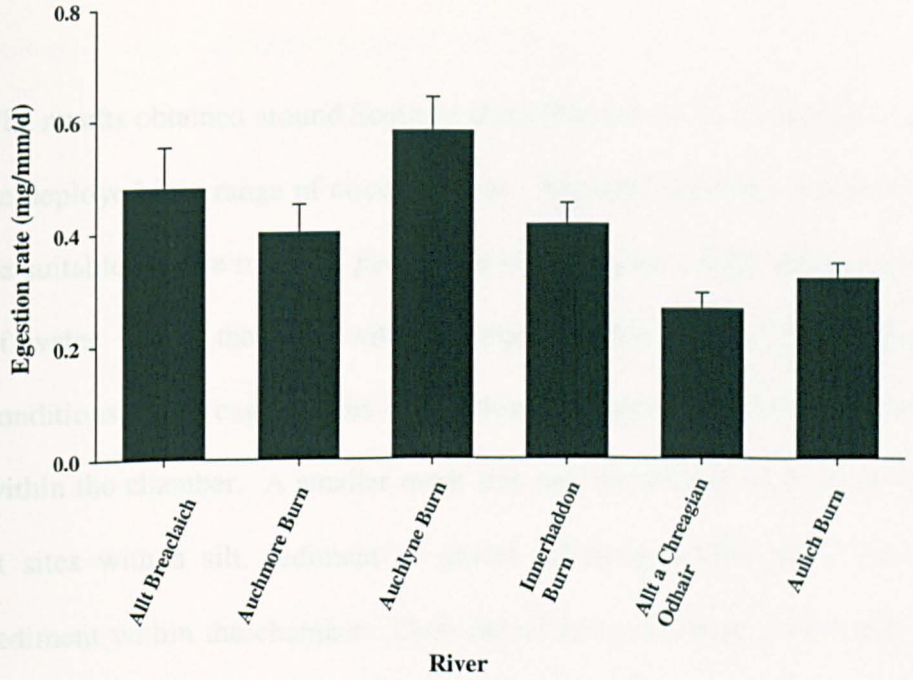


Figure 7.1: Post-exposure egestion rates measured over 24 hours in *L. peregra* following a 48 hour exposure period at control sites (n = 15).

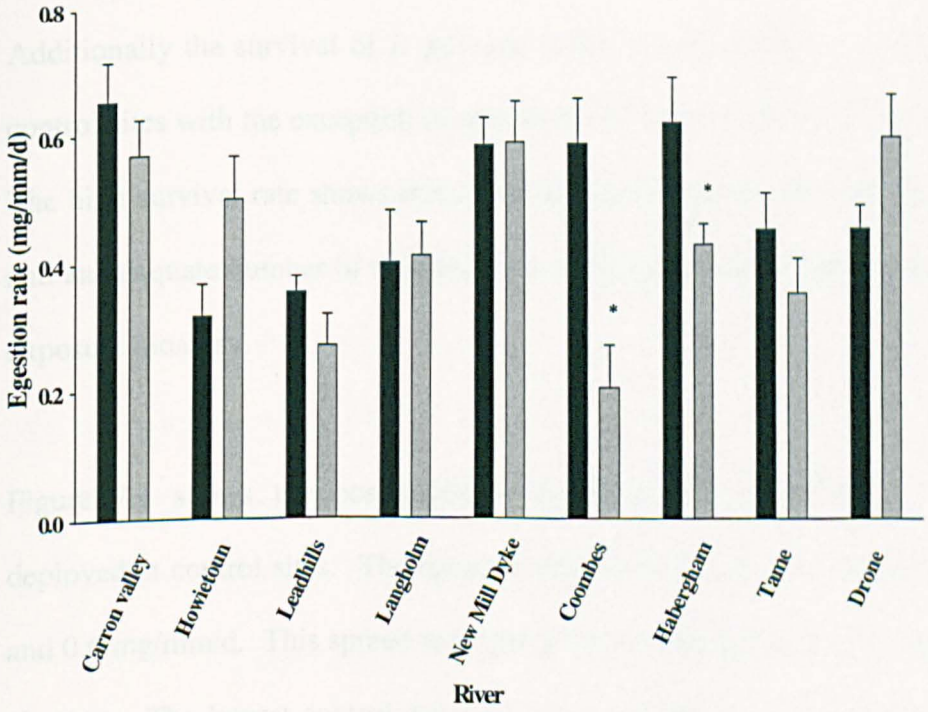


Figure 7.2: Post-exposure egestion rates for upstream and downstream sites at historically polluted sites in the U.K.. Asterisks represent those downstream sites that were significantly different from upstream site ($p<0.05$). Bars represent standard error (n=15).

7.4 DISCUSSION

The results obtained around Scotland show that the *in situ L. peregra* bioassay could be deployed in a range of circumstances. The test chambers constructed proved to be suitable for use in the *L. peregra in situ* bioassay. They allowed the circulation of water within the cage without subjecting the animals to unfavourable flow conditions. The cage and its orientation also prevented the build up of substrate within the chamber. A smaller mesh size and orientation of the cage with the flow at sites with a silt, sediment or gravel substrate, would allow the build up of sediment within the chamber. Over the 48 hour exposure period this would cause stress to the animal through physical damage and under some circumstances may lead to death through excessive build up of sediment.

Additionally the survival of *L. peregra* under *in situ* conditions was 100% for all control sites with the exception of one death (93% survival) at the Innerhadden site. The high survival rate shows that *L. peregra* was suitable for deployment in rivers and an adequate number of animals were consistently recovered for use in the post-exposure bioassay.

Figure 7.1 shows the post-exposure egestion rates calculated for *L. peregra* deployed at control sites. The egestion rates for control sites ranged between 0.25 and 0.6 mg/mm/d. This spread was typical of control egestion rates reported in past chapters. The lowest control egestion rates were observed in Chapters 2 and 4 in the region of 0.2 mg/mm/d and the highest in Chapter 2 in the copper control animals at 0.67 mg/mm.d. The variation in post-exposure egestion rates observed at

the control sites was not unexpected. Due to the differences in egestion rates between control sites and animals, establishing a baseline egestion rate to be used as a reference for all further deployments would not be practical at this stage. Until further work is carried out on the feeding behaviour of *L. peregra* the results from bioassays should be evaluated on an individual basis rather than comparing them to a baseline rate. Comparison of results with a baseline rate, calculated from only a few experiments and which has high variation in the data, will produce false conclusions.

Survival of *L. peregra* deployed at the upstream sites was calculated at greater than 85%. At the downstream sites survival was greater than 90% at all sites with the exception of Coombes Brook which showed only 66% survival. Again recovery was sufficient at all sites to complete the post-exposure section of the bioassay.

Figure 7.2 shows post-exposure egestion rates for all upstream and downstream sites. Coombes Brook and Habergham Clough were the only two sites exhibiting a significant decrease ($p<0.05$) in post-exposure egestion rate as compared with control rate. The main contaminants at Coombes Brook were cadmium, copper, zinc and aluminium (Table 7.6). The cadmium concentration measured at the upstream site was double the EC_{50} value estimated for egestion in Chapter 3 (5.6 µg/L) and exceeded the EQS value. The copper concentration measured also exceeded the EQS value but was less than the EC_{50} value estimated for egestion in Chapter 3 (13 µg/L). The cadmium and copper concentrations measured at the upstream site, should have resulted in a decrease in post-exposure egestion rate following the 48 hour exposure period. However, the egestion rate from the

upstream site was 0.58 mg/mm/d, which was quite high for a control value, showing no evidence of metal contamination on *L. peregra*. The post-exposure egestion rate for the downstream site was statistically significantly lower ($p<0.05$) at 0.20 mg/mm/d. The cadmium and copper concentrations at the downstream site were 13.1 μg/L and 0.01 mg/L respectively, again exceeding the EQS set for freshwater in the U.K. In addition the concentration of zinc measured at the downstream site, while not above the EQS (0.175 mg/L), was substantially higher at 0.108 mg/L than at the upstream site (0.003 mg/L). This was also true for aluminium concentrations.

Water hardness measured at the Coombes Brook downstream site was substantially higher (269.3 mg$CaCO_3$/L) than at the upstream site (50.6 mg $CaCO_3$/L upstream), which alters the EQS of some metals at this site. At between 50 and 100 mg/L hardness the EQS for copper is 6 μg/L whereas at above 250 μg/L hardness it is 28 μg/L. With this in mind, copper no longer becomes a pollution issue at the downstream site. The only remaining metal exceeding its EQS at the downstream site is copper. Therefore the upstream site was more contaminated than the downstream site in terms of metal pollution, when environmental factors are taken into account. The substantial difference in egestion rates for *L. peregra* at the downstream site did not reflect this.

A significant decrease in post-exposure egestion rate was also seen at the downstream Habergham Clough site when compared with the upstream site. Abandoned surface mines can be a continuous source of pollution with the major stressors being low pH and sometimes elevated concentrations of heavy metals (Pereira *et al.*, 1999). However, no metal analysis was done at this site as the major

pollutant was believed to be from coal mine drainage. There were no anomalies between sites in terms of environmental parameters measured, with the pH at the downstream site 6 units higher than at the upstream site.

Water samples from Leadhills and New Mill Dyke (Table 7.6) were analysed for metal content. The downstream Leadhills site showed a 23% decrease in post-exposure feeding rate (0.35 mg/mm/d upstream, 0.27 mg/mm/d downstream), which was not significantly different from the upstream site. Table 6.6 shows that the downstream site was contaminated with cadmium (12.3 μg/L) and copper (0.01 mg/L). The slight decrease in post-exposure egestion rate may have been due to the elevated levels of cadmium but not copper as the concentration measured at the downstream site falls below the EC_{50} value obtained in Chapter 3.

The New Mill Dyke site showed no difference in post-exposure egestion rates (Figure 7.2). Egestion rates measured for both upstream and downstream sites were quite high (both at 0.58 mg/mm/d). The upstream site exceeded the EQS values set for cadmium, copper and iron. The aluminium concentration was also very high at the upstream site as was the manganese concentration. At the downstream site cadmium no longer exceeded the EQS value. Copper remained above the EQS and iron was high at 11.2 mg/L. Aluminium is ubiquitous in the environment and is most soluble under acid conditions. At pH 7.4 (New Mill environmental data Table 6.4), most aluminium in the water would exist as the insoluble $Al(OH)_3$. However, Elgovan *et al.* (1997) reported the availability of aluminium to *L. stagnalis* at neutral pH, with significant accumulation in the gut, digestive gland and kidney.

Any effects of increased metal uptake such as reduced activity caused by aluminium (Truscott *et al.*, 1995) should have been observed at the upstream site.

Two additional sites displayed evidence (not statistically significant) of a reduction in post-exposure egestion rates at the downstream site. These were Carron Valley and the River Tame sites. The pollutants under consideration at the Carron Valley site were those related to forestry activity, i.e. erosion and siltation resulting from cultivation, nutrient enrichment, and run-off following aerial fertiliser applications; and the contribution of forests to surface water acidification. There was a 14% decrease in post-exposure egestion rate at the downstream site. Environmental parameters measured at the Carron Valley and River Tame sites were comparable, with a higher pH recorded at the downstream site, so would not have contributed to the difference in egestion rates and display no evidence of increased acidification.

There was a 24 % decrease in post-exposure egestion rate at the downstream River Tame site. The River Tame has historically been degraded with discharge of sewage treatment works effluent. Major pollutants listed in past years have included pesticides, metals and increased ammonia level. Increased organic loading generally produces an increase in conductivity, decrease in oxygen and pH values. Pulmonate snails are often tolerant of organic pollution (Goudreau *et al.*, 1993) and an increase in the numbers of *L. peregra* was reported by Thomas and Daldorph (1994) in nutrient enriched drainage channels. An increase in pH associated with sewage treatment discharge may also be detrimental to molluscs, as it would allow a larger proportion of the ammonia to exist in its ionised form. Environmental factors measured for the upstream and downstream sites were both comparable with

circumneutral pH values. Therefore the cause of the decrease in post-exposure egestion rate at the downstream River Tame site remains unidentified.

The remaining sites Howietoun, Langholm and the River Drone exhibited no evidence of pollution at the downstream sites. Langholm, which has been polluted with sewage treatment works effluent and has been contaminated with pesticides in the past showed no difference in post-exposure egestion rates between upstream and downstream sites.

Both Howietoun and the River Drone sites showed a 37% and a 24% increase in post-exposure egestion rates at the downstream sites. The environmental measurements taken at the upstream and downstream Howietoun sites were comparable. However the pH value recorded at the River Drone upstream site was high at 9.5, which may have influenced the egestion rate of animals at this site bringing it below that at the downstream site.

Feeding variability can be affected by a number of intrinsic and extrinsic factors. Intrinsic factors might include parasite load, source population and body size as well as the overall status of the organism (Maltby, 1999). Extrinsic factors include temperature, dissolved oxygen concentration and pH. Individual combinations of environmental factors at specific sites define the water quality within that site and will affect organisms in a site specific way. Each site should be considered with respect to environmental and chemical conditions present. If ingestion and egestion rates were influenced by environmental factors, it is possible that the between-site

differences in these rates were, to some extent, related to between site differences in water quality.

Table 7.7 shows BMWP and ASPT scores calculated for each site used in this chapter. All control sites scored A1 quality with the exception of Innerhaddon, which may have scored slightly lower due to the decreased diversity often seen at upland Scottish sites.

The two sites showing a significant decrease in the post-exposure egestion rate, Coombes Brook and Habergham Clough were again classified as A1 and A2 quality with the exception of the Coombes Brook downstream site which was given a score of B. Out of all of the sites surveyed for the TARGET project it was concluded, through the use of additional bioassays, that the downstream Coombes Brook site was the most degraded. Despite the discrepancies discussed above with respect to the metal concentrations at this site, it had been degraded and both the water quality classification and the *L. peregra* bioassay reflected this.

All other upstream and downstream sites were given A1 and A2 scores with the exception of both the upstream and downstream sites at the River Drone which both scored B. However results from the *L. peregra* bioassay did not correlate with the degraded nature of this site as defined by the macroinvertebrate community present.

The scores obtained during this study may only be used as a rough guideline, as a minimum of 3 samples are required in advance of the correct classification of any site. It would therefore be ill advised to draw conclusions regarding water quality

from the BMWP/ASPT scores obtained during this study. However, a problem with many indices of ecological quality is that even in the absence of environmental stress, index values will vary with the type of site due to differences in their physical attributes (Clarke *et al.*, 2002). Additionally, scores may be influenced by variation in sampling effort and the individual carrying out the sampling.

Recently, increased emphasis has been placed on the development and use of *in situ* testing techniques to assess toxicity and the desire to incorporate this type of information into ecological risk assessment frameworks (Sibley *et al.*, 1999). The *L. peregra in situ* bioassay was successfully adapted for use in the field. However, it did not function consistently under contaminated conditions and results obtained under laboratory conditions did not translate to field conditions. While some *in situ* bioassays have performed consistently under degraded conditions (e.g. McWilliam and Baird, 2000b) and might be considered for inclusion in these frameworks, the *L. peregra* bioassay will require further work to be included among them.

Past chapters have illustrated the effectiveness of post-exposure egestion rate as an endpoint and the sensitivity of the *L. peregra* bioassay to metal contamination under laboratory and semi-natural conditions. The results obtained from this chapter were inconsistent with respect to results from the *L. peregra* bioassay deployed at metal contaminated sites. Only two sites out of 9 showed a significant decrease in egestion rates. The difference required between egestion rates at the upstream (control) site and the downstream (contaminated) site were above 31% in both cases. The other 3 sites showing a decrease in egestion rate would have produced a significant result had the test been more sensitive (i.e. if the variation in the egestion

rate data were smaller). A 25-30 % decrease in the feeding rate of *G. pulex* can result in significant reductions in individual growth rate and reproduction (Maltby, 2000). The decreases in egestion rate calculated for the sites mentioned above were between 13% and 23%. Although not statistically significant, this decrease could be detrimental to a natural population.

Further work is required on the feeding behaviour of *L. peregra*. Additionally the possibility that environmental parameters might persist through the exposure period and affect the feeding behaviour of *L. peregra* should be investigated.

CHAPTER 8

GENERAL DISCUSSION

Recently, interest in the use of snails as a test species and especially as indicators of degraded ecosystems has increased (e.g. 2000; Gomot-de-Vaufeury and Pihan, 2000; Oehlmann *et al.*, 2000). The purpose of this thesis therefore, was to study the effects of contamination on ingestion and egestion rates of the gastropod *L. peregra.* This was accomplished through the creation and development a post-exposure feeding toxicity test under laboratory, semi-natural and field conditions, which attempted to fulfill the requirements set out in section 1.6. This test can be used in the laboratory to explore other facets of the toxicological responses of *L. peregra*, this was demonstrated in Chapter 5 through investigation of the major route of cadmium uptake. The following discussion will be divided into three areas:

- Laboratory toxicity test
- *In situ* bioassay
- Overall conclusions

8.1 LABORATORY TOXICITY TEST

L. peregra is a good candidate for use in bioassays and toxicological studies. It is easy to handle, widely distributed and easily identified. Lymnaeids have been extensively studied and *L. peregra* can be cultured successfully although there may be some cause for concern with regards to overall condition of animals kept under laboratory conditions for an extended period of time. Many papers have reported

the successful culture of *L. stagnalis* under laboratory conditions for many successive generations. However, these studies have failed to investigate the possible decline in condition of the animals over time, when compared with wild caught individuals. Additionally, they do not consider the possibility that laboratory cultured animals may become conditioned to their environment, therefore altering their response under experimental conditions. Sourcing considerable numbers of animals for a large-scale experiment becomes impractical and repeated collections are potentially detrimental to the source population. Ideally *L. peregra* used for laboratory studies should be cultured in a micro or mesocosm closely approximating natural conditions, which would continue to allow control over abiotic conditions.

The bioassay developed for use in the laboratory, allows the measurement of both during and post-exposure ingestion and egestion rates. As reported in Chapter 2, egestion may be used as a surrogate measure of ingestion as the two were closely correlated for animals under control conditions. Measuring egestion rate only also decreases the time taken to perform experiments and therefore reduces cost.

Chapter 3 described the response of the *L. peregra* laboratory bioassay to cadmium and copper. The survival rate was 100% during the laboratory bioassay for both cadmium and copper, showing that *L. peregra* is suitable for use in toxicity tests under these conditions. The post-exposure ingestion and egestion rates measured for *L. peregra* exposed for 48 hours to cadmium and copper exhibited a dose dependent decrease in both cases. Laskowski and Hopkin (1996) maintain that when exposed to permanently contaminated food, snails are endangered by a prolonged decrease in consumption rate that eventually may lead to death by

starvation rather than contamination. There is some evidence for this in Chapter 3, however this effect is difficult to assess in a short ecotoxicological experiment.

Overall Chapter 3 indicates that egestion rate could be used as a sublethal endpoint as the EC_{50} values obtained for cadmium and copper were lower than the lethal levels for these metals. Lam (1996) confirms the sublethal effects of cadmium on ingestion in the freshwater tropical gastropod, *Radix plicatulus* reporting a 96-hour LC_{50} of 2.5 mg/L and an NOEC value for consumption of 0.15 mg/L. In a review of current literature regarding the effects of various metals (Cd, Pb, Ni, Hg, V) on freshwater pulmonate snails Ravera (1991) reported that most were performed using *Biomphalaria galbrata* and *Physa acuta* and a few studies involved *L. stagnalis* and *Radix auricolaria japonica.* Current trends in literature with regard to studying the effects of metals on snails have favoured the terrestrial *Helix aspersa* (i.e. Gomot-De Vaufleury, 2000) and the aquatic gastropod *Potamopyrgus* sp. (i.e. Jensen *et al.*, 2001).

As discussed in Chapter 3, aquatic snails have been extensively studied in the literature largely as an indicator of metal contamination due to their capacity to accumulate these pollutants. There is little evidence however, of the consequences of organic pollution on aquatic snails outside of the secondary effects of herbicides (i.e. Cuppen *et al.*, 1997) and the possible effect of hormone disrupting chemicals (i.e. Oehlmann *et al.*, 2000). This is supported by the results from experiments carried out in Chapter 4. No concentration below saturation of either pyrene or lambda cyhalothrin produced lethal effects over a 96 hour period for *L. peregra.* Additionally no concentration below saturation produced a significant decrease in

egestion rates compared to control. The length of time over which the experiment was run may not have allowed for effects of prolonged exposure to be observed. Additionally, there is no facility to explore the possibility of secondary effects on *L. peregra* such as those seen during prolonged exposure to herbicides.

The laboratory bioassay was employed again in Chapter 5 to look at the uptake and accumulation of cadmium as well as the possible effects of exposure on ingestion and egestion rates and growth. These experiments should be treated as preliminary but indicate that water may be a more important source of cadmium to *L. peregra* than food. The results from Chapter 5 also raised some questions concerning the laboratory bioassay. The lack of effect of cadmium on ingestion and egestion rates in the short and long term experiments at levels that had produced effects in Chapter 3, cast doubt on the repeatability of this test under laboratory conditions.

Further work which is required to take the laboratory bioassay forward include:

- Establishment of a stable source population possibly through the use of outdoor mesocosms. This would provide animals of consistent quality and quantity, which should lead to a decrease in variation of feeding and egestion rates. Additionally, this would provide the ability to perform regular positive control tests to ensure the animals used react predictably to a known contaminant.
- Investigation into factors affecting ingestion and egestion rates is required such as parasite load, seasonality, and developmental stage.
- Further testing with a large number of chemicals with differing modes of action and routes of uptake. In addition, an investigation should be

performed into the possibility that a longer exposure period might result in clearer post-exposure effects.

8.2 *IN SITU* BIOASSAY.

As reported in Chapter 2, the egestion rate of *L. peregra* measured under control conditions correlates closely with ingestion rate. This indicates that egestion rate is as good an indicator of feeding behaviour as ingestion rate. The *in situ* bioassay developed in Chapters 6 and 7 uses the post-exposure egestion rate as an indicator of effects as the measurement of ingestion under field conditions is logistically difficult. Egestion rate has been used previously as a surrogate for ingestion in aquatic snails (Calow, 1981; Brown *et al.*, 1994; Blandenier and Perrin, 1998). The measurement of post-exposure effects relies on the persistent influence of pollutants on the ingestion and egestion rates of *L. peregra* following exposure as demonstrated in several chapters of this thesis. The Standard Operating Procedures (SOP) for the *in situ* bioassay itself can be found in Appendix 3.

Ecotoxicological tests should attempt to meet the basic requirements set out in section 1.6. For any scientist developing a new bioassay adherence to these points will produce a more robust procedure. The following criteria can be applied to both the laboratory and *in situ* bioassays. However, they will be discussed in general terms here with conclusions including both tests to follow.

RELEVANCE

Calow (1996) understands relevance to mean the extent to which changes in the test relate to adverse changes in the ecological system under study. A reasonable ecological criterion of the condition of an individual, population, community or ecosystem is that they should persist. So any parameter providing an indication of this would be appropriate for ecotoxiololgical observation. In terms of an individual species, it should be sensitive and contribute towards the functional integrity of a community or be representative of a particular trophic group. Additionally the endpoint should be ecologically relevant and it should be possible to extrapolate effects to higher trophic levels (Cairns and Pratt, 1989).

L. peregra can be seen as an ecologically relevant species representative of primary consumers. They are highly studied and are easily identifiable, they can be sampled easily and have a wide distribution range. Their biological and ecological characteristics are known, they strongly accumulate pollutants and they can be easily kept in the lab (Gomot De Vaufleury, 2000). *L. peregra* have shown a high survival rate in both laboratory and *in situ* testing and can be deployed in a range of environments. Snails are an important contributor to the functional integrity of a freshwater community in both lotic and lentic systems as they transfer energy generated by primary production to higher trophic levels and facilitate the breakdown of detritus.

Ecological relevance was tested initially in the microcosm studies (Chapter 6) where as in Chapters 3 and 4, *L. peregra* showed a decrease in egestion rate when exposed to metals but not organics. The decrease in post-exposure egestion rate in the zinc microcosm concurred with a decrease in feeding rate of *G. pulex* and a slight

decrease in the *D. magna* bioassay. This shows that egestion rate data from *L. peregra* could be linked to data from other trophic levels when exposed to metals, so demonstrating ecological relevance. Despite the correlation between *in situ* and laboratory results due to the nature of the *L. peregra* bioassay, secondary effects such as those elicited by herbicides are not accounted for.

Post-exposure egestion rates collected from the *in situ* deployment of the *L. peregra* bioassay showed some correlation with community structure. At the two sites showing a significant difference in post-exposure egestion rates between the up and downstream sites, the water quality classification showed some degradation. However this argument is weakened by the fact that none of the sites chosen for use were classed as below water quality B (i.e. good).

While the *L. peregra* bioassay was consistently sensitive to metals the relevance of this test in relation to community structure or effects on other invertebrates was not high.

RELIABILITY

According to Calow (1996) reliability refers to an ability to make observations in a controlled way and with confidence. Reliability implies that the results can be repeated and reproduced with little variability i.e. the same test performed with the same substance should give comparable results. For regulatory purposes, tests must be sufficiently simple and standardized in order that governmental, academic and private laboratories of widely varying capabilities can carry them out (Cairns and

Pratt, 1989). This can be partly achieved through the use and availability of test organisms in appropriate quantity and quality.

Chapter 2 endeavoured to address the majority of reliability issues by attempting to minimise variability through the standardisation of test conditions. This allows increased reliability or confidence in the detection of effects through changes in egestion rates. The standardization of testing conditions for both the laboratory based test and the post-exposure section of the *in situ* bioassay produced egestion rates with coefficients of variation of 23% for snails greater than 8 mm shell length. This was in good agreement with McWilliam (2001) who found a 24% coefficient of variation for baseline *D. magna* feeding rates.

Chapter 2 also looked at the difference in performance between the use of snails from the third generation of culture and those collected from the field. This test concluded that the snails collected from the field had a consistently higher egestion rate than those from culture.

Early indications with respect to minimising the variation around the baseline egestion rate to produce repeatable results in the laboratory were encouraging. Looking at egestion rate results from all chapters from control animals measured over 24 hours in the laboratory (Figure 8.1), it is clear that the variability in control egestion rates was not consistently as low as for those experiments discussed in Chapter 2. Figure 8.1 also shows variation in control egestion rates among the 8 experiments. Control egestion rates appear quite constant for all experiments with the exception of number 3 (copper from Chapter 2). From this graph it could be

concluded that generally the egestion rate for *L. peregra* under these conditions was approximately 0.27 (SD = 0.2) mg/mm/d. However, in Chapter 7 results were presented for the post-exposure egestion rates of animals deployed at control sites (Figure 7.1), which produce an average of 0.41 (±0.21, sd) mg/mm/d. *L. peregra* therefore does not seem to produce consistent baseline control data. All egestion rates illustrated in Figure 8.1 were measured using animals collected from the Devon site, whereas those shown in Figure 7.1 were from snails collected at the Howietoun site. It appears, with the exception of egestion rates calculated for the copper experiment from Chapter 4, that there was some difference in egestion rates of *L. peregra* collected from the two sites. Whether this was a genetic or environmental difference would be work for a future study.

One aim of the present study was to evaluate the potential use of *L. peregra* as a candidate for use in a laboratory and field bioassay. Control egestion rates measured show high variability within experiments and differences between animals collected from different sites. Therefore a reliable source population would be recommended before employing this test. This may involve further exploration of factors affecting baseline egestion rates as outlined in section 2.3.1, including the effect of body size, parasite load and functional feeding rate (is there an upper limit?), additionally seasonality may affect feeding and in turn egestion so should also be considered.

The *L. peregra* post-exposure egestion rate bioassay could therefore be a reliable test, but further work on the sources of variability in baseline rates needs to be addressed, before further work with contaminants can be carried out.

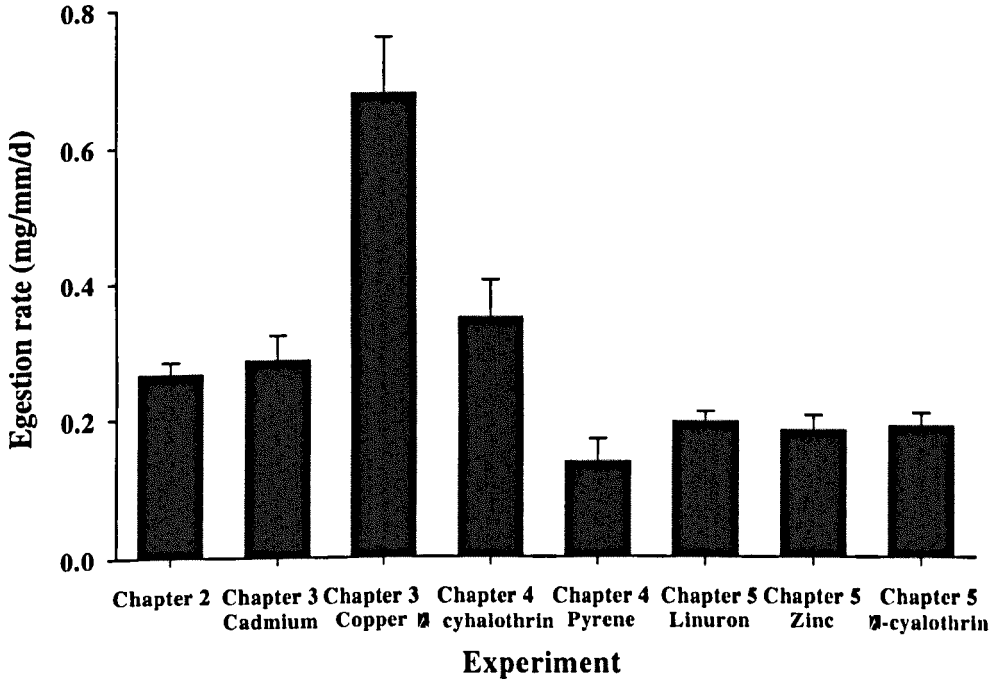


Figure 8.1: Comparison of egestion rates using data collected from control animals in the 24 hour post-exposure period.

SENSITIVITY

In order to demonstrate the sensitivity of a novel endpoint such as the post-exposure egestion rate of *L. peregra*, a significant response to chemicals with different modes of action should be demonstrated. Additionally, the response should be sufficiently sensitive to avoid excessive false negatives (Cairns and Pratt, 1989). As discussed in Chapter 2, none of the numbers of replicates tested reached the desired power of 0.8 when 20% was the specified minimum detectable difference between egestion rate means. The number of individuals chosen per test was 15, which gave a power of 0.55. Logistical constraints dictate the number of individuals chosen per treatment. Despite the use of 12 individuals (power = 0.42) in tests carried out in Chapter 3, this number of animals proved to be suitable as the statistical analysis of

data detected effects on egestion rate. However there still remains a very high (58%) probability of committing a type II error. As no more than 15 individuals were chosen per treatment, there remains the very high probability (50%) of committing a type II error. At high statistical power the minimum detectable difference between control and treated egestion rates will be quite low. Therefore without using a large number of individuals per treatment there is little confidence in the ability to detect 0small differences between egestion rates.

The ability of a bioassay to detect small differences between egestion rates will be important in field studies, where the contaminant may be present in low concentrations or decreased bioavailability due to environmental conditions (McWilliam and Baird, 2002a). As discussed by Cairns and Pratt (1989), the sensitivity of a response may have little meaning unless it is correlated with observable adverse ecological effects. Chapter 7 shows some correlation between degraded sites and a decrease in post-exposure egestion rate. The two sites showing significant reduction in post-exposure egestion rate (Coombes Brook and Haberham Clough) were historically polluted with metals. A further 3 sites showed some decrease in post-exposure egestion rate that was not statistically significant (see Table 8.1). As discussed in the above section on reliability, the high variability of post-exposure egestion rate data (Figure 7.2) could have prevented diction of a significant difference for these three sites. An increase in the number of individuals deployed at each site would increase the power, but perhaps not be practicable. The sensitivity of the *in situ* assay deployed at field sites was therefore questionable.

Table 8.1. Summary table of *in situ* sites producing a decrease in post-exposure egestion rates at the downstream site. STW = sewage treatment works

Site	Percent decrease in post - Exposure egestion rate	Pollutant	Significant ($p<0.05$)
Carron Valley	13	Forestry runoff	No
Coombes Brooke	65	Metals	Yes
Haberham Clough	31	Metals	Yes
Leadhills	23	Metals	No
River Tame	22	STW effluent	No

COST AND TIMING

Cairns and Pratt (1989) describe cost as a function of the time necessary to conduct the test, the space required and the level of expertise needed both to perform the test and to evaluate the results. The bioassay protocol developed (see Appendix 3) was simple to carry out and requires only 3 days to complete, but is very labour intensive during this period. This however does not include the creation of spinach mats for feeding, the preparation of filter paper for gravimetric analysis of faeces or the field collection of animals. Including preparation and collection the bioassay requires 5 days to complete in both the laboratory or *in situ*. A great deal of space and expertise is not required to carry out the test either in the field or in the laboratory. No specialized equipment is required to carry out this bioassay with the exception of a balance capable of measuring milligrams accurately.

8.3 OVERALL CONCLUSIONS AND SUMMARY

The laboratory based bioassay has proven to be a fast and simple tool for investigating the toxicological responses of *L. peregra* ingestion and egestion rate. Ingestion and egestion rates proved to be pertinent and sensitive endpoints when investigating the toxic response to metals. The test however has little application in

investigating the effects of organics on *L. peregra*, but would be a useful tool to look at the uptake kinetics of these compounds. This was illustrated through the use of the laboratory assay to investigate the uptake route of cadmium to *L. peregra* in Chapter 5.

Some questions remain regarding the reliability and sensitivity of the *L. peregra* bioassay. The failure of this test to pick up small changes in ingestion or egestion rates in both the laboratory and under field conditions was a consequence of low power. This is a consequence of too few replicates and high variation around ingestion and egestion rates. Further investigation into the feeding behaviour of *L. peregra* through the establishment and use of a stable source population could provide insight into the associated sources of variability.

The *L. peregra in situ* bioassay was successfully adapted for use in the field, however, it did not function consistently under contaminated conditions and results obtained under laboratory conditions did not translate to field conditions. The short exposure period may mean that pulse events are missed. But as the *L. peregra* bioassay alone will not give an accurate assessment of water quality in a riverine system, other bioassays deployed simultaneously with longer exposure times (e.g. 6 day exposure *G pulex* feeding bioassay, (Maltby *et al.*, 1990) would be more likely to detect these events. The *L. peregra in situ* bioassay could be useful as one of a suite of bioassays employing organisms from different trophic levels that contribute toward the assessment of water quality and ecosystem function.

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APPENDIX 1

SNAIL WEIGHTS AND SHELL LENGTHS

Table A1: Wet weights, dry weights and shell lengths used to determine the sell dimension to use as a measure to size.

Length (mm)	Wet weight (g)	Dry weight (g)
11	0.3180	0.0178
9.6	0.2058	0.0207
12.3	0.3637	0.0283
9.3	0.1961	0.0183
8.1	0.1690	0.0142
9.3	0.2019	0.0171
10	0.2116	0.0151
8.4	0.1389	0.0103
9.7	0.2182	0.0165
11.2	0.3158	0.0155
10	0.2269	0.0169
9.5	0.1663	0.0106
9.7	0.2177	0.015
11.7	0.2663	0.019
12.6	0.3971	0.0247
10	0.2322	0.0205
8.7	0.1580	0.0122
9.9	0.2165	0.0156
10.1	0.2397	0.0229
10.5	0.2723	0.0216
12.2	0.3664	0.0156
10.5	0.1353	0.0223
9.1	0.1836	0.014
9.2	0.1377	0.0076
9	0.1758	0.0069
9.4	0.1979	0.018
8.2	0.1327	0.0108
10.6	0.3483	0.0263
10.1	0.2317	0.0166
8.9	0.1574	0.0048
12.3	0.4347	0.0357
13.4	0.5599	0.0384
11.6	0.3253	0.0305
10.9	0.2906	0.0229
8.2	0.1303	0.0094
9.55	0.2030	0.0149
9.1	0.1618	0.0128
10.5	0.2675	0.0137
12.6	0.4513	0.0415
8.7	0.2268	0.0114
12.9	0.5102	0.0158

12.2	0.3503	0.0242
10.5	0.2630	0.0246
11.2	0.3674	0.0288
12.9	0.4271	0.021
9.6	0.1803	0.0137
11	0.2867	0.0223
9.4	0.2078	0.0197
9.8	0.1912	0.0131
11	0.3327	0.0248
9.9	0.2239	0.0181
10.3	0.2774	0.0124
9.3	0.1709	0.0126
9.9	0.2203	0.0202
8	0.1227	0.0102
9.4	0.3019	0.0158
9.3	0.2092	0.0205
8.1	0.1095	0.0086
12.6	0.4291	0.0377
10.6	0.2681	0.0211

APPENDIX 2

BIOLOGICAL WORKING PARTY (BMWP) AND AVERAGE SCORE PER TAXON (ASPT) SCORES FOR FIELD STUDY SITES

Table A2: Numbers of individuals within families of benthic macroinvertebrates sampled from control study sties and calculated BMWP, ASPT and water quality scores.

Family	Allt Breaclaich	Achmore	Allt a Chreagain Odhair	Aulich	Achlyne	Innerhaddon
Ancylidae				35	3	
Baetidae	77	56	16		48	59
Caenidae			2	11	1	
Chironomidae	8	4			1	
Chloroperlidae	1	2		6		1
Coleopter larvae				6	20	
Cordulegasteridae				1		
Elmidae	34	17		6	13	1
Empididae	1					
Gammaridae	28	21				
Glossophoniidae					1	
Goeridae	3	2				
Heptagenidae	102	36	12	29	33	26
Hydropsychidae		5	3	2		1
Hydroptilldae						1
Leptophlebiidae				1	1	
Leuctridae	23	47	1	5	21	3
Limnephilidae	2	4		1	1	
Nemouridae	8	16	3	2	7	2
Odontoceridae	3					
Oligochaetae	7	11		14	3	2
Perlidae	3	7	1			3
Perlodidae	1	1	1			
Polycentropodidae	1	1	4	1		
Rhyacophilidae	1	4				1
Scirtidae			5	12		
Sericostomatidae	1	1				
Simuliidae	6	3	2			2
Tabanidae				17	12	
Taeniopterygidae	1	1				
Tipulidae	2	2		3	4	1
BMWP	146	242	80	101	80	85
ASPT	6.95	7.05	7.27	6.31	5.71	6.54
Water quality	A1	A1	A1	A1	A2	A1

Table A3: Numbers of individuals within families of benthic macroinvertebrates sampled from upstream and downstream sites at Carron Valley and calculated BMWP, ASPT and water quality scores.

Family	Upstream Burnhouse	Downstream Bin Burn
Baetidae	68	84
Chironomidae	8	125
Chloroperlidae		5
Dytiscidae	7	
Elmidae	23	
Ephemerellidae	22	7
Gammaridae	5	
Goeridae	1	
Heptagenidae	5	9
Leuctridae	7	22
Limnephilidae	1	
Oligochaetae	17	9
Scirtidae	5	
Simulidae		9
Tipulidae		3
BMWP	75	67
ASPT	6.25	6.53
Water Quality	A1	A1

Table A4: Numbers of individuals within families of benthic macroinvertebrates sampled from upstream and downstream sites at Howietoun and calculated BMWP, ASPT and water quality scores.

Family	Upstream Loch Coulter	Downstream Loch Coulter
Ancylidae	1	
Aselliidae	1	4
Baetidae	59	15
Caenidae	2	
Chironomidae	37	4
Chloroperlidae	2	
Collembola		1
Dytiscidae	2	
Elmidae	26	7
Empididae	1	2
Ephemerellidae	57	3
Erpobdellidae		11
Gammaridae	16	95
Heptagenidae	20	
Hydropsychidae	9	12
Leuctridae	123	
Limnephilidae	9	19
Odontoceridae	6	
Planorbidae		3
Polycentropodidae	9	
Rhyacophilidae	4	8
Sericostomatidae	3	
Simuliidae		4
Sphaeriidae		9
Tipulidae		32
Valvatidae		3
BMWP	124	71
ASPT	6.53	4.18
Water Quality	A1	A2

Table A5: Numbers of individuals within families of benthic macroinvertebrates sampled from upstream and downstream sites at Leadhills and calculated BMWP, ASPT and water quality scores.

Family	Upstream Mennock	Downstream Glengonnar
Baetidae	641	146
Chironomidae	254	108
Chloroperlidae	1	3
Collembola	1	
Dytiscidae	13	
Elmidae	63	5
Ephemerellidae	690	4
Gammaridae	46	108
Goeridae	2	
Heptagenidae	190	5
Hydropsychidae	1	
Leuctridae	66	163
Nemouridae	1	1
Perlidae	8	
Perlodidae	1	1
Polycentropodidae	1	
Rhyacophilidae	29	24
Simuliidae	149	5
Tabanidae	5	5
Tipulidae	4	8
BMWP	128	98
ASPT	6.4	6.53
Water Quality	A1	A1

Table A6: Numbers of individuals within families of benthic macroinvertebrates sampled from upstream and downstream sites at Langholm and calculated BMWP, ASPT and water quality scores.

Family	Upstream Esk	Downstream Esk
Ancylidae	1	
Aselliidae	1	
Baetidae	172	124
Caenidae	11	59
Chironomidae	96	60
Coleopter larvae		11
Dytiscidae	2	17
Elmidae	34	10
Empididae	1	
Ephemerellidae	101	119
Gammaridae	9	39
Glossiphoniidae	1	
Goeridae	2	
Heptagenidae	99	140
Hydropsychidae	4	
Lepidostomatidae	1	1
Leptoceridae	1	4
Leuctridae	64	53
Limnephilidae	3	1
Nemouridae	3	
Perlidae	1	
Rhyacophilidae	4	
Sericostomatidae	2	3
Tabaninae		1
Tipulidae	10	1
BMWP	128	98
ASPT	6.4	6.53
Water Quality	A1	A1

Table A7: Numbers of individuals within families of benthic macroinvertebrates sampled from upstream and downstream sites at Jackson Bridge and calculated BMWP, ASPT and water quality scores.

Family	Upstream New Mill	Downstream New Mill
Aselliidae	1	2
Baetidae	16	14
Ceratopogonidae		4
Chironomidae	261	58
Chloroperlidae	3	2
Collembola		3
Dytiscidae	11	3
Elmidae	3	5
Empipidae	1	
Ephemerellidae	6	
Gammaridae	6	2
Halipidae		1
Leptophlebiidae	1	
Leuctridae	9	13
Psychodidae	3	11
Rhyacophilidae	1	1
Sialidae	1	
Simuliidae	3	2
Taeniopterygidae		1
Tipulidae	1	1
BMWP	152	6.61
ASPT	101	6.73
Water Quality	A1	A1

Table A8: Numbers of individuals within families of benthic macroinvertebrates sampled from upstream and downstream sites at Coombes Brook and calculated BMWP, ASPT and water quality scores.

Family	Upstream Coombes	Downstream Coombes
Baetidae	80	36
Chironomidae	2	25
Chloroperlidae	3	
Elmidae	2	1
Empipidae	1	
Ephemerellidae	25	8
Gammaridae	84	9
Heptagenidae	10	
Hydrophilidae		4
Hydropsychidae	15	
Leptoceridae	1	
Leuctridae	16	
Limnephilidae	2	
Philopotamidae	1	
Psychodidae		2
Psychomyiidae (Ecnomidae)	1	
Rhyacophilidae	18	
Simuliidae	8	3
Tipulidae	2	2
BMWP	112	42
ASPT	6.59	4.67
Water Quality	A1	B

Table A9: Numbers of individuals within families of benthic macroinvertebrates sampled from upstream and downstream sites at Hapton Colliery and calculated BMWP, ASPT and water quality scores.

Family	Upstream Habergham	Downstream Habergham
Baetidae	616	12
Caenidae	3	2
Chironomidae	23	9
Donaciinae	1	
Dytiscidae	18	9
Elmidae	13	4
Ephemerellidae	137	104
Gammaridae	1	
Halipidae	1	
Heptagenidae	16	
Hydropsychidae	1	
Leuctridae	4	3
Notoridae	17	1
Psychodidae	1	
Psychomyiidae (Ecnomidae)	1	
Rhyacophilidae	4	
Simuliidae	57	1
Tipulidae	9	4
BMWP	94	94
ASPT	5.88	5.88
Water Quality	A1	A2

Table A10: Numbers of individuals within families of benthic macroinvertebrates sampled from upstream and downstream sites at River Tame and calculated BMWP, ASPT and water quality scores.

Family	Upstream Tame	Downstream Tame
Aselliidae	106	
Baetidae	179	312
Chironomidae	20	62
Dytiscidae	3	
Elmidae	1	
Empididae		1
Ephemerellidae	501	302
Gammaridae	124	7
Glossiphoniidae		1
Heptagenidae	31	8
Hydropsychidae		1
Leuctridae	62	19
Rhyacophilidae	2	2
Simuliidae	10	122
Tipulidae		1
BMWP	67	70
ASPT	6.09	5.38
Water Quality	A1	A2

Table A11: Numbers of individuals within families of benthic macroinvertebrates sampled from upstream and downstream sites at River Drone and calculated BMWP, ASPT and water quality scores.

Family	Upstream Drone	Downstream Drone
Aselliidae	106	1357
Baetidae	179	29
Chironomidae	20	418
Dytiscidae	3	
Empipidae	1	
Ephemerellidae		
Gammaridae	501	
Glossiphoniidae	124	1
Oligochaetae		9
Polycentropodidae		
Simuliidae	31	496
Tipulidae		
BMWP	50	50
ASPT	4.55	5
Water Quality	B	B

APPENDIX 3.

STANDARD OPERATING PROCEDURE FOR IN SITU POST-EXPOSURE EGESTION INHIBITION BIOASSAY FOR THE SNAIL, *LYMNAEA PEREGRA*

(i) PURPOSE:

Standardisation of procedure for field deployment and processing of the *Lymnaea peregra* egestion rate bioassay

(ii) SCOPE:

To be applied in all *in situ* egestion inhibition tests utilising *L.peregra.*

(iii) REFERENCES:

Crichton CA, Conrad AU, Baird DJ (2003) Assessing Stream Grazer Response to Stress: A Post-Exposure Egestion Bioassay Using the Freshwater Snail *Lymnaea peregra* (Müller). Submitted to *Bull Environ Contamin Toxicol*

(iv) PROCEDURE:

Materials (per field site)

30 L Snail medium
10 Adult *L. peregra*
1 (10-L) bucket with lid
10 PVC capped cages
1 wire mesh cage
2 bricks
10 zippy ties
Scissors
20 glass microfibre filters (Whatman, 47 mm GF/C 1822 047)
30 filter papers (Whatman #1 filter papers, 47 mm, 1001042)
20 x 60 ml wide mouthed glass jars
20 x 25cm^2 square mesh (500 μm) pieces
20 rubber bands
2 x 7 L plastic tanks
Drying oven
Balance (0-1 g accurate to ± 0.001 mg)
Snail spoon (made by cutting half the bulb of a 3 ml plastic pipette)
Filter apparatus
Tweezers
Organic spinach (150 g)
Hand held blender
Heating plate
Measuring cylinder 100 ml
1 aeration pump
Callipers

2.0 Method

Acclimation of *L.peregra*

1. Field collect the animals a few days before *in situ* bioassay.

2. Keep the snails in a 7-L tank in snail medium under aeration at 16°C. Place no more then 15 snails in each tank.

3. Add 0.5g of fish food flakes (Tetramin) to each tank.

Field deployment

1. Transfer 10 *L.peregra* to a 10-L bucket containing snail medium for transport to field. If the journey to the site is expected to take longer then 1 hour or is undertaken during warm weather, a portable aeration device should be used.

2. At the field site place one snail in each cage and cover the cage with a mesh cap.

3. Secure 10 cages within the wire mesh basket and close the basket with zippy ties. Place the cages with the bore of the cage parallel with the direction of the water flow (see Figure 2). Secure baskets containing cages onto the bottom of the river using bricks and string.

4. Remove the baskets containing cages after 2 days. Fill the 10-L bucket with site water and submerge the snail cages with the snails. Transport the animals back to the lab.

5. In the lab record status of each snail (alive or dead) and place live snails in a wide-mouthed jar with a spinach disk at the bottom.

Preparation of spinach disks

1. Simmer 100 g of fresh organic spinach in 100 ml of water for 10 minutes until the spinach has reduced in size and is dark green in colour.

2. The mixture is then left cool at room temperature. Once cooled the mixture is blended using a hand blender for 5 minutes.

3. The spinach mixture is then diluted 3:1 using snail medium and 15 ml of this mixture is filtered onto a Whatman GF/C 47 mm circle.

4. The spinach mats are then placed into an oven at 60°C for 30 minutes. Drying the mats ensures that the spinach will adhere to the filter paper. These mats are then ready for use. Mats can be frozen for up to 3 months.

Measurement of egestion rates

1. One spinach mat is placed into each of twenty glass jars and the jars are filled with snail medium. The snails are then randomly placed one in each of ten jars. The jars are sealed with mesh to keep the snails from escaping.

2. All the jars are placed into a fish tank and snail medium added so that the jars are well submerged beneath the water surface. The water is then gently aerated.

3. The snails are left for 24 hours at constant temperature (18°C to 22°C).

4. After 24 hours the jars are removed from the tanks and the snails removed from the jars and set-aside for measuring with callipers. Snail length is recorded as the longest axis across the snail shell (see Figure 1).

5. The contents of each jar (controls and snail jars) is filtered onto a piece of Whatman GF/C filter paper of which the dry weight was recorded.

6. These filter papers are dried at 60°C for overnight and reweighed. The dry weight is recorded.

7. Faecal production is measured as follows.

$$E_r = (Ff - Fi) / T \times W$$

Where:
E_r = Egestion rate (mg/mm/day or mg/mg/day)
Fi = initial filter paper dry weight (mg)
Ff = final filter paper dry weight (mg)
T = exposure time (days)
W = length of snail (mm)

Figure 1: Measurement of the longest shell axis of *L. peregra*