Feeding behaviour of *Lumbriculus variegatus* as an ecological indicator of *in situ* sediment contamination

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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

Previous studies have demonstrated that the feeding behaviour of *Lumbriculus variegatus* may be significantly inhibited during exposure to toxic substances. The potential use of an *in situ* sediment bioassay, using *L.variegatus* post-exposure feeding inhibition as an endpoint, was investigated. The bioassay consisted of exposing animals in the field for a six-day exposure period and feeding rates were measured immediately afterwards over a twenty-four hour post-exposure period. The bioassay methodology developed in the laboratory produced a consistent baseline response that was reliable and repeatable.

Endpoint sensitivity was demonstrated under laboratory conditions, where bioassay organisms exhibited delayed recovery from feeding inhibition after previous exposure to sediment-associated contaminants. The apparent insensitivity of the bioassay to sediment-associated metals means that the technique should only be used as part of a suite of bioassays that employ representative deposit feeders. The ecological relevance of the bioassay endpoint was also demonstrated by comparing short-term measures of post-exposure feeding inhibition with the longer-term effects of a toxicant on *L.variegatus* populations.

The bioassay methodology was successfully adapted for *in situ* use. Post-exposure feeding inhibition was detected at contaminated field sites. However, the consistent baseline response produced in the laboratory could not be replicated during deployments of the bioassay at upstream ("clean") field sites. Increased environmental "noise" may have

been a result of a number of confounding factors that could limit the sensitivity of the bioassay endpoint if not adequately controlled. Despite the above concerns, the *in situ* bioassay is suggested to represent a useful tool, which uses a more realistic field exposure scenario to investigate the effects of sediment-associated toxicants with an important functional component of aquatic ecosystems.

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1 CHAPTER 1 - INTRODUCTION

1.1 GENERAL INTRODUCTION

Many of the environmentally persistent chemicals that are found in freshwaters become associated with particulate material that eventually settles to the bottom sediment. As a consequence aquatic sediments can act as a sink for a variety of organic and inorganic chemicals. Once associated with the sediment they may be directly toxic to benthic organisms, thereby causing changes to community structure and function. Benthic fauna may also mediate the transfer of contaminants back into the water column and into higher levels of the aquatic food chain (U.S.E.P.A 1997). In recent years a significant number of the persistent chemicals that were found to accumulate in aquatic sediments have been banned from use (Stronkhorst *et al.* 2004). However, many chemicals may remain in sediments at potentially toxic concentrations long after the source of contamination has been controlled. Alongside historical problems, a large number of modern industrial and household chemicals are released into the aquatic environment which may subsequently accumulate in the underlying sediment (Long *et al.* 1998).

In a recent report to the US congress the United States Environmental Protection Agency (U.S.E.P.A) estimated that 10% of freshwater sediments in the United states were sufficiently contaminated to pose a significant health risk to fish, wildlife and humans (U.S.E.P.A 1997). In Europe the issue of sediment contamination is less well defined as a result of non-standardised assessment techniques and monitoring programmes between

individual countries (Fleming *et al.* 1997). The incoming European Union Water Framework Directive (WFD) seeks to manage aquatic resources on a river basin scale. It has therefore been suggested that the current period represents a very good opportunity to integrate sediment assessment and management approaches across Europe (European Sediment Research Network 2004).

In order to make decisions regarding the ecological risks posed by sediment-associated chemicals, environmental managers require robust, ecologically relevant and cost-effective risk assessment tools. Most sediment monitoring programmes and site-specific assessments use chemical analysis, invertebrate community surveys and laboratory-based toxicity bioassays, either singularly or in combination (Burton 1991). The following section briefly reviews each method in turn, prior to moving on to the reasoning behind the development of *in situ* bioassay techniques.

1.2 ESTABLISHED SEDIMENT ASSESSMENT TECHNIQUES

1.2.1 Chemistry-based assessments and sediment quality values

Definitive chemical targets as a surrogate measure of environmental "quality" have long been a favourite tool of environmental scientists and regulators (Maher *et al.* 1999). They are attractive for a number of reasons, not least because they offer a clearly defined target for monitoring and compliance. Within the field of sediment ecotoxicology the development of chemical "benchmarks" or sediment quality values (SQV's) (Chapman *et*

al. 2001) has received a good deal of attention in recent years (U.S.E.P.A 1998;Macdonald *et al.* 2000). While there is considerable variation between individual approaches, chemical-specific SQV's are usually derived from a combination of chemical partitioning models and laboratory-based toxicity studies using representative benthic invertebrates (Macdonald *et al.* 2000). This data is then used to predict the effects of a given level of chemical contaminant measured in field-collected samples (Macdonald *et al.* 2000). If a pre-determined SQV is exceeded the sediment sample is predicted to be harmful to resident biota. If deemed necessary further laboratory toxicity tests may then be carried out to further "validate" predictions.

The scientific defensibility of the SQV approach has recently been questioned for a number of reasons (Chapman *et al.* 2001;Burton 2001). Firstly, the relation between chemical concentration in the sediment and impacts on ecological quality is complex and site-specific. For instance, the fact that a SQV for a given chemical is exceeded does not necessarily result in significant biological harm, as a result of limited contaminant bioavailability e.g (Paine *et al.* 1996). Models of chemical partitioning behaviour between water and important binding sites in the sediment e.g organic carbon (Ditoro *et al.* 1991) are often used in SQV techniques, in order to predict the proportion of a chemical that will be bioavailable to organisms. However, numerous studies have demonstrated that the bioavailability of sediment-associated chemicals is determined by a large number of additional chemical, physical and biological factors that are not considered with chemical partitioning models (e.g Boese *et al.* 1990;Kaag *et al.* 1997;Loonen *et al.* 1997;Forbes *et al.* 1998;Selck *et al.* 1998;Lee *et al.* 2000).

The SQV approach is also reliant on the use of laboratory toxicity test data to derive effect concentration estimates, which as discussed below have their own significant limitations (Burton 1999). Finally, chemical-specific SQV's are problematic because most chemicals occur in the environment as complex mixtures, which may act additively, antagonistically or synergistically with one another. With the above limitations in mind it has been suggested that SQV's should not be used as a stand-alone tool for decision-making (Chapman *et al.* 2001;Burton 2001).

1.2.2 Macro-invertebrate community analysis

Ecological assessments of sediment toxicity have commonly used resident biota as an indicator of sediment quality (La Point and Fairchild 1992). Although a variety of indicator species/groups have been developed, benthic macro-invertebrates represent the most commonly used fauna (Rosenberg *et al.* 1997). Benthic species are found in close association with sediments for most of their life cycle, which means they are particularly susceptible to the effects of sediment contamination (La Point and Fairchild 1992). As individual species and/or families vary in their sensitivity to pollution, variations in abundance may be used to integrate the biological effects of pollution over time. As a group benthic macro-invertebrates fulfil a number of important roles in ecosystem functioning, and also form a very significant component of aquatic food webs. Therefore, the results of such assessments are both biologically interpretable and ecologically relevant (Clements 1997).

A variety of approaches have been developed for surveying and analysing macro-invertebrate communities. For example, in the United Kingdom the standardised method that is used for general water quality monitoring is the River Invertebrate Prediction and Classification System (RIVPACS) (Wright *et al.* 1993). RIVPACS is a multivariate technique that combines measures of species/family abundance with habitat data, in order to account for the impact of site-specific characteristics on macro-invertebrate distributions patterns. By using an extensive reference site database RIVPACS provides a means of identifying potentially contaminated sites that deviate from the predicted community composition (Wright *et al.* 1993).

Despite their widespread use in water quality monitoring, benthic community analysis techniques are subject to a number of important limitations related to their discriminatory power. For example, the macro-invertebrate community at a particular site may be significantly impacted when compared to reference conditions, but without additional lines of evidence it is difficult to discriminate between the effects of water column and sediment pollution. This problem may be particularly acute in flowing waters, where water column pollution tends to be more periodic in nature and therefore potentially difficult to detect with chemical screening of water samples (Tucker and Burton 1999).

The discriminatory power of benthic community analysis is also very much dependent on how well responses due to chemical contamination can be distinguished from those due to site-specific environmental conditions (Rosenberg *et al.* 1997). For example, benthic

species distributions may vary significantly in response to seemingly minor changes to sediment particle size, texture and organic content (La Point and Fairchild 1992). In addition, the effects of high/low flow periods and natural seasonal variation in recruitment patterns may also be very difficult to control for when using benthic community surveys (Power *et al.* 1988). Finally, on a more practical note spatial heterogeneity in depositional areas is often high, which may necessitate the use of large numbers of samples in order to properly account for the variability in macro-invertebrate distributions (Rosenberg *et al.* 1997). This may in turn increase the costs associated with an individual site assessment.

1.2.3 Laboratory-based invertebrate bioassays

The development and standardisation of laboratory-based bioassays has been a major focus of sediment ecotoxicology research (Power and Chapman 1992). A wide variety of bioassays (i.e species, endpoints) have been developed previously (Burton 1991). Most standardised techniques to date involve the use of single-species tests with representative benthic macro-invertebrates e.g (U.S.E.P.A 2000;O.E.C.D 2001). As highlighted above, benthic macro-invertebrates are in close association with sediments for most of their life cycle, and can thus be employed in the development of ecologically relevant sediment assessment tools. During laboratory toxicity tests organisms are exposed to field-collected or artificially-“spiked” sediments under controlled conditions, thereby allowing for the separation of toxicant effects from additional environmental variables. Laboratory bioassays are also relatively rapid and straightforward, which enables the processing of a

large numbers of samples in a short space of time, thereby reducing the associated costs (Ankley 1997).

A principle criticism with laboratory bioassays is their lack of ecological realism (Burton 1995). Organisms in the field are naturally exposed to variable abiotic and biotic conditions that may interact with the effects of sediment-associated contaminants e.g pH, dissolved oxygen. Laboratory bioassays are simply unable to integrate all of the possible interactions that can take place. For example, the toxicity of sediment-associated polycyclic aromatic hydrocarbons (PAH's) was enhanced *in situ* as a result of exposure to ultra-violet light (Monson *et al.* 1995), yet standard laboratory lighting does not typically produce the appropriate wavelengths that are required for photo-activation of PAH's (U.S.E.P.A 2000).

Laboratory bioassays are also subject to a number of important methodological uncertainties. During field collection naturally-occurring physical and chemical gradients that occur in sediments may be destroyed (Luther 2001), thereby altering contaminant partitioning, bio-availability and toxicity e.g (Sasson-Brickson and Burton 1991;Pereira *et al.* 2000). Most benthic communities reside in the thin upper layer of substrate and if the source of pollution has ceased this top layer may be relatively clean (Chapman *et al.* 1992). Under these circumstances the benthic community may actually be less exposed to toxicants than would be predicted by disruptive field sampling and subsequent laboratory testing. In addition, the individual methods used to collect and process sediments prior to testing may further influence contaminant bio-availability and toxicity (Stemmer *et al.* 1990;West *et al.* 1994;Defoe and Ankley 1998;U.S.E.P.A 2001;Van Hoof *et al.* 2001).

When combined together the artefacts associated with field collection and sample processing in the laboratory may result in sediment that bears little or no resemblance to the substrate found *in situ*.

1.2.4 Integrated "weight of evidence" approaches

Recognition of the uncertainties associated with each single assessment methodology described previously has led to the formalisation of integrated, "weight of evidence" frameworks for sediment assessment e.g sediment quality "triad" (Chapman 2000;Burton *et al.* 2002). In this approach no single line of evidence determines decision-making; rather chemical measures, biological community analysis and bioassays are used in combination, as each method may provide potentially unique information (Burton *et al.* 2002). The development of integrated assessments was a key recommendation from a recent European Union technical working group that was established to address the issue of contaminated sediment assessment and management across the European Union (European Sediment Research Network 2004). While an integrated approach seems to provide a logical progression from over-reliance on any one technique, the success of such an approach will still be dependent on the usefulness of the information gained from the individual techniques that are used. For instance, when referring to bioassay techniques, Chapman *et al* (1997) emphasised the need to incorporate more ecologically-relevant toxicity measurement endpoints into the sediment quality triad as they are developed.

1.3 FIELD (*IN SITU*) BIOASSAYS

As highlighted previously in Section 1.2.3, natural field conditions are difficult to replicate in the laboratory. Therefore, a logical extension of laboratory toxicity testing was to develop bioassay methods that are suitable for use in the field (*in situ*). The development of *in situ* bioassay techniques has become a very active area of research in aquatic ecotoxicology, and has been extensively reviewed by Chappie and Burton (Chappie and Burton 2000). Bioassay organisms are supplied from laboratory cultures or collected from uncontaminated field sites, prior to deployment at reference and impacted sites. Depending on the specific test design organisms may be exposed *in situ* singularly or in groups with the aid of relatively simple, non-standardised holding chambers. Table 1.1 contains examples from previous studies with *in situ* bioassay techniques. This is by no means a complete review, but is rather an attempt to highlight the diversity of species, bioassay endpoints and contaminant exposure routes that have been used previously.

Table 1.1 Examples of previous *in situ* bioassay studies that have used a variety of test species, contaminant exposure routes and bioassay endpoints

Test organism	Exposure route	Bioassay endpoint	Reference
Phytoplankton, *Chlorella vulgaris*	Water column	Growth	(Moreira-Santos *et al.* 2004)
Seaweed, *Fucus vesiculosis*	Water column	Growth	(Telfer and Wilkinson 1997)
Zooplankton, *Daphnia magna*	Water column	Survival and Post exposure feeding rate	(Mcwilliam and Baird 2002b)
Mussel, *Mytilus edulis*	Water column	Survival	(Warren *et al.* 1995)
Mussel, *Mytilus edulis*	Water column	Filtration rate, oxygen uptake, nutrient absorption efficiency and growth	(Widdows and Page 1993)
Amphipod, *Gammarus pulex*	Water column	Survival and Feeding rate	(Crane and Maltby 1991)
Midge larvae, *Chironomus riparius*	Water column and sediment combined	Survival and Growth	(Sibley *et al.* 1999)
Midge larvae, *Chironomus riparius*	Sediment only	Acetylcholinesterase and glutathione S-transferase activity	(Olsen *et al.* 2001)
Amphipod, *Hyalella azteca*	Water column, against sediment surface, sediment	Beta-galactosidase activity	(Hatch and Burton 1999)
Speckled sanddab, *Citharichthys stigmaeus*	Water column	Mixed function oxidase	(Rice *et al.* 1994)
Brook trout, *Salvelinus fontinalis*	Water column	Survival	(Simonin *et al.* 1993)

1.3.1 Pros and cons with *in situ* bioassays for sediment assessment

There are a number of potential benefits associated with the use of *in situ* bioassays for sediment assessments. Firstly, environmental realism will be improved by exposing organisms to naturally varying field parameters such as pH, dissolved oxygen, nutrients, photoperiod, ultra violet light intensity, temperature and hydrological conditions (Chappie

and Burton 2000). Through the use of different tests species and chamber designs it is also possible to study exposure-response relationships under more realistic field conditions. For example, benthic species can be used to study the toxicity of sediments separately from the overlying water e.g (Crane *et al.* 2000). *In situ* exposures can also avoid artefacts associated with field collection and processing of sediment samples for laboratory bioassays (Burton 1995;U.S.E.P.A 2001).

As with all experimental studies there are a number of potential artefacts associated with the use of *in situ* bioassays (Chappie and Burton 2000). Principle concerns include the effects of transporting animals to field sites and the rapid change in environmental conditions between the laboratory and field. Containing organisms in cages or chambers during the exposure period may also reduce water exchange (and hence water quality), increase sedimentation and alter food availability. The presence of indigenous biota may also alter the effects of toxicants on bioassay organisms (Reynoldson *et al.* 1994). Some of these issues may be controlled via the use of appropriate reference performance and acclimation periods, although caging artefacts are to some extent inevitable (Chappie and Burton 2000). Finally, equipment loss or damage due to flooding and vandalism may also significantly disrupt deployments. Despite the above concerns it is suggested that on balance the improved realism that is gained with *in-situ* bioassays may outweigh their limitations (Chappie and Burton 2000).

A number of previous studies have directly compared the responses of *in situ* and laboratory sediment bioassays. For example, enhanced toxicity in a laboratory bioassay was

suggested to be a result of increased contaminant bioavailability, as a result of the disruption of field sediments during collection and processing (Sasson-Brickson and Burton 1991). A study with the juvenile clam, *Mercenaria mercenaria* found evidence of enhanced toxicity *in situ*, which was suggested to be a result of the interactive effects of contaminants and site-specific habitat characteristics (Ringwood and Keppler 2002). In a third study the differences in laboratory and *in situ* comparisons were site-specific (Tucker and Burton 1999). For example, toxicity of urban wastewater was enhanced in the laboratory when compared to *in situ* exposures, whereas *in situ* toxicity at an agricultural site was enhanced when compared to laboratory exposures. These studies highlight that valuable information that can be gained from the use of *in situ* techniques.

1.3.2 Single species *in situ* bioassays and sub-lethal endpoints

The effects of toxicant "stress" may be manifested at all levels of biological organisation (Maltby 1999). Experiments using measurements on individual organisms are the emphasis of most standardised laboratory sediment bioassays e.g (U.S.E.P.A 2000;O.E.C.D 2001). Individual endpoints are easier to measure when compared to population and/or community-level endpoints. In addition, individual effects may be manifested more rapidly when compared to effects on populations or communities, so they may act as an early warning signal of biological impairment (Maltby 1999). Lethality has commonly been used as an endpoint for *in situ* bioassays previously e.g (Ireland *et al.* 1996;Schulz 2003;Phillips *et al.* 2004). However, sub-lethal responses to toxicant stress are also common and may indicate the impairment of important organism functions prior to

death (Walker *et al* 2001). Further, contaminant concentrations found in field situations are often quite low, so bioassays that rely solely on lethality may miss potentially important sub-lethal effects (Chapman 1989).

A number of sub-lethal endpoints have previously been developed with *in situ* macro-invertebrate bioassays. These include reproduction (Pereira *et al.* 2000), growth (Sibley *et al.* 1999), "scope for growth" (Maltby *et al.* 1990b;Widdows *et al.* 1995), morphological deformities (Meregalli *et al.* 2000) and feeding rate (Crane and Maltby 1991). Changes in organism behaviour as a result of toxicant stress integrate complex sub-lethal changes to biochemical and physiological functions (Atchinson *et al.* 1996). Although many animal behaviours may be susceptible to the effects of toxicants, because of its importance to individual growth and development most work has been carried out with foraging (Walker *et al* 2001).

1.3.3 Feeding inhibition as a bioassay endpoint

Feeding inhibition is suggested to be a general response to toxicant stress. Table 1.2 below contains examples of studies where toxicant-induced feeding inhibition has been found previously. This list is by no means complete, but is rather used to highlight that feeding inhibition can occur in a diverse collection of aquatic organisms as a result of exposure to a wide variety of contaminant classes commonly found in aquatic systems. Feeding inhibition may occur over relatively short time frames, thereby allowing for a more rapidly measured response when compared to effects on growth, fecundity and survival.

This point may be particularly relevant for *in situ* bioassays, where time spent in the field is an important consideration.

Table 1.2 Examples of previously published studies on toxicant-induced feeding inhibition with a variety of aquatic organisms, toxicants and feeding behaviours

Organism	Toxicant	Feeding behaviour	Reference
Rainbow trout, *Oncorhynchus mykiss*	Various Metals	Prey strikes per minute	(Woodward *et al.* 1995)
European frog, *Rana esculenta*	Fungicide	Time spent foraging	(Semlitsch *et al.* 1995)
Gastropod, *Potamopyrgus antipodarum*	Cadmium	Egestion rate	(Jensen *et al.* 2001)
Bivalve, *Mytilus edulis*	Insecticide	Filtration rate	(Donkin *et al.* 1997)
Cladoceran, *Daphnia magna*	Various metals and organics	Ingestion rate	(Mcwilliam and Baird 2002a)
Oligochaete, *Limnodrilus Hoffmeisteri*	Polycyclic aromatic hydrocarbons (PAH's)	Egestion rate	(Lotufo and Fleeger 1996)

Feeding inhibition can also be a very ecologically relevant bioassay endpoint. The acquisition of nutritional resources through feeding is of fundamental importance to individual organism energetic budgets. Toxicant-induced feeding inhibition may reduce individual growth and development rates, fecundity and survival, which in turn may cause changes to the dynamics of populations (Calow and Sibly 1990;Allen *et al.* 1995). Observed reductions in feeding rates may be used to predict effects on individual growth and reproductive performance, which may then be incorporated into population models in order to predict effects on population dynamics (Calow and Sibly 1990). Predictions from population models may then be compared to distributions of the same species under similar levels of contamination *in situ* e.g (Klok and De Roos 1996). Therefore, an energetics-

based approach provides a very useful link between the individual-level effects of feeding behaviour and its potential consequences at the population level.

Along with direct effects on individual energetic budgets feeding inhibition of a particular species or functional group may also have important consequences for wider aquatic ecosystem functioning. For example, bacterial grazing is important in the cycling of nutrients, and algal grazing may be key to the maintenance of light conditions that are conducive to the growth of submerged macrophytes (Baird *et al.* 2001). The effect of toxicant-induced feeding inhibition of the phytoplankton grazer, *Daphnia magna*, was to cause an increase in phytoplankton biomass in a model ecosystem study (Slijkerman *et al.* 2004). The inhibition of *Gammarus pulex* feeding behaviour *in situ* (an amphipod leaf "shredder") was also correlated with a reduction in the rate of *in situ* leaf decomposition and the diversity of the macro-invertebrate community (Maltby *et al.* 2002). Therefore, feeding inhibition of individuals within a population can also be linked to effects on key ecological processes.

1.3.4 Feeding inhibition as an *in situ* bioassay endpoint

The feeding rates of a number of representative macro-invertebrate species have been successfully developed as *in situ* bioassay endpoints previously (Crane and Maltby 1991;Crane *et al.* 1995;Hatch and Burton 1999;Mcwilliam and Baird 2002b;Crichton 2003). However, so far this approach has not been extended to a true deposit-feeding organism. Since the emphasis of this work was to develop an *in situ* bioassay to assess

sediment contamination, selection of a deposit feeding organism that spends most or all of its life in intimate contact with sediment through burrowing and feeding will arguably improve the overall relevance of the bioassay.

1.4 SELECTION OF FEEDING INHIBITION OF *Lumbriculus variegatus* AS AN *IN SITU* BIOASSAY ENDPOINT

A wide variety of benthic macro-invertebrates have been selected for sediment toxicity studies previously (Burton *et al.* 1992). The use of the freshwater oligochaete, *Lumbriculus variegatus* has a relatively long history e.g (Bailey and Liu 1980). Sub-lethal toxicity endpoints that have been developed in previous laboratory studies with *L.variegatus* include growth (Fisher *et al.* 1999), reproduction (Nebeker *et al.* 1989;Chapman *et al.* 1999;Fisher *et al.* 1999), sediment avoidance behaviour (West and Ankley 1998), metabolic rate (Penttinen and Kukkonen 2000), escape reflex (Drewes 1997) and feeding rate (Landrum *et al.* 2002;Landrum *et al.* 2004a;Landrum *et al.* 2004b). For the current work, selection of *L.variegatus* and the use of feeding behaviour as a bioassay endpoint were based on the fulfilment of a number of important criteria discussed below.

1.4.1 Basic ecology

L.variegatus is a member of the Lumbriculid family of freshwater oligochaetes. It is a widely distributed species across Europe and North America (Brinkhurst and Jamieson 1971). *L.variegatus* has previously been found at densities of up to 11,000 individuals/m$^{2\ -1}$

(Cook 1969) in a wide variety of aquatic habitats including small, oligotrophic streams (Cook 1969), peat marshes (Healy 1987), agricultural drainage streams (Verdonschot 1999) and man-made trout farm ponds (Wahab 1986).

As a group the oligochaetes are an important component of freshwater food webs, both as consumers of periphyton, detritus and the associated micro-fauna (Brinkhurst *et al.* 1972) (Chauvet *et al.* 1993;Brust *et al.* 2001), and also as prey species for a variety of invertebrates, fish and waterfowl (Zaranko *et al.* 1997;Wallace *et al.* 1998;Marsden and Bellamy 2000). Oligochaetes also form an important link in the food chain transfer of persistent environmental contaminants (Egeler *et al.* 2001;Wallace *et al.* 1998).

Reproduction with *L.variegatus* is most commonly via asexual fission or architomy, and is suggested to be controlled by the size of individual worms (Christensen 1984;Cook 1969). Architomy results in small body fragments that survive and readily regenerate missing head or tail sections, thus forming a new individual. The advantages of this form of reproduction may include surviving predatory attack (Drewes and Fourtner 1990) and rapid exploitation of available food resources (Christensen 1984;Cook 1969).

No previously published studies are available on the diet of *L.variegatus*, but like most oligochaetes it probably feeds on a diverse mixture of small food particles that accumulate in benthic environments (e.g live algae, decaying plant material, bacteria and fungi) (Brinkhurst and Jamieson 1971). When inhabiting soft substrates *L.variegatus* burrows into the sediment and feeds in a head-down fashion. Sediment is ingested, the digestible portion

is assimilated, and the undigested remainder is egested onto the sediment surface as faecal pellets (Appleby and Brinkhurst 1971). Because feeding rates are relatively high the so-called "conveyor-belt" feeding exhibited by many oligochaetes (including *L.variegatus*) results in the regular reworking of the top layer of sediment, which can have profound effects on the properties of sediments and overlying waters (Robbins 1982).

1.4.2 Ecological relevance of *L.variegatus* feeding inhibition

The inhibition of *L.variegatus* feeding behaviour is suggested to be an ecologically relevant bioassay endpoint for a number of reasons. Firstly, feeding inhibition is likely to have a direct impact on individual energetic budgets, which may then be translated into reduced vital rates of individual organisms (i.e growth, fecundity and survival). A reduction in any one of these rates may then be subsequently expressed as a reduction in population biomass. Since oligochaetes are an important component of food webs, a reduction in population biomass may have important implications for the transfer of energy from detritus-based food webs to higher levels of the food chain.

Toxicant-induced feeding inhibition of *L.variegatus* may also have wider impacts on ecosystem function. The sediment reworking behaviour of oligochaetes has previously been shown to cause significant changes to the biological, chemical and physical characteristics of sediments and overlying waters (McCall and Tevesz 1982). For example, oligochaetes have previously been demonstrated to alter organic matter processing rates, nutrient availability, solute transport, pH and redox potential profiles within sediments (Davis

1974;Chatarpaul *et al.* 1980;Fisher and Matisoff 1981;Chauvet *et al.* 1993;Wang and Matisoff 1997;Mermillod-Blondin *et al.* 2000). Physical disruption of the sediment as a result of oligochaete feeding behaviour has also been correlated with changes to the distribution and bioavailability of contaminants within sediments and overlying waters (Karickhoff and Morris 1985;Reible *et al.* 1996;Peterson *et al.* 1996).

Finally, the burrowing and feeding behaviour of *L.variegatus* brings it into contact with all three potential routes of exposure for sediment-associated toxicants (i.e ingestion of particulates, sediment pore water and overlying water). Ingestion of contaminated sediment by *L.variegatus* has also been shown to increase the uptake and accumulation of hydrophobic toxicants when compared to water-only exposures (Leppanen and Kukkonen 1998b). Therefore, changes to *L.variegatus* feeding rate may alter the degree of contaminant exposure and uptake, and thus susceptibility to toxic effects and bioaccumulation potential.

1.4.3 Practical considerations with *L.variegatus*

Starter cultures of *L.variegatus* may be easily sourced from a number of organisations in Europe and North America. Culture techniques that produce sufficient quantities of healthy *L.variegatus* individuals are relatively simple to establish and maintain using inexpensive, non-specialised equipment and techniques e.g (Phipps *et al.* 1993;U.S.E.P.A 2000). *L.variegatus* is also large enough to provide sufficient tissue mass for chemical analysis if required and its size permits easy handling and separation from sediments. The

fact that *L.variegatus* has been recorded in a variety of aquatic habitats suggests that it is tolerant of a range of environmental conditions, which may be particularly useful when deploying the animal *in situ*.

1.4.4 Measuring the feeding behaviour of *L.variegatus in situ*

The feeding behaviour of *L.variegatus* has previously been used as a sub-lethal response to toxicants, but only in laboratory-based bioassays (Landrum *et al.* 2002;Landrum *et al.* 2004a;Landrum *et al.* 2004b). In order to carry out an exposure to *in situ* sediments, *L.variegatus* must be held within some form of holding chamber. This technique has been successfully developed with *L.variegatus* in a number of previous studies that have employed survival and bioaccumulation as endpoints (Monson *et al.* 1995;Sibley *et al.* 1999;Greenberg *et al.* 2002). However, the measurement of *in situ* feeding behaviour that is proposed in the current work presents some difficulty. As previously described, *L.variegatus* is likely to feed on a mixture of detritus and sediment. Therefore, it would be very difficult to measure the rate of ingestion of this material under field conditions. Likewise, measuring the amount of material egested by the worms is also problematic, as it would not be possible to determine the faecal pellets that had originated from *L.variegatus* versus those produced by indigenous invertebrates.

When the cladoceran, *D.magna* and the freshwater snail, *Lymnaea peregra* were exposed to a variety of toxic chemicals in the laboratory, their feeding behaviour was found to be significantly inhibited during a subsequent post-exposure period under clean

conditions (Taylor *et al.* 1998;Villarroel *et al.* 1999) (Mcwilliam and Baird 2002a) (Crichton *et al.* 2004). Post-exposure feeding inhibition of both species has since been successfully used as a bioassay endpoint in artificial ecosystems (McWilliam 2001;Crichton 2003) (Slijkerman *et al.* 2004) and with *in situ* bioassays, where feeding behaviour could not otherwise be measured directly (Mcwilliam and Baird 2002b;Crichton 2003). The use of a post-exposure measurement could therefore provide a means of using *L.variegatus* feeding behaviour as an endpoint with an *in situ* sediment bioassay. *L.variegatus* could be exposed to field sediments for a given period, and then immediately transferred to uncontaminated sediment in beakers in order to measure post-exposure feeding rate over a defined period.

1.5 BIOASSAY DEVELOPMENT CRITERIA

The successful development of a novel post-exposure feeding bioassay with *L.variegatus* will be dependent on the relevance, reliability and cost-effectiveness of the technique (Calow 1996).

1.5.1 Bioassay relevance

If the primary goal of sediment assessments is to protect ecological integrity, the ecological relevance of any assessment technique should be a priority (Clements 1997). To be ecologically relevant the selected species should be sensitive to contaminants, or representative of an important trophic group that contributes to the functional properties of

the system that is being studied. It should also be possible to extrapolate the effects of the bioassay endpoint to effects at higher levels of organisation i.e populations, communities (Calow 1989). Along with ecological relevance, the sociological relevance of bio-assessment techniques is also significant. For example, the importance of a functioning benthic invertebrate community may be easier to convey if their role as a food source to recreational fish species is highlighted (Clements 1997).

1.5.2 Bioassay reliability

The reliability of a bioassay technique is defined as "observations made in a controlled way and interpreted with confidence" (Calow 1996). Important points include the ability to provide a regular supply of bioassay organisms that are of a consistent physiological quality. Providing organisms of consistent quality will avoid confounding bioassay results with variation in the response that is solely due to organism condition. The bioassay endpoint should also respond in a reproducible fashion under controlled environmental conditions. This may be tested with appropriate controls and regular reference toxicity testing (U.S.E.P.A 2000). Reliability also refers to the ability of the bioassay to detect the effects of a variety of toxicants that are commonly found in the environment. If the bioassay is only responsive to certain classes of contaminants its wider applicability may be limited. Finally, variation in the bioassay endpoint that is due to naturally varying environmental parameters should be kept to a minimum, in order to avoid masking the bioassay "signal" with environmental "noise".

1.5.3 Cost and timing

The costs associated with a bioassay technique are largely dependent on the equipment, facilities, time (i.e labour) and skill level required. A test procedure that employs little or no specialised equipment that is carried out over relatively short time periods is desirable. However, there is always likely to be some form of trade-off between costs and timing, and the relevance and reliability of the bioassay.

1.6 THESIS AIMS

The purpose of this thesis was to develop and test an *in situ* sediment bioassay using the post-exposure feeding behaviour of *L.variegatus* as the bioassay endpoint. The specific aims for each chapter of work were as follows:

- Chapter 2 – To develop a consistent and reliable methodology for measuring post-exposure feeding behaviour of *L.variegatus* with uncontaminated conditions in the laboratory
- Chapter 3 – To test the sensitivity and reproducibility of post-exposure feeding inhibition after exposure to a variety of sediment-associated contaminants in the laboratory
- Chapter 4 – To adapt and test the bioassay for *in situ* use during exposures at a variety of field sites

- Chapter 5 – To compare short-term measures of toxicant-induced feeding inhibition with longer-term effects on *L.variegatus* populations

2 CHAPTER 2 - GENERAL METHODS AND BIOASSAY DEVELOPMENT

2.1 INTRODUCTION: GENERAL METHODS

Prior to carrying out experimental work to develop post-exposure feeding inhibition as a bioassay endpoint a reliable supply of test organisms was required. As a result of the widespread use of *L.variegatus* in bioaccumulation testing, simple and reliable methods for culturing have been developed and widely used (Phipps *et al.* 1993;U.S.E.P.A 2000). These methods can be adapted to the facilities that are available within a particular laboratory, as they require no specialist equipment. During distinct stages of this work three slightly different methods were used for culturing *L.variegatus*. The following general methods section will describe each of these in chronological order.

Sediment was required as a feeding substrate at all stages of this work in order to carry out bioassay development (this Chapter), laboratory toxicity tests (Chapter 3), *in situ* bioassays (Chapter 4), and population studies (Chapter 5). Previous sediment toxicity studies have commonly used field-collected samples. Principal limitations to their use include the potential for pre-existing contamination, the presence of indigenous organisms, variability in their physico-chemical characteristics, year-round collection problems and changes in sediment physico-chemistry during storage (Kemble *et al.* 1999).

As an alternative to field sediments, formulated or artificial sediments have been developed for toxicity and bioaccumulation testing (Suedel and Rodgers 1994;Harrahy and

Clements 1997) (Kemble *et al.* 1999;Ribeiro *et al.* 1999). These consist of defined quantities of reference materials that provide a more standardized and reproducible substrate. However, because the substrate is artificial, chemical binding phases that play an important role in contaminant bio-availability in natural sediments may be reduced or absent, which may lead to enhanced contaminant bio-availability (Harrahy and Clements 1997;Chapman *et al.* 1999). Conditioning or aging artificial sediment prior to use may at least partly address the problem of enhanced bioavialbility, but this process will require more time to carry out (Kemble *et al.* 1999).

In consideration of the above points it was decided to use field-collected sediments throughout this work in order to try to more closely approximate a natural substrate. As this required the collection and processing of sediment at each stage of this project, details of the three slightly different methods that were used are described in the following general methods section.

2.2 AIMS: GENERAL METHODS

The aims of this section were:

- To describe the methods for culturing and maintaining *L.variegatus* under laboratory conditions
- To describe the methods for the collection and processing of field sediments that were used in laboratory and field studies

2.3 MATERIALS AND METHODS: GENERAL METHODS

2.3.1 Maintenance of *L. variegatus* cultures

2.3.1.1 Stirling 2002-2003 (Chapters 2 and 3)

Cultures were established at Stirling University in January 2002 with approximately 300 *L.variegatus* individuals from a culture held at WRc, Medmenham, United Kingdom. This culture had been held on-site since 1997 from a stock originating from the United States Environmental Protection Agency (U.S.E.P.A), Duluth (A.Conrad, *pers comm*). The methods that were used for culturing worms were adapted from previously published work (Phipps *et al.* 1993;U.S.E.P.A 2000). Duplicate static cultures were held in 15 L plastic aquaria containing hard artificial water (A.S.T.M 1980; Table 2.1) and a 1cm layer of shredded brown paper towel substrate (unbleached, non-dyed, Lotus professional range). A 1g ration of finely ground Tetramin fish food (Tetrawerk, Germany) was added to each aquarium three times per week. It was found that using a static culture system necessitated closely balancing the quantities of food required to produce sufficient animals for testing with maintenance of overlying water quality.

Complete water exchange was carried out prior to the addition of food, and the water was continually aerated. Cultures were maintained at $20^{o}C \pm 0.5^{o}C$ using a constant temperature room with a 12:12 light:dark photoperiod. Once a month each culture was split

approximately in half, one half was discarded while the other half was added to a fresh layer of paper towel substrate. Regular harvesting of worms and replacement with fresh substrate promoted better individual condition and population growth. Under these conditions estimated population doubling rate was approximately 35-40 days.

Table 2.1 Analar-grade salt solutions used to make 10L of hard ASTM artificial water for culturing *L.variegatus* in Stirling 2002-2003

Salt	Stock concentration	Volume of stock to make 10 L hard ASTM
KCL	3.2 g/L^{-1}	25 ml
$MgSO_4.7H_2O$	49.1g/L^{-1}	50 ml
$NaHCO_3$	38.4g/L^{-1}	50 ml
$CaSO_4.2H_2O$	9.82g/L^{-1}	500 ml

2.3.1.2 New Brunswick 2003 (Chapter 4)

A culture was established at the University of New Brunswick, Fredericton, New Brunswick in May 2003 with approximately 1000 *L. variegatus* individuals purchased from Aquatic Research Organisms (Hampton, NH, U.S.A). Animals had been held on-site since 1986 and were originally supplied by the Eastman Kodak Corporation (Rochester, NY) and identified by Dr.R.O Brinkhurst (A.R.O, *pers comm*). After previously using a static system for culturing animals in Stirling during 2002-03, it was decided to construct a simple pre-heated flow-through system using a supply of de-chlorinated municipal water. The flow through system provided better water quality, which in turn allowed for increased feeding levels and therefore resulted in a more plentiful supply of worms.

Duplicate 20 L aquaria were used to hold worms and the same paper towel substrate was used as in Section 2.3.1.1. Water was supplied at a flow rate of approximately 130 ml/min from a pre-heated 100 L header tank ($20^{o}C \pm 1.0^{o}C$, 300 Watt heater). Additional aeration was used to ensure proper mixing in the header tank and the aquaria. Cultures were fed 3 times per week with 2g of finely ground Tetramin fish food. At the time of feeding water supply was diverted for 60 minutes in order to let the added food settle out. Cultures were held in a constant temperature room at $20^{o}C$ under a 12:12 light:dark photoperiod. Substrate and worms were harvested once a month and substrate was renewed as in Section 2.3.1.1. Under these conditions estimated population-doubling rate was approximately 14 days.

2.3.1.3 Stirling 2003-2004 (Chapter 5)

Cultures were established in November 2003 with approximately 300 worms originating from the culture maintained in New Brunswick during 2003. A flow-through system was used to supply pre-heated ($20^{0}C \pm 0.5^{o}C$), de-chlorinated tap water at a flow rate of 100 ml/min to a single 40L aquarium. Feeding and substrate renewal were carried out in the same way as that described in Section 2.3.1.1 and cultures were held under a 12:12 photoperiod. Under these conditions population-doubling rate was approximately 18 days.

2.3.2 Water quality analysis of cultures

2.3.2.1 Stirling 2002-2003 (Chapters 2 and 3) and 2003-2004 (Chapter 5)

Dissolved oxygen, pH, and conductivity of culture water were measured weekly using a WTW Multiprobe 340i portable meter (WTW, Weilheim, Germany). Triplicate samples of culture water were taken for analysis of total ammonia and total hardness 8 weeks after cultures were established. Total hardness was determined using a standard titration method with ethylenediaminetetra-acetic acid disodium salt (EDTA, Stirling 1985).

Total ammonia was determined using a colormetric Berthelot reaction method supplied by Bran-Luebbe (autoanalyser manufacturer). The blue-green complex formed from the reaction of reagents with ammonia in water samples is enhanced with the use of sodium nitroprusside. Samples were then measured using a Bran Luebbe Autoanalyser 3 (spectrophotometer) using a wavelength of 660nm. This method calculates the total ammonia in a water sample, which is comprised of ionised and unionised ammonia. As unionised ammonia can be toxic to aquatic animals it was this form of ammonia that was of concern (Stirling 1985). The proportion of unionised ammonia was determined by taking the pH and water temperature at the time of sampling and by using Equation 2.1 below:

Equation 2.1 **% unionised ammonia = 100/ [1+antilog (pKa -pH of sample)]**

where pKa = negative logarithm of the ionisation constant, which depends on temperature at the time of sampling (Stirling 1985). This formula calculates the %

unionised ammonia concentration as a proportion of total ammonia, which was subsequently converted to mg/L.

2.3.2.2 New Brunswick 2003 (Chapter 4)

Dissolved oxygen, pH, and conductivity were measured using YSI portable meters (YSI Inc,Yellowsprings, OH) once weekly. Samples of culture water were taken for ammonia and total hardness analysis 8 weeks after cultures were set-up. Total hardness was determined using the method described in Section 2.3.2.1. Total ammonia concentration was determined using a phenol-hypochlorite colormetric method (Stirling 1985). Ammonia in water samples reacts with re-agents to give indophenol blue. The colourmetric reaction is enhanced with the use of sodium nitroprusside. Sample absorbance was determined with a Milton Roy 301 spectrophotometer. Ammonia concentrations of samples were obtained from the fitted absorbance curve of ammonia standard solutions. The proportion of unionised ammonia was determined by measuring pH and temperature at the time of sampling and was then calculated by using Equation 2.1 (Section 2.3.2.1).

Table 2.2 Water quality parameters for cultures held at Stirling and New Brunswick, standard deviations are in brackets, n = 3 unless otherwise indicated

Water quality parameter	Stirling 2002-2003	New Brunswick 2003	Stirling 2003-2004
Total hardness (mg/L^{-1})	152 (5.3)	102 (9.7)	23.2 (0.2)
Total ammonia (mg/L^{-1}) Unionised (mg/L^{-1})	0.82 (0.09) 0.0080 (0.0017)	0.12 (0.01) 0.0011 (0.0004)	0.13 (0.06) 0.0014 (0.0014)
Dissolved oxygen (%)	71 (6.2,n=10)	85 (7.6,n=10)	87 (9.1,n=10)
pH	7.3 (0.41,n=10)	7.3 (0.1,n=10)	6.9 (0.26,n=10)
Conductivity (uS)	196 (44.1,n=10)	261 (29.3,n=10)	67 (13.6,n=10)

2.3.3 Collection of field sediments

2.3.3.1 Stirling 2002-2003 (Chapters 2 and 3)

Sediment for work in Chapters 2 and 3 was collected on the 12th March 2002 from the Goodie River, near Thornhill, Central Scotland (Ordnance Survey grid reference 682977, Figure 2.1). This is a small tributary (<5m channel width) of the Forth river system that largely drains protected marshland. Sediment was collected from the upper 5 cm layer of substrate using an adapted hand-held Ekman-type sediment grab. Sediment was taken to the laboratory and thoroughly homogenized using a cement mixer. Larger particles, debris and macro-organisms were removed by sieving the sediment through a 500μm steel mesh sieve. Sediment was then settled for 24 hours before removing overlying water, prior to distribution to 1L high-density polyethylene bottles in 750g portions. Bottles were stored frozen at -18°C for a period of 4 weeks in order to kill any remaining macro-invertebrates that may have interfered with feeding experiments. Sediment was then stored at 4°C prior to use.

Prior to freezing triplicate sub-samples of sediment were taken for moisture content, particle size, organic carbon and nitrogen analysis. Moisture content was determined by drying sediment at 60°C for 48hrs. Carbon and nitrogen analysis was carried out using a Perkin Elmer series II 2400 CHNS/O elemental analyser. In order to correct for inorganic carbon sub-samples were combusted at 550°C for four hours and then processed along with

un-combusted samples. Sediment was analysed for particle size by placing wet samples in a 63µm sieve, the fraction passing through the sieve was caught in a tray below. This tray was then dried to a constant weight and this portion made up the <63µm fraction. The fraction held within the 63µm sieve was dried for approximately 2 hours at 60°C and subsequently shaken for 10 minutes through 500, 250, 125 and 63µm sieves using a Fritsch Analysette 3 shaker (Oberstein, Germany).

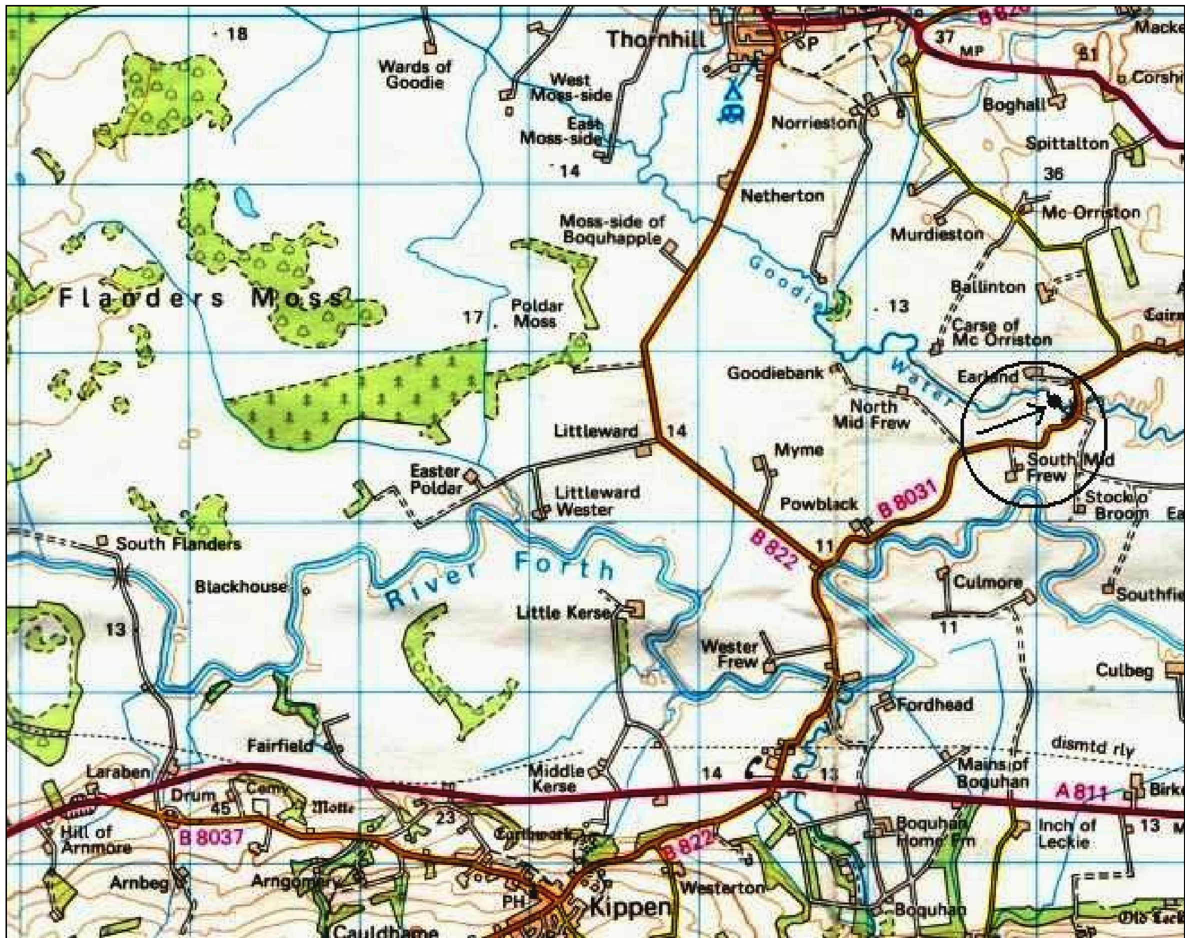


Figure 2.1 Location (marked by arrow) of the sediment collection site on the Goodie River, Forth Valley, Stirlingshire, March 2002, Ordnance Survey national grid reference 682977

2.3.3.2 Stirling 2003-2004 (Chapter 5)

The sediment used in Chapter 5 was collected from the same site on the Goodie River near Stirling on the 22nd November 2003 (Figure 2.1). Identical methods were used to process and characterise the sediment as those described previously in Section 2.3.3.1. Sediment physico-chemical characteristics are given in Table 2.3.

2.3.3.3 New Brunswick 2003 (Chapter 4)

The sediment used as a feeding substrate for *in situ* studies was collected on the 16th May 2003 from Little Magaguadavic Lake, approximately 25 kilometres west of Fredericton, New Brunswick, Canada (Figure 2.2, Canterbury 21G/14 map). The lake was in an isolated location with no obvious sources of pollution in the surrounding area. Sediment was collected from an area approximately 5 meters deep using an Ekman sediment grab. Sediment was then taken back to the laboratory and subsequently processed and stored as described previously in Section 2.3.3.1, except that a cement mixer was unavailable to mix sediment so this had to be carried out by hand. Samples for carbon, nitrogen and particle size analysis were dried and subsequently analysed in Stirling during autumn 2003 using the same methods described in Section 2.3.3.1. Sediment physico-chemical characteristics are given in Table 2.3.

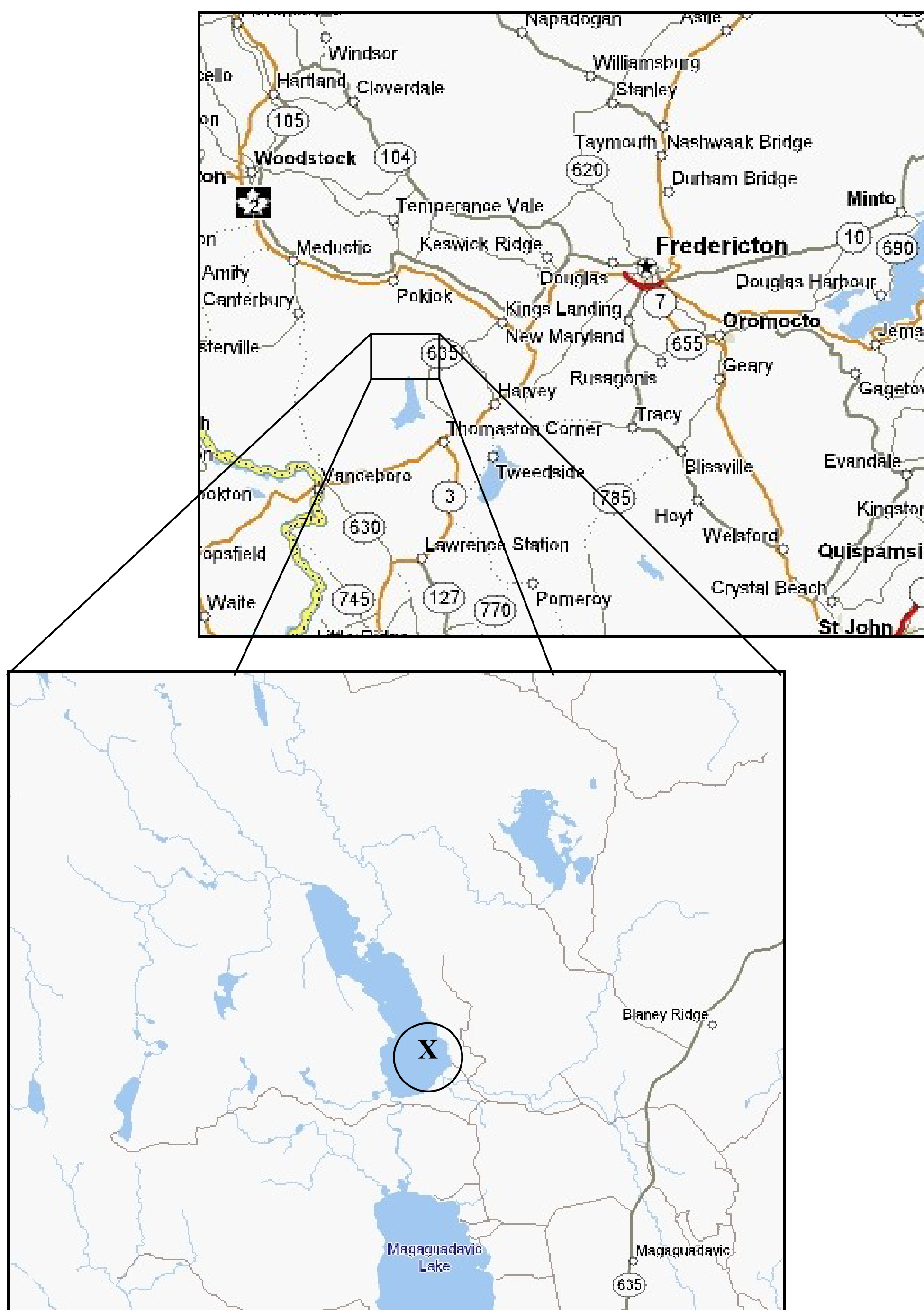


Figure 2.2 Location (x) of the sediment collection site at Little Magaguadavic Lake, nr Fredericton, New Brunswick, Canada, grid reference 45 ° 47.62'N, 67 ° 13.48'W

Table 2.3 Major physico-chemical characteristics of sediments used in distinct sections of this study

	Goodie River, Stirling 2002-2003	**Magaguadavic Lake, New Brunswick 2003**	**Goodie River, Stirling 2003-2004**
Dry matter (%)	52.2 (0.7), n=10	41.2 (1.5),n=5	55.5 (0.89), n=10
Organic Carbon (%)	1.6 (0.19), n=3	7.4 (0.3), n=3	1.47 (0.17), n=3
Total Nitrogen (%)	0.18 (0.05), n=3	0.05 (0.02),n=3	0.11 (0.02), n=3
Particle size as % of dry weight, n=3			
<63μm	24.9 (1.0)	57.3 (2.4)	19.2 (2.3)
63-125μm	12.9 (0.7)	32.8 (1.4)	17.8 (1.1)
125-250μm	25.1 (0.2)	7.0 (0.3)	27.6 (0.5)
250-500μm	32.5 (1.4)	3.0 (0.05)	29.2 (3.1)
>500μm	4.5 (0.1)	0	6.2 (0.2)

2.4 INTRODUCTION: BIOASSAY DEVELOPMENT

As egestion rate was not measured directly in the field, the bioassay design employed a post-exposure approach (Mcwilliam and Baird 2002a), where egestion rate was measured in clean (uncontaminated) sediment. Prior to testing the endpoint under contaminated conditions experimental work was carried out to develop the methodology under clean (uncontaminated) conditions. Baseline variation in feeding rates was minimised in order to reduce background "noise" and thus improve the statistical power of the endpoint. Manipulating or controlling for biotic and abiotic experimental variables that have previously been shown to influence feeding rate can reduce baseline variation and thus increase sensitivity. Bioassay sample size should also be large enough to detect significant changes in the measured endpoint. While attempting to improve the reliability and

sensitivity of the endpoint, practical considerations related to the use of the technique in the laboratory and field must also be considered.

2.4.1 Factors influencing the feeding rate of *L.variegatus*

The feeding rate of deposit feeders varies in response to a complex array of intrinsic and extrinsic variables (Cammen 1980). During previous studies with oligochaetes reproduction, body size, population density, ambient temperature, dissolved oxygen concentration and sediment composition have all been found to influence feeding rates (Robbins *et al.* 1984)White *et al.* 1987;Lotufo and Fleeger 1996;Leppanen and Kukkonen 1998a;Landrum *et al.* 2004a;Landrum *et al.* 2004b). Each of these factors is briefly considered below with particular reference to the development of the bioassay endpoint.

2.4.1.1 Reproduction

Reproduction of *L.variegatus* is most commonly via asexual fission or architomy (Christensen 1984;Cook 1969). During the fragmentation process the ingestion of sediment is halted so that worms can empty their gut prior to splitting (Leppanen and Kukkonen 1998c). Newly formed head and tail segments are unable to resume feeding until fully regenerated, which takes approximately 2 or 7 days respectively (Leppanen and Kukkonen 1998c). A similar interruption to feeding was also linked to the fragmentation of the lumbriculid oligochaete, *Stylodrilus heringianus* (White *et al.* 1987).

Based on the above, the effects of worms reproducing during the current work could be very significant. Firstly, if reproduction occurred during the contaminant exposure period those worms affected would not be exposed to a potentially significant route of uptake via ingestion. This could result in underestimation of the toxic effects of chemicals. Secondly, the use of reproducing worms would increase the likelihood of false positive results, as a reduction in feeding may be due to reproductive behaviour rather than contaminant exposure. Reproductive behaviour would also increase the variability in baseline (control) feeding rates and, therefore, reduce the statistical power of the bioassay. Therefore, an attempt should be made to control for the effects of reproduction throughout this work.

Reproduction in *L.variegatus* is suggested to be primarily controlled by the size of individual worms, although fragmentation may be artificially induced via the application of gentle pressure to the body surface (Cook 1969;Drewes and Fourtner 1990). Previously it was found that individual *L.variegatus* between 5-9mg wet weight at the start of experiments did not reproduce during a 28-day period in clean lake sediment, whereas larger worms (>9mg wet weight) reproduced at least once (Leppanen and Kukkonen 1998c). An initial experiment was therefore carried out with individual worms below 9mg wet-weight to study if reproduction could be controlled through the use of a size-selection procedure (Experiment 1, Section 2.6.1). Standardised invertebrate bioassays are usually started with animals of a defined age- or stage-structure to control for individual variability in the measured response (U.S.E.P.A 2000;O.E.C.D 2001). As the reproductive strategy used by *L.variegatus* makes age or stage selection very difficult, starting the bioassay with worms of a defined size range could also improve overall standardisation.

2.4.1.2 Worm size

Individual worm size may also be an important determinant of feeding rate (Cammen 1980). When the amount of material egested by deposit feeders is related to body size a significant positive relationship is usually found i.e larger animals ingest more material per unit of time when compared to smaller animals. However, in a previous study with *L.variegatus* the relationship between worm dry weight and egestion rate was not consistent between two separate experiments (Leppanen and Kukkonen 1998a). A significant correlation was found in the first experiment (no *r* value given) but in the second no correlation was found. This would suggest that the relationship is subject to considerable variability even when using the same population of worms. The work in Experiment 1 (Section 2.6.1) helped to determine the utility of normalising *L.variegatus* feeding rates to a measurement of worm size.

2.4.1.3 Population density

The population density of worms in experimental beakers may affect feeding rates via intra-specific competition for limited resources e.g food and/or space. Previously densities of 24,900 and 49,750 individual *L.variegatus* per square metre were found to cause a significant reduction in individual egestion rates (Leppanen and Kukkonen 1998a). Worms were observed to have processed approximately half of the entire sediment within beakers after a 7-day period. Exhaustion of sediment (as a food resource) was also linked to a

reduction in feeding rate at the later stages of an experiment with a tubificid oligochaete (Davis 1974).

The sediment reworking rate of the lumbriculid oligochaete, *Stylodrilus heringianus* was not reduced as a function of densities of up to 134,000 individuals per square metre, but the depth to which worms fed increased with increasing density (White *et al.* 1987). The change in depth of feeding was suggested to be a result of the complete processing of the top layer of sediment. Therefore, it seems that oligochaetes are able to feed normally at high starting densities, but may respond to increasingly limited food resources (as sediment is processed) by changing their depth of feeding. This would suggest that as long as the quantity of sediment in beakers does not become limiting during the experimental period the effects of population density on feeding rates might not be significant. The effects of different worm densities on individual feeding rates were investigated in Experiment 2 (Section 2.6.2).

2.4.1.4 Temperature

Many studies have previously found a strong relationship between temperature and the feeding activity of oligochaetes, including *L.variegatus* (Appleby and Brinkhurst 1971;Davis 1974;White *et al.* 1987;Leppanen and Kukkonen 1998a;Landrum *et al.* 2004a;Landrum *et al.* 2004b). Generally as temperature increases feeding activity also increases, but for a given population it is likely that there will an optimum temperature range above and below which feeding rates are reduced (Appleby and Brinkhurst 1971).

In the laboratory the effect of temperature on feeding rate may be controlled with the use of acclimation procedures and constant temperature facilities. However, controlling for the effects of temperature in field studies may be more difficult. Temperatures during the post-exposure feeding period might be controlled with portable incubators, but it is likely that ambient temperatures during the exposure period at field sites will vary considerably. Therefore an important consideration in this work was the potential effects of different exposure period temperatures on subsequent post-exposure feeding rates. This was investigated in Experiment 3 (Section 2.6.3).

2.4.1.5 Additional considerations

Additional factors that could affect the feeding rates of worms include water quality parameters (e.g dissolved oxygen, pH, ammonia), sediment composition (particle size and organic matter content) and the length of the post-exposure feeding period. Water quality can be determined during laboratory and field studies and extreme values can be identified. Sediment for laboratory experiments was standardised, therefore differences due to factors such as particle size and organic matter content should not be significant. It was possible that transferring worms from exposure (contaminated) to post-exposure (clean) sediments in both laboratory and field studies might cause significant disruption to their feeding activity. Therefore, Experiment 2 (2.6.2) was also used to identify any differences in the variability of feeding rates with different lengths of time for the post-exposure feeding period

2.5 AIMS: BIOASSAY DEVELOPMENT

The principle aims of this section were to:

- Conduct a series of experiments with a view to developing and standardising a method for measuring post-exposure egestion rate of *L.variegatus*
  - Experiment 1 – Controlling for reproduction and worm size measures versus egestion rate
  - Experiment 2 – The effects of the number of animals per replicate and length of the post-exposure feeding period on endpoint variability
  - Experiment 3 – The effects of four exposure temperatures on post-exposure egestion rate
- Determine the statistical power of the proposed bioassay methodology to detect a specified minimum difference between treatment means
  - Experiment 4 – Statistical power of the proposed bioassay method

2.6 MATERIALS AND METHODS: BIOASSAY DEVELOPMENT

2.6.1 Experiment 1- Controlling reproduction and worm size versus egestion rate

There were a number of objectives to Experiment 1. Firstly, reproduction over the course of a six-day exposure period and a subsequent twenty-hour post-exposure period was followed. The use of individual worms in each replicate beaker would help to

determine if selecting worms below 9mg wet-weight was a successful method to control for the effects of reproduction on feeding rates. A six-day exposure period was anticipated to be the maximum amount of time worms would be exposed to contaminated sediments. Shorter time periods might reduce the sensitivity of the bioassay, whereas longer time periods might increase the risk of equipment losses in the field due to flooding and vandalism. Twenty-four hours was anticipated to be the maximum length of time for the post-exposure feeding period, as longer time periods might allow for significant recovery of worm feeding rates. Experiment 1 also developed the basic method for measuring egestion rate during both the exposure and post-exposure periods. Finally, the relationship between post-exposure egestion rate and worm size measures was also studied with a view to normalising egestion rates to worm size.

2.6.1.1 Set-up and identification of non-feeding worms

Twenty-four hours prior to the start of Experiment 1 30 tall glass beakers (60ml, Fisher,U.K) were filled with 20g of wet sediment and topped up with hard ASTM water. Beakers were left overnight at 20°C to settle prior to exchanging overlying water the next day. This period allowed sufficient settling time and equilibration of beakers to the test temperature. On the same day as beaker set-up approximately 100 worms were selected from cultures. All worms were estimated to be below 9mg wet-weight (based on preliminary observations of worm weights in relation to body size), with fully formed head and tail parts. They were then randomly placed in triplicate 200ml glass beakers containing 50g of wet sediment and filled with hard ASTM water. Worms were then left to feed

overnight at 20°C. The purpose of this acclimation period was to identify worms that were not actively feeding i.e had empty guts.

The next day, all of the worms were separated from the sediment using a 250µm sieve. They were then pooled together and visually examined. Non-feeding worms were easily identified by their empty guts, which appear transparent against a white background. Once actively feeding worms were identified they were placed in individual foil cups containing 20ml of hard A.S.T.M for 8 hours to empty their guts prior to weighing. Previously *L.variegatus* has been shown to empty 98% of gut contents during a 6 hour period in water (Mount *et al.* 1999). After the gut-purging period, 30 worms were randomly selected and weighed individually. Excess water was removed by blotting dry the weigh pan rather than the worms themselves. Worms were then added to individual beakers to start the experiment.

Experimental beakers were held for six days in complete darkness at 20°C. This is termed the exposure period, as it is envisaged that this would be where worms will be exposed to contaminated sediments in the laboratory and field in subsequent chapters. Beakers were observed daily to check for mortalities and behaviour of worms. Overlying water was exchanged on Days 2 and 4, prior to exchange dissolved oxygen, temperature and pH of overlying water was measured in triplicate.

On Day 5 of the exposure period 30 separate beakers were set up to measure post-exposure egestion rate from Days 6 to 7 of the experiment. Beaker set-up was the same as

that described previously for the exposure period. On Day 6 worms were sieved from the exposure beakers and the number of worms was counted. Individual worms were then added to the post-exposure beakers where egestion rate would be measured. Once worms had fully burrowed into the sediment (<30 minutes) measurement of post-exposure egestion rate was started. The methodology for measuring egestion rate is described below and was adapted from a previous study (Leppanen and Kukkonen 1998a).

2.6.1.2 Measurement of *L.variegatus* egestion rate

A thin (approximately 1 mm) layer of acid-washed quartz sand (Ottawa 20-40 mesh, Fisher, UK) was deposited on top of the sediment containing burrowed worms. Worms quickly protruded their tails through the sand layer and began egesting faecal pellets onto the surface. This can be seen in Figure 2.3. Egestion rate was then measured over a twenty-four hour period. At the end of the feeding period faecal pellets were collected from the sand layer using a Pasteur pipette. The use of a relatively coarse sand allowed for the very effective separation of sand grains from faecal pellets within the pipette. In order to standardize the effort expended per beaker, a 4-minute collection period was used, which was more than an adequate time period to collect even the largest quantities. Three procedural blanks (sand layer but without worms) were used to correct for the very small (<0.1mg) contribution from any suspended materials settling onto the sand layer after its addition. Faecal pellets were then filtered onto 47-mm glass fibre filter papers (MF200,Fisher,U.K), dried at 60°C for 48 hours and weighed. The egestion rates of individual worms were calculated as the quantity of dry faecal pellets (mg)/hour.

START **AFTER 24 HOURS**

Figure 2.3 The sand layer method for measuring egestion rate used throughout this work. The left hand picture shows the sand layer at the start of the 24-hour feeding period, with worms protruding their tails above its surface. In the right hand picture worms have been left to feed for 24 hours, faecal pellets are clearly visible on top of the sand layer

Worms were sieved from beakers immediately after the collection of faecal pellets. The wet weight of worms was then taken after using the 8-hour gut-purging period described previously. Worm lengths were then taken by narcotising the worms in water saturated with carbon dioxide using a soda stream drinks maker. The use of carbon dioxide prevented muscular contraction while taking measurements. Triplicate repeated measurements of individual length were taken and the mean calculated. Worms were then placed in individual foil containers and dried at 60°C for 48 hours prior to weighing.

The relationship between post-exposure egestion rate and worm size in Experiment 1 was firstly examined with standard correlation to determine the significance of any relationships (Minitab version.13). The value of r required to achieve statistical significance with standard correlation is rather low, so egestion rate was regressed against worm size measurements to determine the amount of variation in egestion rate explained by worm size (r^2) (Dytham 1999). While worm size measurements cannot be considered as true independent variables (as they are not fixed values and cannot be measured without error), analysis of residuals suggested that the magnitude of error did not vary with the magnitude of worm size measurements (Sokal and Rohlf 1995).

2.6.2 Experiment 2 – Number of animals per replicate and length of the post-exposure feeding period

The second experiment looked at the variability in post-exposure egestion rates of various numbers of worms per replicate beaker over varying time periods with a view to reducing variability in the bioassay endpoint. The use of more than one animal per replicate might reduce variability in the measured endpoint, as it will be an average of the feeding rates of x number of animals. However the use of more than one worm per replicate may result in density-dependent effects on individual feeding rates.

The numbers of animals per replicate was 1,2,4,5, and 7. Selection of this range was based on what could be supplied from cultures on a regular basis. Time periods of 4,6 and 24 hours were chosen for measuring post-exposure egestion rate in an attempt to balance a

number of issues. Firstly, worms may take a certain amount of time to acclimate and begin feeding after addition to post-exposure beakers. If the post-exposure feeding period were too short increased variability in egestion rates could result. Secondly, as the post-exposure feeding period is in clean sediment too long a time period could result in worm feeding rates recovering completely after previous inhibition under contaminated conditions. Lastly, the chosen time period should fit within the constraints of a realistic working day in the laboratory or in a field situation.

Twenty-four hours prior to the start of Experiment 2 20g of wet sediment was distributed to 75 tall glass beakers (60ml, Fisher,U.K) and filled with hard ASTM water. Beakers were left to settle overnight at 20°C and overlying water was exchanged the following day. On the same day as beaker set-up a sufficient number of worms estimated to be below 9 mg wet weight were selected for experiments from cultures. The procedure described in Experiment 1 (Section 2.6.1.1) was then used to identify feeding and non-feeding worms.

The experiment had a two-factor design with numbers of worms per beaker and length of post-exposure feeding period as factors. 1,2,4,5 and 7 worms were added to 5 replicate beakers. This was equivalent to a starting density of 1042, 2083, 4166, 5208 and 7291 individual worms $m^{2\ -1}$ respectively. These treatments were then repeated for one of the three time periods used to measure post-exposure egestion rate (4,6 and 24 hours). Five replicate beakers were used in this experiment as it was anticipated that this would be the minimum number of replicates used throughout subsequent experiments.

The exposure and post-exposure periods were carried out exactly as in Experiment 1 (Section 2.6.1.2). On Day 5 beakers for the post-exposure period were set-up and settled overnight. On Day 6 worms were sieved from exposure jars and counted. Only those worms with fully formed heads and tails and full guts were then added to post-exposure beakers. Post-exposure egestion rates were then measured over 4,6 and 24-hours. Post-exposure egestion rates were calculated as the quantity of dry faeces (mg) egested per individual worm per hour. The variability in egestion rates with different treatments was expressed as the co-efficient of variation (CV) (Zar 1999). The CV expresses the variability in a data set that is independent of magnitude.

2.6.3 Experiment 3 – The effects of exposure temperature on post-exposure egestion rate

In order to test for the effects of previous exposure temperature on post-exposure egestion rates, *L.variegatus* were exposed to a range of temperatures for six days. Post-exposure egestion rates were then subsequently measured at $20^{o}C \pm 0.5\ ^{o}C$. Temperatures for the exposure period were 5, 10, 15 and $20^{o}C \pm 0.5\ ^{o}C$. The range of temperatures selected was based on what might be expected under field conditions during the summer. The $10^{o}C$ and $20^{o}C$ treatments were controlled through the use of separate controlled temperature rooms. The $5^{o}C$ and $15^{o}C$ treatments were controlled via the use of a standard refrigerator and a laboratory incubator respectively. All experiments were carried out in complete darkness. The $20^{o}C$ treatment was designated as the control as worms were

normally cultured at this temperature. This experiment would also determine if any mortality was associated with a sudden change in temperature.

The methods for setting up experimental beakers and selecting feeding worms were identical to those described previously in Experiment 1 (Section 2.6.1.1). The beakers used to identify feeding worms were held at 20°C overnight. The beakers for the actual exposure period were settled overnight at one of the four designated temperatures in order to ensure that temperatures were properly equilibrated when the worms were added. The experiment was started by the addition of 5 worms with full guts to one of five replicate beakers. This was repeated for each of the four temperature treatments. No temperature acclimation regime was used. Beakers were then held at their respective temperatures for six days. Overlying water was exchanged on Days 2 and 4. The water used for exchange was cooled to test temperature overnight before use.

On Day 5 post-exposure beakers were set-up and allowed to settle overnight at 20°C. In order to compare egestion rates during exposure with those from the corresponding post-exposure period at 20°C, egestion rates were measured over a 24-hour period between Days 5 and 6 using the sand layer method (Section 2.6.1.2). At the end of the exposure period (Day 6) faecal pellets were collected (to calculate exposure egestion rate), and worms were transferred to fresh beakers to measure post-exposure egestion rate at 20°C (Section 2.6.1.2).

Egestion rates for both periods were calculated as the quantity of dry faeces (mg) egested per individual worm per hour. Mortalities and worm behaviour were noted throughout the exposure and post-exposure periods. Comparisons of egestion rates between treatment groups for each feeding period were analysed separately using analysis of variance (Mintab version 13). Fisher's least significant difference test was used to identify which treatments were significantly different from the 20°C control for each feeding period. Any comparisons of exposure and post-exposure egestion rates at a given temperature were carried out with paired t-tests as the data are paired observations of the same worm populations.

2.6.4 Experiment 4 - Statistical power of the proposed bioassay methodology

The proposed bioassay methodology developed in Experiments 1-3 consisted of five individual worms per replicate beaker feeding for a 24-hour post-exposure period. Statistical analysis of the bioassay endpoint in future chapters involved comparing the mean and variance of replicates within treatment groups. Therefore, a sufficient number of replicates per treatment group should be used to enable detection of a specified minimum difference between treatment means.

The statistical power of a procedure (1-β) refers to the ability to detect a significant difference (usually at p=0.05) between samples, or an association between variables when one exists i.e to avoid type II errors where we fail to reject the null hypothesis when it is false (Wheater and Cook 2000). Increasing the number of replicates per treatment group

will increase the power of a procedure, which will in turn reduce the likelihood of committing type II errors (Zar 1999). The choice of replicate numbers for a test is usually limited by the practical constraints associated with culturing sufficient numbers of animals, equipment requirements and the time to carry out a procedure. So there is often a trade-off between statistical power and logistical constraints.

In order to estimate the statistical power of the proposed bioassay methodology, analysis was performed on 30 independent replicate beakers each containing 5 worms. The beakers were held at 20°C for six days (exposure period), worms were then transferred to beakers to measure post-exposure egestion rate. A bootstrapping technique was used to randomly assign post-exposure egestion rate data from each of the thirty beakers to a set of replicates ranging in size from 4 to 10, which were re-sampled 100 times. The minimum specified difference in egestion rates was initially set to 20%. A critical value was derived for each of the 100 re-samplings and the mean was used to calculate the power for a given number of replicates when analysis of variance is employed at the $\alpha = 0.05$ significance level (Zar 1999). After initially determining the power values using between 4 and 10 replicates to detect a 20% change in post-exposure egestion rate, further bootstrapping was carried out with the same number of replicates but with a 25% and 29% minimum detectable difference specified.

2.7 RESULTS

2.7.1 Experiment 1

The twenty-four hour acclimation period was very useful for identifying worms with full guts. Out of the 100 worms that were originally selected from cultures, 6 were found to have empty guts. The removal of non-feeding worms should reduce the overall variability in feeding rates.

Prior to carrying out the acclimation period worms were selected by a visual estimation of their size, which was then confirmed by wet weighing. However, weighing each individual worm prior to starting the bioassay is not feasible due to the time involved. The wet-weights of the 30 worms selected for use in Experiment 1 are shown in Figure 2.4. Mean individual wet weight was 5.16 mg (min= 2.4mg,max = 8.8mg,sd = 1.6). This highlights that it was possible to select worms that were consistently below the maximum limit of 9mg wet-weight by a simple visual estimation of size, without the need to confirm with weighing.

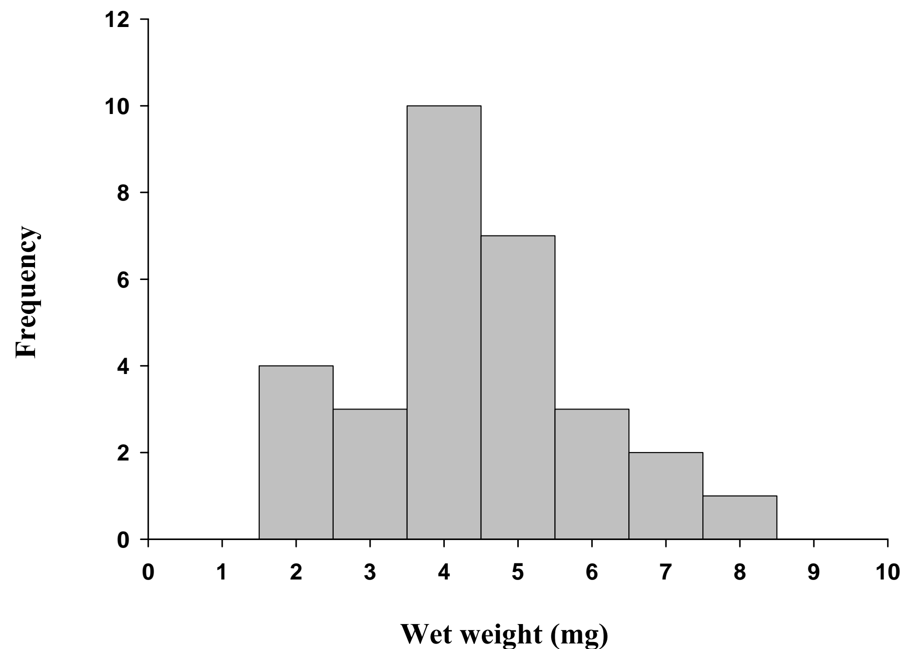
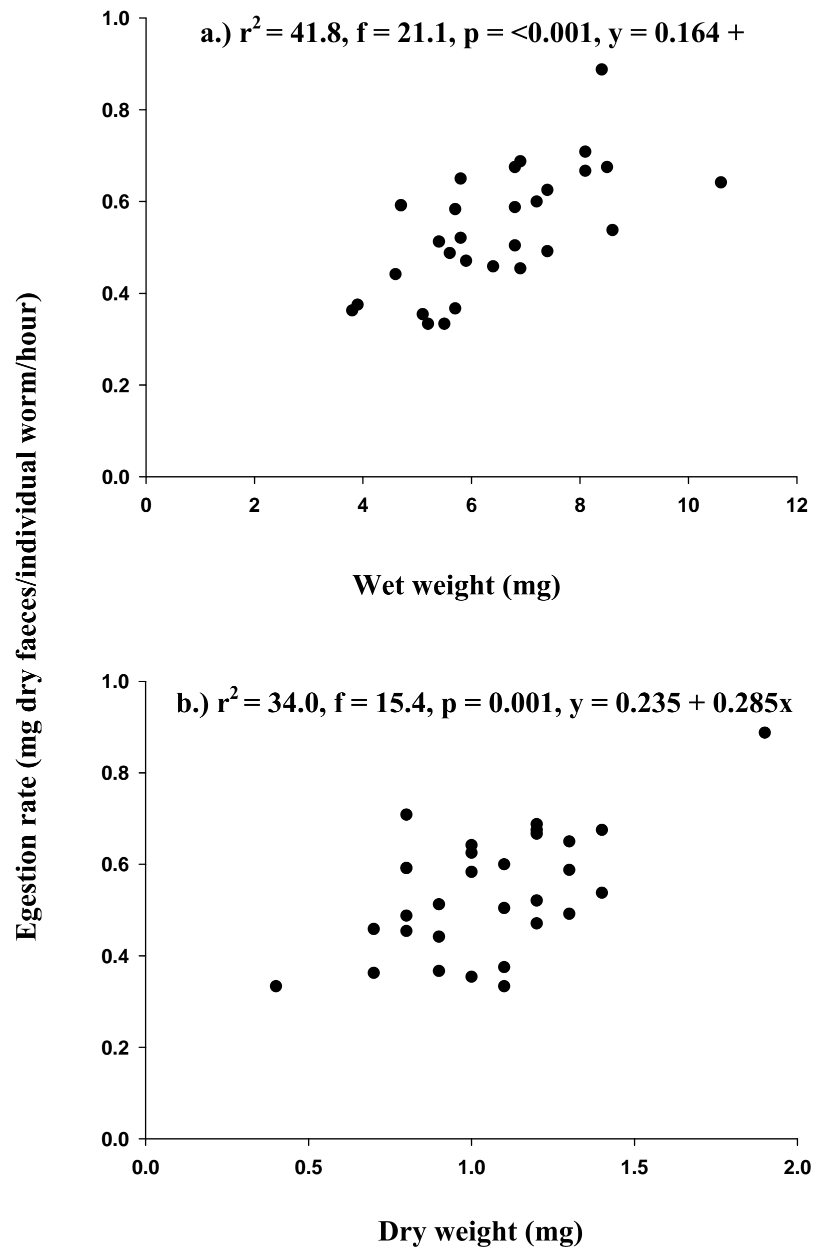


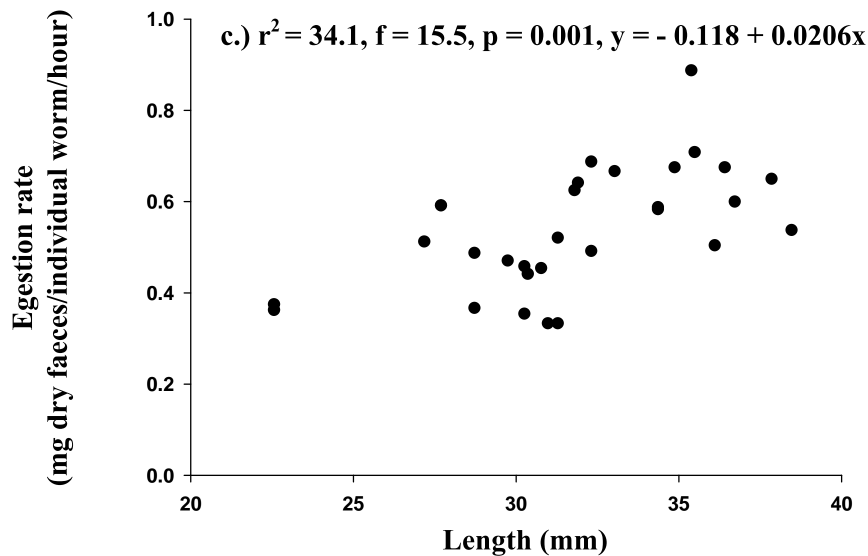
Figure 2.4 The weight distribution of *L.variegatus* selected for Experiment 1, n = 30

By the end of the exposure period in Experiment 1 dissolved oxygen levels of overlying water were always above 70% saturation (mean 73.9,sd=2.4,n=3) and pH was deemed to be within an acceptable range (mean = 7.31,sd=0.18,n=3). Unfortunately during the course of the experiment one of the replicate beakers was dropped, so egestion rate data is based on 29 rather than 30 replicates. There was no evidence of reproduction throughout Experiment 1 as all 29 beakers contained only one worm.

Post-exposure egestion rates from Experiment 1 were quite variable (mean=0.54mg dry faeces/hour, coefficient of variation = 25%). The relationships between post-exposure egestion rates and worm size measures are shown in Figures 2.5a-c. All three measures were significantly correlated to egestion rate. Of the three measures wet weight provided

the best fit to egestion rate data, but this only explained 42% of the variance in egestion rates (Figure 2.5a).





Figures 2.5a-c The relationship between *L.variegatus* egestion rate and size of individual worms (n=29) from Experiment 1, regression equations, *r*-squared, f and p statistical values for each regression are shown

2.7.2 Experiment 2

Dissolved oxygen was above 70% saturation in all of the measured beakers (mean 71.9,sd 1.55) and pH was between 7 and 7.6. This confirmed that increasing the number of worms from 1 to 5 per replicate did not cause a significant reduction in water quality (at p=<0.005). By the end of the six-day exposure period no fragmentation had occurred in any of the beakers and all worm guts contained sediment.

The coefficients of variation for post-exposure egestion rates with different lengths of time spent feeding and number of animals per replicate are shown in Figure 2.6. Both the time spent feeding and the number of animals per replicate had a significant effect on the variability of the bioassay endpoint. The use of a 24-hour post-exposure feeding period

reduced variability when compared to the 4 and 6-hour periods. Increasing the number of worms per replicate from 1 to 5 reduced the variability in egestion rates from 24.8% to 16.9% respectively during a 24-hour feeding period. A further increase to 7 animals per replicate did not significantly reduce variation further. The use of 5 worms per replicate that fed for 24 hours did not affect individual feeding rates when compared to beakers that contained single worms (t-value = -0.52,p-value = 0.616).

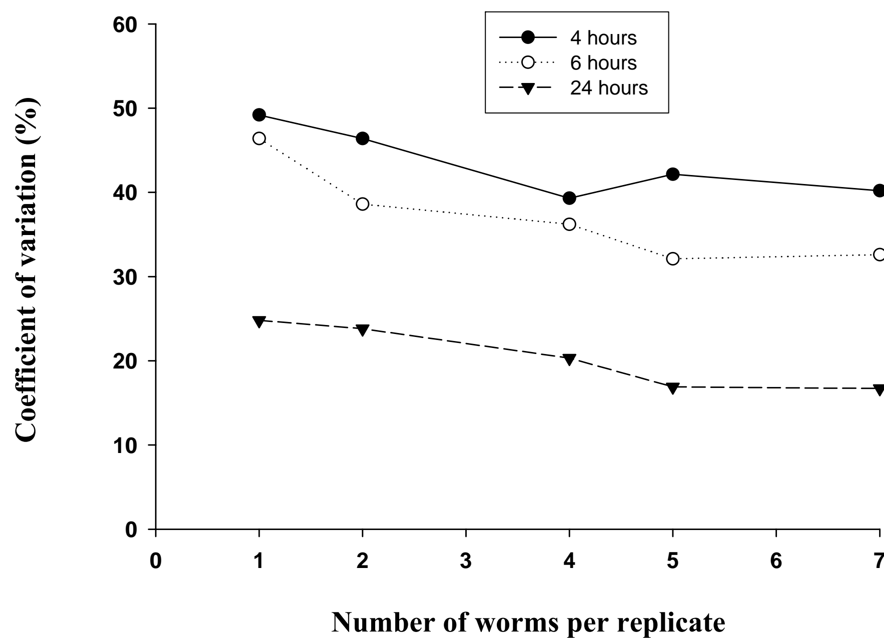


Figure 2.6 The coefficient of variation in post-exposure egestion rate of *L.variegatus* with differing numbers of worms per replicate feeding over 4,6 and 24 hour periods

2.7.3 Experiment 3

As can be seen in Figure 2.7 temperature had a significant effect on both exposure and post-exposure egestion rate. No mortalities or reproduction occurred in any of the treatment groups throughout the experimental period. The egestion rate of worms held at 5°C during

the exposure period could not be measured, as the majority of the worms did not burrow into the sediment. Once 5°C worms were added to post-exposure beakers (held at 20°C) they burrowed into the sediment and began to feed, but egestion rates over the post-exposure period were significantly lower than the 20°C control ($p=<0.001$). Therefore, previous exposure to 5°C had a persistent effect on the proposed bioassay endpoint.

Egestion rates at 10°C and 15°C were not significantly different from each other, but were both significantly lower than the 20°C control ($p=0.001$) during the exposure period. However, both 10°C and 15°C treatments recovered to control rates during the post-exposure period, so previous exposure to 10°C and 15°C did not have a sufficiently persistent effect on worms to be detected during the post-exposure feeding period at 20°C.

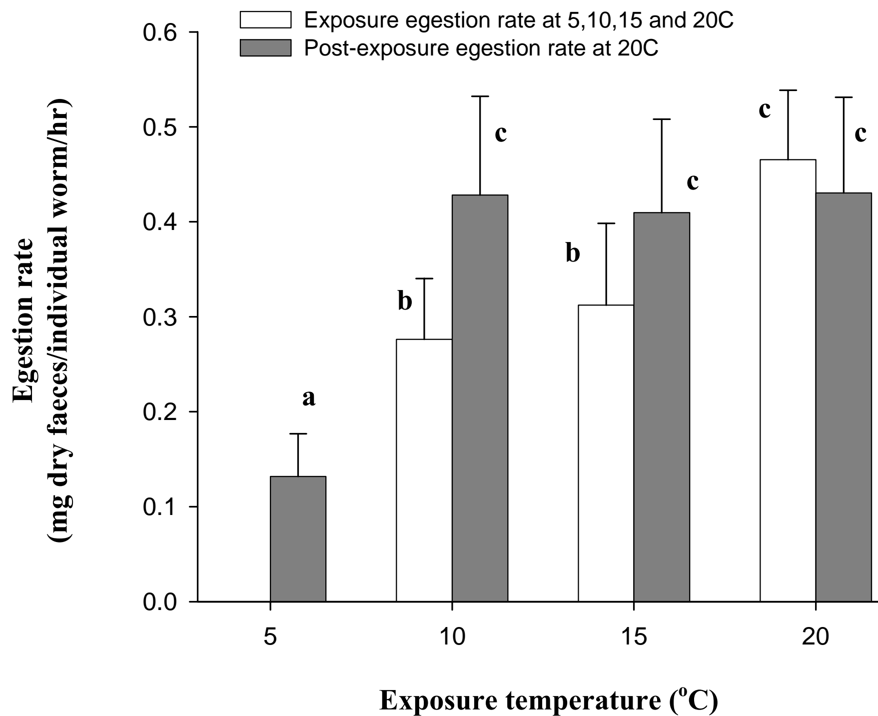


Figure 2.7 The egestion rate of *L.variegatus* during exposure to four temperatures and subsequently during a post-exposure period at 20°C, individual letters (a-d) denote significant differences, error bars represent 95% confidence intervals, egestion rates could not be measured during exposure to 5°C (see text for explanation)

2.7.4 Experiment 4

Figure 2.8 shows the change in the statistical power of analysis of variance when the proposed method for measuring egestion rate (i.e five worms per replicate feeding for a 24-hour post-exposure period) was used with between 4 and 10 replicates and 20,25 and 29% minimum detectable differences specified. As would be expected an increase in the number of replicates increased power. The power of the test to detect a 20% change in egestion rate increased from 0.44 to 0.73 when replicate numbers were increased from 4 to 10. However,

no number of replicates within the range selected reached a power of 0.8 when 20% was specified as the minimum detectable difference. Further analysis revealed that eleven replicates were required to reach a power of 0.8 when 20% minimum detectable difference was specified. When the minimum detectable difference was increased from 20% to 29% the power of 5 replicates increased from 0.53 to 0.80.

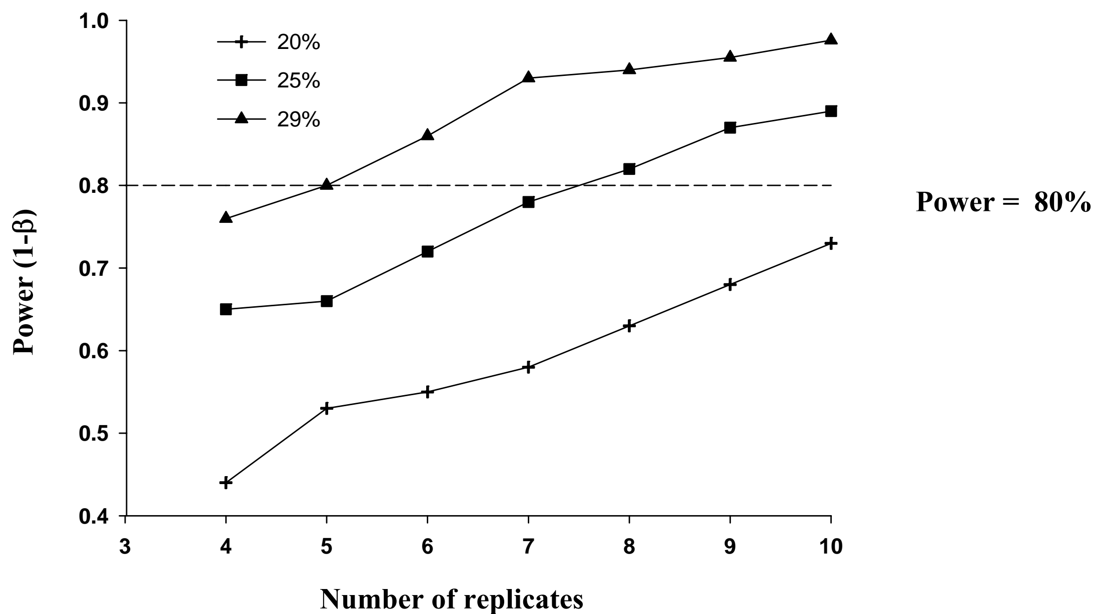


Figure 2.8 The number of replicates required for at least 80% probability of detecting a specified minimum difference of 20,25 and 29% (at α = 0.05 significance) in post-exposure egestion rate of *L.variegatus* using the method developed in Experiments 1-3

2.8 DISCUSSION

2.8.1 Experiment 1

In Experiment 1 the use of a 24-hour period prior to starting the experiment proved to be a very simple way of identifying worms that were not actively ingesting sediment. The selection of worms less than 9mg wet-weight was also effective in controlling for reproduction over the course of a 7-day period. This finding is supported by the conclusions of an earlier study (Leppanen and Kukkonen 1998c). Based on the results from Experiment 1 the proposed bioassay methodology involved starting exposures with a twenty-four hour period in clean sediment that was used to identify feeding and non-feeding worms. Further, because of the time involved, weighing individual worms prior to starting the bioassay was deemed to be unfeasible. Therefore selection of worms for the bioassay in all subsequent work was based on a visual estimation of their size, which was shown to be an accurate and consistent method of selecting animals below 9mg wet-weight (Figure 2.4).

Experiment 1 also studied the relationship between egestion rate and worm dry weight, wet weight and length. At best only 42% of the variance in egestion rates was explained by worm size (wet weight). This finding was significant, as it was envisaged that egestion rate would be normalised to at least one of the three worm size measures. However, based on the above findings it was decided to dispense with normalizing feeding rates to worm size for laboratory studies with the bioassay. As worms will be selected from a defined size range to control for reproduction, the effects of size variation should be limited. Subsequent

results from Experiment 2 would seem to confirm that the variability in individual egestion rates using this approach is within acceptable limits (i.e <20%).

Studies using a variety of marine deposit feeders have suggested that feeding or egestion rates increase as a function of body size to a power of less than one (Hargrave 1973;Kudenov 1982;Forbes and Lopez 1987). In other words, as individual animal size increases their proportional feeding rate decreases. This may be a result of feeding rates being limited by a physiological surface area such as the respiratory surface or gut (Forbes and Lopez 1987). One hypothesis suggests that larger individuals in a population may feed at lower relative rates because their ability to digest and absorb food is greater than their ability to obtain sufficient oxygen for metabolism from the respiratory surface (Forbes 1987). While specific studies using freshwater oligochaetes are limited, the above would seem to highlight that the size scaling of deposit feeders is often more complex than that which can be described by a simple linear relationship.

2.8.2 Experiment 2

In Experiment 2 increasing both the number of animals per replicate and the time spent feeding caused a reduction in the variability of the measured endpoint. As the CV for 5 animals feeding for 24 hours was 16.9%, a 20% difference between egestion rates would allow the minimum detectable difference to exceed baseline variation i.e baseline variation would not mask toxic effects. On this basis, five worms per replicate beaker feeding for a twenty-four hour post-exposure period were chosen as the bioassay methodology.

Feeding periods of less than twenty-four hours would have been more preferable, as the bioassay might have been carried out over the course of a single day. A short post-exposure period in clean conditions would also mean there would be less potential for recovery of feeding rates. Unfortunately the use of four and six-hour periods produced unacceptably high variation in egestion rates (>30%). High variability will reduce the ability to detect feeding inhibition under contaminated conditions, and is probably a result of the disruption associated with the transfer of worms to post-exposure beakers. While it is possible that variability might be acceptable for 12 and 16-hour feeding periods, the choice of 24 hours as the next longest period was based on what would be deemed practical in the field.

The use of 5 worms per beaker (or 5208 individuals/m^2) did not affect the egestion rate of individual worms over 24 hours, which confirmed there was no density-driven change in egestion rate. Previously the individual egestion rate of *L.variegatus* was found to be unchanged at densities of up to 12,450 individuals/m^2(Leppanen and Kukkonen 1998a).

2.8.3 Experiment 3

In Experiment 3 a decrease in temperature resulted in a decrease in feeding activity (Figure 2.7). This is in line with previous studies of the effects of temperature on the feeding activity of *L.variegatus* (Leppanen and Kukkonen 1998a;Landrum *et al.* 2004a;Landrum *et al.* 2004b). The persistent effects of previous exposure temperature on post-exposure egestion rates were only evident in the 5°C treatment. Clearly this could

present problems if worms are exposed to 5°C in the field as false positive results may occur. However, as fieldwork testing of the bioassay is planned for the summer it is envisaged that ambient temperatures will be higher than 5°C. On this basis the proposed bioassay methodology should not be subject to any persistent effects of previous exposure temperature. During the experiment animals were transferred directly from 20°C to test temperatures. It is not known if the worms may have been more able to cope with exposure to 5°C if a more gradual acclimation regime had been used.

2.8.4 Experiment 4

The results from Experiment 4 suggest that 11 replicates would be required to reach the desired power of 0.8 when 20% was specified as the minimum detectable difference in post-exposure egestion rates (Figure 2.8). Based on the previous experiments in this chapter, 5 replicates was deemed to be the maximum possible in terms of the time required to carry out the bioassay and animals required from cultures. The proposed bioassay method with five replicate beakers produced a power of 0.53 at 20% minimum detectable difference. This would mean there would be a 47% probability of committing a type-II error (failure to detect effects). While this level of error is undesirable, the choice of replicate numbers is a compromise between what is practical to use and that which achieves greater statistical power (Wheater and Cook 2000). The minimum difference that five replicates could detect with 80% power was 29%, which could be an acceptable level of discriminatory power. Unfortunately, no previous studies have been carried out with *L.variegatus* to determine the implications of a specified reduction in feeding rate.

2.9 SUMMARY

A summary of the proposed bioassay methodology developed in this chapter is shown in Figure 2.9. This was used in subsequent chapters to study post-exposure feeding inhibition in the laboratory (Chapters 3 and 5) and field (Chapter 4). In addition to the post-exposure bioassay, egestion rate was also measured during the exposure period for contaminant studies in the laboratory (Chapter 3). This enabled comparisons of exposure and post-exposure egestion rates under artificially contaminated conditions in the laboratory, in order to determine the sensitivity and persistence of feeding inhibition as a bioassay endpoint.

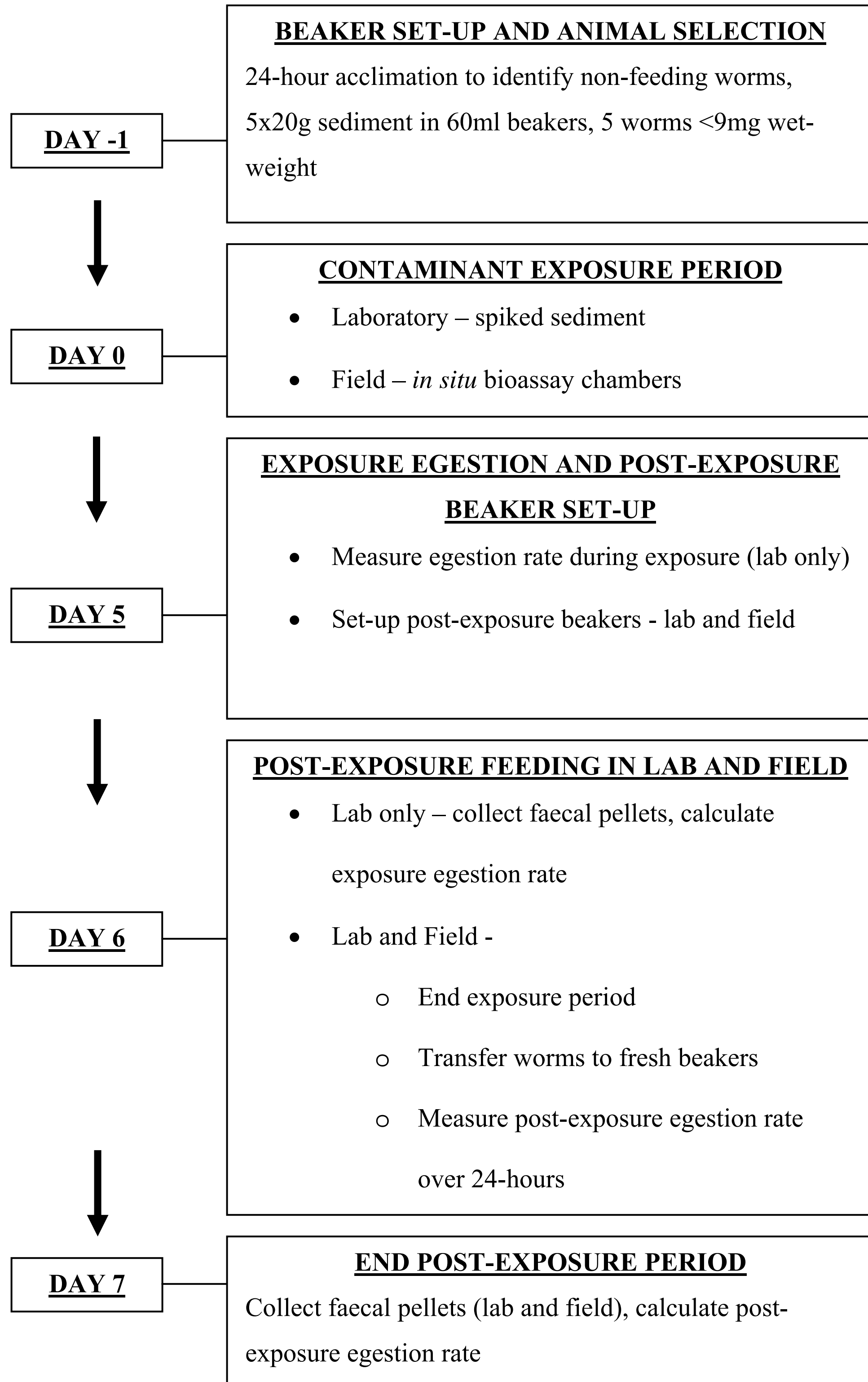


Figure 2.9 Summary of the proposed bioassay methodology for use in both the laboratory and field (*in situ*)

3 CHAPTER 3 – CONTAMINANT-INDUCED FEEDING INHIBITION IN THE LABORATORY

3.1 INTRODUCTION

As post-exposure feeding inhibition of *L.variegatus* is a novel bioassay endpoint it must be shown to be suitable for bioassay use. Prior to testing the proposed bioassay *in situ*, the endpoint was tested in the laboratory to determine its relative sensitivity and reproducibility. This required the bioassay response to be detectable during the post-exposure period in clean sediment, after previous exposure to a variety of sediment-associated contaminants. Comparison of feeding rates during contaminant exposure with those post-exposure highlighted any patterns in the recovery of feeding rates during the post-exposure period. While feeding behaviour during the contaminant exposure period could not be measured in subsequent *in situ* studies, comparisons between feeding behaviour and lethality during contaminant exposure helped to determine the relative sensitivity of *L.variegatus* feeding inhibition. Laboratory toxicity studies were therefore required to study exposure and post-exposure feeding rates under controlled exposure conditions using artificially contaminated ("spiked") sediments.

As was previously highlighted in Section 1.3.3 feeding inhibition is considered a general response to toxicant stress with a variety of aquatic organisms and contaminants. Feeding inhibition may be caused by the impairment of chemoreception, by an alteration in feeding behaviour, by a reduction in ingestion rates through direct poisoning or by a

combination of these. Table 3.1 contains examples of studies where toxicant-induced feeding inhibition has been demonstrated with deposit feeding invertebrates previously. As can be seen from the reported effect concentrations in Table 3.1 feeding inhibition usually occurs at contaminant concentrations significantly below those that cause mortality. A reduction in feeding rate may translate into effects at the population level via a reduction in the energy available for growth and reproduction (Section 1.3.3). Deposit feeders such as *L.variegatus* are also important prey items in aquatic food webs, and their feeding activity has previously been demonstrated to alter the physical and chemical dynamics of sediments and overlying waters (Section 1.4.2). Therefore, measuring the feeding behaviour of *L.variegatus* may provide a sensitive and ecologically relevant response to sediment contamination.

Table 3.1 Examples of feeding inhibition studies with a variety of deposit feeders, where possible feeding effect concentrations and reported mortalities are given for comparison

Species	Contaminant(s)	Effect levels	Reference
Stylodrilus heringianus	Endrin	Sediment reworking reduced approx 5 orders of magnitude lower than LC_{50}	(Keilty *et al.* 1988b)
Nereis diversicolor	Various hydrocarbons	None calculated	(Gilbert *et al.* 1994)
Arenicola cristata	Kepone	No effect levels calculated, reduction in feeding at 2.8µg/L with no mortality	(Rubinstein 1979)
Heteromastus filiformis	DDT	*EC_{50} burial rate 37nmol/g	(Mulsow *et al.* 2002)
Limnodrilus hoffmeisteri	Pyrene and Phenanthrene	Pyrene egestion rate IC_{25} - 51mg/kg, mortality-<20% in all treatments	(Lotufo and Fleeger 1996)
Abra alba	Diesel oil	EC_{50} egestion rate-55mg/kg mortality-none observed	(Stromgren *et al.* 1993)
Lumbriculus variegatus	Tetrachlorobiphenyl	*EC_{50} burial rate–96nmol/g *EC_{50} biomass–386nmol/g *LOEC mortality–880nmol/g	(Landrum *et al.* 2004)
Limnodrilus hoffmeisteri	Pyrene, phenanthrene and dibenzofuran	Egestion reduced by a factor of 2-3 in 90mg/kg pyrene, no mortality reported	(Reible *et al.* 1996)

*effect concentrations reported on a body residue basis (nmol chemical/g wet tissue), LOEC = lowest observable effect concentration for mortality, LC_{50} = estimated concentration causing 50% mortality, EC_{50} and IC_{25} = effect and inhibition concentration reducing measured endpoint by 50% and 25% respectively

As was previously highlighted in Section 1.4.4, measuring the feeding behaviour of *L.variegatus* directly *in-situ* would be very difficult, so measuring feeding behaviour during a post-exposure period after previous exposure to field sediments may circumvent this problem. Most of the studies listed in Table 3.1 have only measured feeding behaviour during direct exposure to contaminants with two notable exceptions. Stromgren et al (1993)

found a contaminant-induced reduction in the egestion rate of the bivalve, *Abra alba* to be sufficiently persistent to be detectable up to 4 days after transferring animals into clean sediments. Similarly, Lotufo and Fleeger (1996) found a depression in post-exposure egestion rate with the tubificid oligochaete, *L.hoffmeisteri* up to 72 hours after exposure to phenanthrene. Although previous work is limited, the two studies above suggest that recovery from feeding inhibition in deposit feeders may be sufficiently delayed so as to allow detection during a post-exposure feeding period. Both of these studies selected hydrocarbons as examples of common sediment contaminants. Further work would also identify if post-exposure feeding inhibition occurs with a greater variety of contaminant classes.

3.1.1 Selection of test substances

3.1.1.1 Organic contaminants

Table 3.2 gives details of the 3 organic substances used in this chapter and their important physio-chemical characteristics. Pyrene is a polycyclic aromatic hydrocarbon (PAH), a group of environmentally persistent and ubiquitous organic compounds. While point-source releases such as oil spills and effluent discharges may be significant on a local scale, anthropogenic enrichment of aquatic systems is largely attributed to the combustion of fossil fuels and subsequent atmospheric deposition (Walker *et al* 2001;Van Metre *et al.* 2000). As a result of their relatively low solubility in water and high environmental stability PAH’s such as pyrene have a tendency to accumulate in aquatic sediments where they may

reach levels that are harmful to aquatic invertebrates (Van Metre *et al.* 2000;Monson *et al.* 1995).

Cypermethrin is a synthetic pyrethroid insecticide that is widely used for crop and textile protection. Due to their low solubility in water, pyrethroids show a tendency to rapidly and strongly bind to particulate matter when released into the aquatic environment (Solomon *et al.* 2001). Significant accumulation of cypermethrin and permethrin was recently reported in river sediments within the Humber River catchment in the United Kingdom (Long *et al.* 1998). While the pyrethroids are considered to be far less persistent in the environment when compared to many of the insecticides they were designed to replace, concern has been raised regarding the biological effects of such accumulations in aquatic sediments (Maund *et al.* 2002).

Chlorothalonil is a broad-spectrum organo-chlorine fungicide commonly applied to agricultural crops to protect from blights, leaf spots and mildews. It is among the top five fungicides sold in North America and is widely used in potato production to protect from early and late blight (Caux *et al.* 1996). Chlorothalonil is relatively soluble in water but a number of environmental fate studies have suggested that it may be rapidly removed from the water column as it becomes associated with particulates (Ernst *et al.* 1991;Thomas *et al.* 2002).

Studies concerning the potential accumulation of chlorothalonil in sediments are limited, but a number of previous studies have suggested that degradation of chlorothalonil

in the water column is moderately fast (<30 days) (Caux *et al.* 1996). However, the regular (every 7-10 days) prophylactic re-application of chlorothalonil has raised concerns regarding the potential for accumulation in the environment (Ernst *et al.* 1991). For instance, significant suppression of chlorothalonil degradation was found after repeated applications, due to the toxicity of break down products to the soil microbial community (Motonaga *et al.* 1998).

Table 3.2 Details of the 3 organic substances used in this chapter and their important physico-chemical characteristics

Test substance	Chemical class	Solubility limit in water	Log Kow[1]	Log Koc[2]
Pyrene	Polycyclic aromatic hydrocarbon (PAH)	200 μg/l [a]	4.9 [a]	4.8 [a]
Cypermethrin	Pyrethroid insecticide	4 μg/l @ pH 7 and 20°C [b]	6.6 [c]	5.4 [d]
Chlorothalonil	Broad spectrum fungicide	900μg/l at 25°C [e]	2.64-4.38 [e]	3.1 [e] *

[1] Log Kow = log octanol-water partition co-efficient used to describe a compounds partitioning behaviour between octanol and water, essentially an index of a substances hydrophobicity (Walker *et al* 2001). Generally more hydrophobic substances have higher log Kow values

[2] Log Koc = the log affinity of a compound for the organic carbon fraction of soil or sediment which is the water-sediment partition coefficient (usually denoted K_d) normalised to organic carbon (OC) content of the sediment (Walker *et al* 2001).

[a] (Conrad 2000)

[b] (Tomlin 1994)

[c] (Royal Society of Chemistry 1991)

[d] log of original Koc value of 238,000 based on field-collected aquatic sediment with 1% total organic carbon content (Maund *et al.* 2002)

[e] after review by (Caux *et al.* 1996) *for silty clay loam soil

3.1.1.2 Metal contaminants

Metal compounds are commonly found in association with aquatic sediments as a result of natural geo-chemical weathering processes (Boudreau 1999;Helland 2001). Metals present in the environment are considered non-biodegradable as they cannot be broken down into less harmful components (Walker *et al* 2001). Like most metals, significantly elevated levels of copper and cadmium in aquatic sediments are usually a result of anthropogenic activities such as mining and heavy industry that may lead to long-term sediment contamination problems (West *et al.* 1993;Klerks and Levinton 1989). Copper is termed an essential metal in that trace quantities are required to maintain normal organism function but once these requirements are exceeded toxicity may result. Cadmium is a non-essential metal that may exert direct toxicity to organisms or may cause deficiencies in essential elements via antagonistic interactions at biological uptake sites (Walker *et al* 2001).

3.2 AIMS

The aims of this chapter were:

- To study the feeding rates of *L.variegatus* during direct exposure to contaminants. This enabled direct comparisons to be made with separate lethality tests, in order to determine the relative sensitivity of the bioassay.
- To study the post-exposure feeding behaviour of *L.variegatus* following exposure to a variety of sediment contaminants in the laboratory. This was carried out using the bioassay methodology developed in Chapter 2
- Measuring feeding rates during- and post-exposure also enabled the study of any recovery in feeding rates during the post-exposure period

3.3 MATERIALS AND METHODS

3.3.1 Experimental animals

All animals used in this section were supplied from the culture previously described in Section 2.3.1.1. Prior to the start of all feeding and lethality experiments worms were selected using the methodology described previously in Section 2.6.1.1, to ensure that actively feeding worms were used.

3.3.2 Sediment for laboratory toxicity testing

All sediment used in this work was from the same field-collected sample described previously in Section 2.3.3.1. Sediment was stored for a maximum period of one year from the date of collection. Prior to the addition of individual test substances samples were taken for moisture content and organic carbon analysis. Moisture content was used to calculate nominal target concentrations of tests substances on a dry weight basis. All concentrations of test substances are therefore referred to on a dry weight basis (i.e mg test substance/kg dry sediment).

3.3.3 Addition of test substances to sediments

The nominal concentrations of test substances used for feeding and lethality tests are shown in Table 3.3. Prior to carrying out exposures test substances were added to sediments ("spiked"). Numerous techniques are available for this purpose and selection is largely determined by the requirements of a particular study (Northcott and Jones 2000). The principle requirement for this work was the introduction of the test substance in a consistent manner to ensure homogenous exposure among replicates.

Table 3.3 Test substances and nominal concentrations used in lethality and feeding tests, all concentrations are given as mg test substance/kg dry sediment, (fc) = full control, (sc) = solvent control

Test substance	Lethality test concentration	Feeding test concentration
Pyrene	0(fc),0(sc),20,30,61,94,300	0(fc),0(sc),20,30,61,94,300
Cypermethrin	0(fc),0(sc),1.5,4,12,35,100	0(fc),0(sc),0.3,0.55,1.0,1.8
Chlorothalonil	0(fc),0(sc),0.2,0.75,2.8,10,40	0(fc),0(sc),0.2,0.75,2.8,10,40
Cadmium	0 (fc), 5,13,38,107,300	0 (fc), 5,13,38,107,300
Copper	0 (fc), 5,13,38,107,300	0 (fc), 5,13,38,107,300

3.3.3.1 Spiking of organic chemicals

A solvent carrier was used to spike organic chemicals into the sediment. The desired quantity of pyrene, cypermethrin and chlorothalonil (98% purity, Sigma-Aldrich, Germany) was added to 10ml of HPLC-grade acetone (Fisher Chemicals, UK). The solvent carrier was added to a 10L stainless steel bowl (Hobart food mixer) containing 3g of silica-sand (grain size <100µm, Fisher,U.K). The bowl was then gently rolled for 5 minutes by hand until the acetone had evaporated, thereby coating the sand with test substance. For each treatment group 750g of wet sediment was added to the bowl and thoroughly mixed for 30 minutes at approximately 500 rpm. Full and solvent controls were prepared in exactly the same way without the addition of test substances. Solvent controls were used to test for any toxicity associated with solvent addition.

After spiking, sediments were stored in the dark at 4°C in 1L Pyrex flasks until they were used. Storage times varied for each test substance according to previously published work regarding their partitioning behaviour and bioavailability after spiking in field-

collected sediments (Conrad *et al.* 2002;Leppanen and Kukkonen 2000;Maund *et al.* 2002). Pyrene and cypermethrin were stored for 14 and 2 days respectively prior to use. A storage time of 48 hours was selected for chlorothalonil, although no studies concerning partitioning behaviour in sediments were available.

3.3.3.2 Spiking of metals

Nominal target concentrations for metal exposures are given in Table 3.3. Addition of copper (as Cu (II) $SO_4{:}5H_2O$) and cadmium (as $3CdSO_4.8H_2O$) (AnalR grade, BDH, U.K) was carried out by dissolving the required quantity of metal salt to achieve nominal sediment concentrations in a 100ml solution of distilled water. The solution was then added to 750g of wet sediment using the same food mixer technique described above for organic chemicals. Immediately after spiking, sediments were stored in the dark at 4 ^{o}C in 1L Pyrex flasks for 48 hours prior to use.

3.3.4 Experimental set-up for feeding and lethality tests

For both feeding and lethality tests 24 hours prior to the start of the exposures 20g wet weight of spiked and control sediment (solvent and full) was distributed to 60ml tall glass beakers (Fisher,U.K). Beakers were then filled to the top with hard A.S.T.M water. The overnight settling period allowed for sufficient settling time and equilibration of beakers to test temperature. The next day, overlying water was exchanged in all beakers, prior to the addition of worms for the start of the contaminant exposure period. While it is likely that a

quantity of test substance associated with the water column phase would be removed by doing this, it was necessary, in order to observe worms after addition to test beakers at the start of exposures.

3.3.5 Measurement of exposure and post-exposure egestion rates

Egestion rates for both the exposure and post-exposure periods were measured using the methodology developed in Chapter 2 (Section 2.6.1.2). Five worms below 9mg wet-weight with visibly full guts were added to 5 replicate beakers containing spiked or control sediments. The beakers were then held at 20°C in complete darkness for six days. This constituted the contaminant exposure period. Overlying water was exchanged on Days 2 and 4, prior to each exchange dissolved oxygen, temperature and pH were measured in triplicate using a WTW Multiprobe 340i meter (WTW,Weilheim,Germany). Beakers were observed daily to check for mortalities and behaviour of worms.

Egestion rate during the contaminant exposure period was measured from Days 5-6. On Day 5 the sand layer was added to exposure beakers. Worms quickly protruded their tails above the sand and began egesting faecal pellets onto the sand layer. Egestion rates were then measured by collecting the faecal pellets that had been deposited onto the sand layer during the 24-hour period between Days 5 and 6. Triplicate procedural blanks were used throughout (sediment and sand layer, no worms).

On the same day as the exposure feeding period was started (Day 5), separate beakers were set-up to measure post-exposure egestion rate in clean sediment. Beakers, water and sediment quantities were the same as those used in the exposure period described above. Sediment was allowed to settle overnight and overlying water was removed the following day (Day 6). After collecting faecal pellets from exposure beakers on Day 6, the exposure period was ended by sieving worms from the contaminated sediments. Worms were then counted and five from each exposure beaker were transferred to a corresponding post-exposure beaker containing clean sediment. Once worms were observed to have fully burrowed into sediment (<60 minutes) the sand layer was added and post-exposure egestion rates were measured over a 24-hour period. On Day 7 faecal pellets were collected from post-exposure beakers. Worms were then sieved from the sediment and counted to check for any mortalities or reproduction.

3.3.6 Lethality tests

Nominal concentrations for lethality tests are given in Table 3.3. Beaker set-up, animal selection and exposure conditions were exactly the same as described previously for feeding tests (Section 3.3.4 and 3.3.5). Lethality tests ran for 6 days, beakers were observed daily and mortalities removed. Dead worms were only visible if on the sediment surface. Worms were deemed dead if completely immobilized, non-responsive to touch and pale or white in colour.

3.3.7 Sediment avoidance behaviour

In previous studies, sediment avoidance behaviour has been suggested as a mechanism by which oligochaetes alter their exposure to contaminated sediments (Kukkonen and Landrum 1994;Keilty *et al.* 1988a). During both feeding and lethality tests any worms avoiding the sediment at the end of the exposure period were noted. Worms were deemed to be actively avoiding sediments if visible on the surface but still responding to touch. Avoidance was expressed as the number of complete worms present on the sediment surface as a percentage of the total number of worms added to each jar at the start of test, minus any mortalities collected during the exposure period.

3.3.8 Chemical analysis of sediments

For both organic and metal substances, triplicate samples from the highest concentration were taken immediately after spiking in order to check for the effectiveness of mixing. Due to the problems associated with sampling sediment from the actual exposure beakers, designated analytical beakers were used in all experiments and were treated identically to exposure beakers containing worms. For organic chemical analysis triplicate sediment samples were taken at the start and end of the experimental period from the high, middle and low nominal concentrations along with the control. For metals triplicate samples were taken at the start and end of the experiment from the high and low target (nominal) concentrations. Triplicate procedural blanks (no sediment) were used throughout the extraction and analytical process for both organics and metals.

3.3.8.1 Extraction and analysis of organic substances

Sediment analysis of pyrene, cypermethrin and chlorothalonil was carried out by ultrasonic solvent extraction from sediments followed by high performance liquid chromatography (HPLC). Details of extraction solvents, HPLC columns, mobile phases, detectors, wavelengths and column retention times are given in Table 3.4. Prior to sampling for chemical analysis, moisture content was determined using triplicate sub-samples from analytical beakers. A known wet weight (approximately 2g) of sediment was then sampled and extracted with 20ml of HPLC-grade solvents in a Teflon tube. Samples were hand shaken for 30 seconds and sonicated for a further 30 minutes. Samples were then shaken using a benchtop shaker (IKA Laborteknik HS250, Staufen,Germany) for 30 minutes before sonication for a further 30 minutes. Samples were then centrifuged for 15 minutes at 2500 rpm. A 2ml aliquot of extracted solution was evaporated to dryness under a nitrogen stream evaporator (Model N-vap 112, Organonation Associated). The resulting residue was re-suspended with 2ml of the respective mobile phase. Samples were stored frozen at -20°C prior to HPLC analysis, which was within 1 week of extraction date.

Prior to injection onto HPLC columns the sample was passed through a Sartorius Minisart RC syringe filter (0.2um, Fisher, UK) to reduce potential interference from co-extractives. Filtered and unfiltered samples were analysed in order to check for potential loss of the target chemical through the filtration process, which was found to be negligible. A 50 μl sample was injected onto the HPLC column using a 4100 constametric pump (LDC

analytical) coupled with a Spectrasystem 3000UV detector and FL3000 fluorescence detector (Thermo-Finnigan, U.K). Mobile phase flow rate was 1ml/minute for all analysis. Quantification of measured (actual) concentrations were based on comparisons with peak areas of calibration standards (Sigma-Aldrich,Selze,Germany,>98% purity) using Chromquest computer software (Thermo-Finnigan,U.K). All glassware was thoroughly rinsed with HPLC-grade methanol, and then triple rinsed with Millipore water prior to use.

Table 3.4 Extraction solvents, mobile phases, columns and detectors used for high–performance liquid chromatography analysis (HPLC) for organic chemicals

Test substance	Extraction Solvent(s)	HPLC Column	Mobile Phase	Detector and wavelengths (nm)	Retention time (mins)
Pyrene	Ethyl acetate:MeOH (2:1)	Phenosphere next C18 5-µm 250 x 4.6mm	ACN: H_2O (80:20 v/v)	Flourescence (306*) (410**)	8.2
Cypermethrin	MeOH:DCM (3:2)	Supelcosil LC-ABZ 5-µm 250 x 4.6mm	ACN: H_2O (70:30 v/v)	UV – 210nm	6.9
Chlorothalonil	MeOH:DCM (3:2)	Phenosphere next C18 5-µm 250 x 4.6mm	MeOH:H_2O (60:40 v/v)	UV - 232nm	12.3

MeOH = Methanol, DCM = Dichloromethanol, ACN = Acetonitrile, * = excitation wavelength, ** = emission wavelength

3.3.8.2 Extraction and analysis of metals

Extraction of copper and cadmium from sediments was carried out using a nitric acid-hydrogen peroxide digestion. Approximately 2g wet weight of sediment was dried at 110°C for 24-hours and ground to a fine powder. A 500mg sub-sample was added to a 20ml Teflon screw top digestion vessel. 5ml of concentrated nitric acid (69%, Aristar, BDH,

U.K) was added and the sample was heated to 110°C for 24-hours. Once cooled 3ml of hydrogen peroxide (Aristar, BDH, U.K) was added in 1 ml steps until the sample became totally clear and ceased effervescing. Samples were re-heated at 110°C for a further 2 hours, allowed to cool and made up to 15ml with distilled water and centrifuged at 2000 rpm for 15 minutes.

A 1ml sub-aliqout was analysed by atomic absorption spectrophotometry using a Unicam GF90 graphite furnace (Thermo-Electron, U.K). Detection limits were 0.2 and 0.5ug/l for cadmium and copper respectively. Background levels of cadmium and copper in control sediment were used to adjust measured concentrations. All glassware was soaked overnight in 5% hydrochloric acid (Aristar, BDH, U.K) and then triple rinsed with Millipore water prior to use.

3.3.9 Tissue residue analysis for cadmium

Based on the results of initial lethality tests it was decided to measure the tissue levels of cadmium in *L.variegatus* at the end of a separate 6-day exposure, in order to determine the degree of cadmium uptake by the worms. Separate beakers containing low, medium and high cadmium concentrations (same sediment as feeding tests) were set-up and ran alongside feeding experiments (5,38 and 300mg/kg nominal). Control beakers containing "clean" sediment were used to correct for background cadmium accumulated over the course of the exposure period. Experimental set-up and exposure conditions were the same

as those described for feeding lethality tests, except that only 3 replicates per treatment were used.

At the end of the six-day exposure worms were sieved from each beaker for analysis of tissue cadmium levels. In order to correct for the contribution of sediment-associated metals passing through the gut, worms were placed in water-only beakers containing hard ASTM water for 8 hours to purge their gut contents. *L.variegatus* has previously been demonstrated to empty 98% of its gut content after six hours in water (Mount *et al.* 1999). After purging worms were rinsed with nanopure water, wet weighed and then washed in a solution of EDTA (74mg/l ethylenediaminetetra-acetic acid disodium salt, AnalaR grade, BDH,Poole,UK) for 30 minutes to remove any surface-bound metal. Worm tissue was digested and subsequently analysed using the methods for sediment metals analysis described previously (Section 3.3.8.2). To express tissue cadmium levels on a dry weight basis worm wet weights were converted to dry weights using the average wet:dry weight ratio of 30 worms taken from cultures.

3.3.10 Statistical analysis

For both feeding and lethality tests full and solvent controls were compared using two-tailed t-test's (Zar 1999) to test for any effects of solvent addition. Once it was determined that solvent addition had no significant effect on the measured endpoints solvent control data were excluded from the analysis. Full controls were compared to treatment groups using one-way analysis of variance. Fishers least significant difference test was used for

post-hoc multiple comparisons to identify individual treatments that were significantly different from controls. In cases where after attempting transformations the assumptions of parametric methods were still not met a Kruskall-Wallis test was used to test for treatment effects and a Mann-Whitney U-test carried out to identify those treatment groups that were significantly different from controls. All statistical procedures were carried out using an alpha level of 0.05 with Minitab version 13.

The egestion inhibition concentration (IC_{50}) represented the concentration that reduced egestion rate to 50% of the corresponding full control over the 24-hour exposure period (Days 5-6). IC_{50} values could only be calculated in those experiments where the mean response was reduced by at least 50% in one or more of the treatment groups. The IC_{50} was calculated using linear (pyrene) or sigmoidal dose-response (cypermethrin) regression models in Sigmaplot version 11. Regression significance was determined with analysis of variance and model fit and accuracy were determined by examining standardized residuals versus fitted values, normality tests of residuals and the adjusted co-efficient of determination (r^2). Confidence intervals for linear regression IC_{50} estimates were calculated using an inverse prediction technique (Zar 1999). Those for the sigmoid dose-response IC_{50} estimate were calculated using the regression output in Sigmaplot version 11.

Median lethal concentration (LC_{50}) values and associated 95% confidence intervals were calculated using a standard probit procedure available in Minitab version 13. LC_{50} estimates could only be made where survival was reduced to at least 50% of the controls.

3.4 RESULTS

3.4.1 Water quality analysis

Throughout feeding and lethality tests dissolved oxygen levels of overlying water were always above 70% saturation and temperature and pH were deemed to be within an acceptable range (Temp $20^{o}C \pm 0.2^{o}C$, pH 6.9-7.5).

3.4.2 Sediment chemical analysis

Analysis of sediments immediately after spiking found a coefficient of variation of less than 20% in measured levels of all test substance, which has previously been suggested to indicate an acceptable level of homogeneity (Northcott and Jones 2000). With the exception of pyrene, all test substance concentrations measured at the start of exposures were reduced by more than 10% from nominal target concentrations. Initial concentrations of cypermethrin, chlorothalonil, cadmium and copper were 68%,72%,63% and 61% respectively of nominal concentrations. Therefore measured concentrations were used for all subsequent analysis of experimental results. Actual pyrene concentrations did not significantly decrease over the course of the 6-day exposures. Cypermethrin, chlorothalonil, cadmium and copper concentrations were reduced by 7%, 23%,8% and 8% respectively.

3.4.3 Comparison of endpoint sensitivity

From Figure 3.1(a) it can be seen that during direct exposure to pyrene egestion rates were significantly reduced to less than 50% of controls. However, the estimated IC_{50} for pyrene (Table 3.6) cannot be compared to a reliable LC_{50} estimate as survival was only significantly reduced to 75% of controls in the highest treatment group (300mg/kg). It was not possible to measure feeding rate during exposure to the 300mg/kg pyrene treatment as all worms were on the surface of sediments by Day 6, which prevented the addition of the sand layer.

Direct exposure to cypermethrin reduced both worm survival and egestion rates to less than 50% of controls in separate lethality and feeding tests (Figure 3.1c). This enabled both LC_{50} and IC_{50} values to be calculated (Table 3.6). The feeding IC_{50} for cypermethrin was approximately 38 times lower than the LC_{50}.

Table 3.5 Summary of statistical test results for contaminant effects on exposure and post-exposure egestion rates, F and H values are given from ANOVA and Kruskall-Wallis tests respectively

Test substance	Exposure				Post-exposure			
	F value	H value	df	P value	F value	H value	df	P value
Pyrene	-	44.11	5	<0.001	-	43.7	5	<0.001
Cypermethrin	-	25.23	5	<0.001	30.8	-	5	<0.001
Chlorothalonil	10.48	-	5	<0.001	2.13	-	5	0.075
Cadmium	0.53	-	5	0.75	2.08	-	5	0.096
Copper	1.32	-	5	0.28	1.25	-	5	0.31

Exposure to chlorothalonil had no effect on survival in lethality tests so no LC_{50} could be calculated. Chlorothalonil had a significant effect on the egestion rate of worms during the exposure period at all of the concentrations tested (Figure 3.1e and Table 3.5). However, egestion rates were not reduced below 50% of controls in any of the treatments. Therefore, a reliable estimate of the feeding IC_{50} could not be calculated (Table 3.6).

Exposure to cadmium and copper had no effect on both worm survival and egestion rate (Figure 3.1(g-j) and Table 3.5) during direct exposure. Therefore, no LC_{50} or IC_{50} values could be calculated (Table 3.6).

Table 3.6 Estimated 6-day lethal concentration (LC_{50}) and egestion rate inhibition concentration (IC_{50}) values, > indicates where 50% lethality or feeding inhibition was not achieved at the highest concentration tested, adjusted co-efficients of determination (r^2) from IC_{50} regressions are given where appropriate, ( ) = 95% confidence intervals for estimates.

Test Substance	LC_{50} as mg/kg	IC_{50} (mg/kg)	IC_{50} adj r^2
Pyrene	>300	62.1 (45.7-78.5)	0.89
Cypermethrin	24.6 (19.3-31.9)	0.66 (0.52-0.78)	0.93
Chlorothalonil	>29	>29	-
Cadmium	>173	>173	-
Copper	>184	>184	-

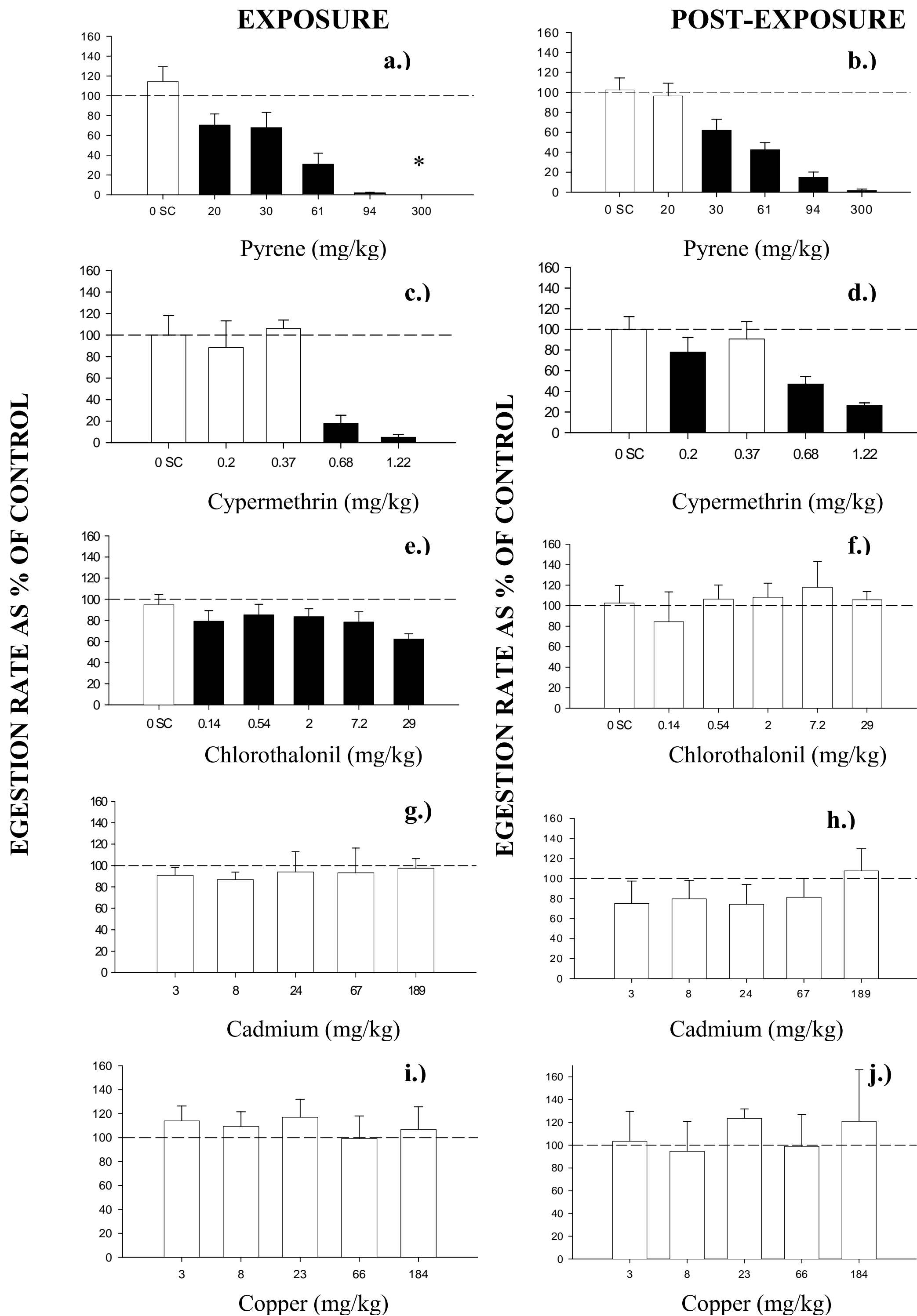


Figure 3.1 (a-j) Exposure and post-exposure egestion rate of *L.variegatus* as a % of control rate. Dashed line is the control feeding rate (100%). Black bars denote where egestion rates are significantly different from controls (p=<0.05), error bars represent 95% confidence intervals, * egestion rate not measured due to worms avoiding sediment, n=5

3.4.4 Post-exposure egestion rates

Both pyrene and cypermethrin had a significant effect on post-exposure egestion rates (Table 3.5). From a comparison of Figures 3.1a,b,c and d, most treatments that significantly reduced egestion rates during exposure also caused a significant reduction post-exposure. There was some evidence of recovery in post-exposure egestion rates with both pyrene and cypermethrin. For example, egestion rates in the 0.68mg/kg cypermethrin treatment were reduced to 18% of controls during the contaminant exposure period, but recovered to 47% of control feeding rates during the post-exposure period. However, post-exposure egestion rates only recovered to control levels in the lowest pyrene treatment (20mg/kg) after previously being inhibited (Figure 3.1b).

For chlorothalonil none of the treatments that had previously caused feeding inhibition during the exposure period were found to cause persistent feeding inhibition during the subsequent post-exposure period (Figures 3.1e and f).

Both cadmium and copper had no significant effect on post-exposure egestion rates (Figures 3.1 g-j). As can be seen from Figure 3.1(h) post-exposure egestion rates were slightly reduced in all but one of the cadmium treatments, but the difference was not statistically significant (Table 3.5).

3.4.5 Sediment avoidance behaviour

There was no observed avoidance of sediments containing chlorothalonil, cadmium and copper in both lethality and feeding tests. There was also no avoidance in feeding tests with pyrene, cypermethrin and chlorothalonil at concentrations that caused significant feeding inhibition during exposure. Sediment avoidance behaviour was observed at the higher concentrations used during lethality experiments with cypermethrin, but avoidance only occurred at concentrations that also caused significant mortalities. Avoidance was only observed at the highest pyrene concentration (300mg/kg), where 100% of worms were on the surface of sediment after 6 days. In all the lower pyrene treatments no worms avoided the sediment, despite significant feeding inhibition.

3.4.6 Tissue residues of cadmium

Figure 3.2 highlights that significant quantities of cadmium were accumulated in *L.variegatus* tissue over the course of six days. This confirmed that the metal was available for uptake and accumulation in worms, and therefore, to potentially cause biological effects (i.e survival, feeding inhibition).

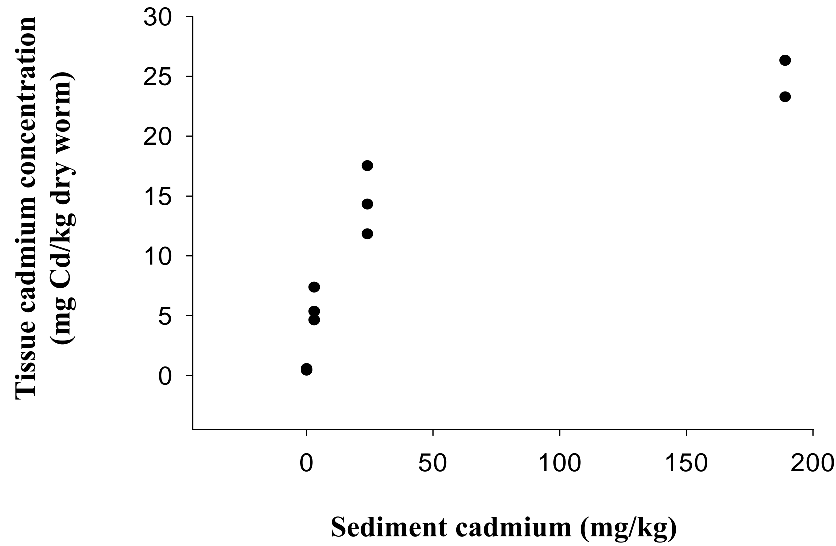


Figure 3.2 *L.variegatus* tissue cadmium levels (n=3) after a 6-day exposure to three concentrations of cadmium-spiked sediments (3,24 and 189mg/kg) and a control (0mg/kg)

Table 3.7 shows the concentrations of cadmium measured in sediment and worms and the calculated biota-sediment accumulation factors (BSAF) for cadmium after 6 days. There is an inverse relationship between sediment cadmium concentrations and BSAF. For instance the lowest measured cadmium concentration (control, 0.004mg/kg) has an approximately 58 times greater BSAF than that of the highest cadmium concentration.

Table 3.7 Sediment and tissue cadmium levels from the cadmium uptake experiment with calculated BSAF shown (BSAF = (tissue cd) / (sediment cd), all concentrations refer to mg cadmium/kg dry sediment or dry tissue, standard deviations in brackets

Sediment cadmium concentration	Tissue cadmium concentration	BSAF
0.064 (0.004) (control)	0.49 (0.07)	7.65
3.0 (0.55)	5.8 (1.41)	1.93
24 (2.6)	14.5 (2.85)	0.60
189 (14.1)	25.3 (1.76)	0.13

3.5 DISCUSSION

3.5.1 Endpoint sensitivity to organic substances

Comparisons of the results from lethality and feeding tests with organic substances confirmed the sensitivity of egestion rate as a sub-lethal endpoint. The IC_{50} for egestion rate with cypermethrin was 38 times lower than the corresponding LC_{50}. The IC_{50} was also below average environmental concentrations of cypermethrin (0.74 mg/kg) reported in a recent study of sediments in the Humber River catchment (U.K) (Long *et al.* 1998). A variety of insecticides have previously been shown to reduce the feeding rates of aquatic deposit feeders at sub-lethal concentrations (Rubinstein 1979), although the effects of pyrethroid insecticides have not been studied to date(Keilty *et al.* 1988b;Meller *et al.* 1998;Mulsow *et al.* 2002). An LC_{50} of 72 mg/kg was found after exposing the terrestrial oligochaete, *Aporrectodea caliginosa*, to cypermethrin-spiked soil (Mosleh *et al.* 2003). During the same study sub-lethal effects on growth were found, but no effect concentrations were reported.

The IC_{50} for pyrene was estimated as 62mg/kg. Due to insufficient mortality at the highest exposure levels, an LC_{50} for pyrene could not be calculated. However, egestion rates were reduced to 2% of corresponding controls in the 94mg/kg treatment while no effects on survival were found. Clearly survival of *L.variegatus* is a far less sensitive endpoint with pyrene exposure when compared to measurements of egestion rate. In a previous study using pyrene a 10-day egestion rate IC_{25} of 51mg/kg was found for the

tubificid oligochaete, *Limnodrilus hoffmeisteri*, whereas mortality was always less than 20% at even the highest concentrations tested (i.e 841mg/kg) (Lotufo and Fleeger 1996). While it was not possible to calculate an LC_{50} for pyrene with the current data it seems reasonable to suggest that it would be substantially higher than the IC_{50} for egestion rate calculated in this work.

The low mortality in high pyrene treatments is in close agreement with previously published work. In the highest pyrene treatment in this study (300mg/kg) mortality was 25% after 6 days exposure. In a previous study with *L.variegatus* mortality was only 17% after a 7-day exposure to 269mg/kg of pyrene (Kukkonen and Landrum 1994). The authors suggested that the low acute mortality was a result of the behavioural modification of toxicant exposure as worms avoided burrowing into the sediment. Similar avoidance behaviour was found in the 300mg/kg treatment.

Feeding during direct exposure to chlorothalonil was reduced in all treatment groups while survival was not affected. Feeding rates were not sufficiently reduced to be able to calculate an IC_{50} for chlorothalonil, but again it seems reasonable to suggest that the IC_{50} would be lower than the LC_{50}. Previous studies on the effects of chlorothalonil on aquatic invertebrates are limited to those concerning acute lethality of species inhabiting the water-column. As no sediments were used in these studies it is difficult do draw any relevant comparisons with previously published work.

3.5.2 Post-exposure feeding inhibition

As highlighted previously, a reduction in egestion rate during exposure should be shown to be sufficiently persistent to be measured during the post-exposure period, in order for the bioassay to be useful for *in situ* studies. Exposure to both cypermethrin and pyrene caused feeding inhibition that was sufficiently persistent to be detected during the post-exposure period. This confirms that for these toxicants post-exposure feeding inhibition is a persistent, sub-lethal response. There was evidence of recovery in egestion rates during the post-exposure period for both pyrene and cypermethrin, but the only occasion where this resulted in egestion rates recovering to control levels was in the lowest pyrene treatment (20mg/kg).

Recovery in the 20mg/kg pyrene group may be a result of the toxic mode of action and/or toxico-kinetics of pyrene. PAH's have previously been shown to reduce feeding rates at sub-lethal concentrations in a variety of aquatic invertebrates (Reible *et al.* 1996;Lotufo and Fleeger 1996;Lotufo 1997;Mcwilliam and Baird 2002a). In the absence of photo-induced toxicity it is suggested that PAH's such as pyrene remain relatively non-toxic until body residues are sufficient to cause non-polar narcosis (Monson *et al.* 1995;Vanwezel and Opperhuizen 1995). After a 7-day exposure to 0.4ug/kg of pyrene elimination by *L.vareigatus* was rapid in the presence of clean sediment and a half-life of 27.1 hours was calculated (Kukkonen and Landrum 1994).

While the concentrations used in the above study are lower than those used in this work, rapidly reversible narcosis and elimination may explain the recovery in egestion rates of the 20mg/kg pyrene treatment. Similar rapid recovery was also found with *D.magna* feeding behaviour in clean media after previous inhibition with fluoranthene (Mcwilliam and Baird 2002a). However, it is important to emphasise that recovery was only seen in the lowest concentration and in the next highest concentration (30mg/kg) egestion rates were sufficiently reduced to enable detection during the post-exposure period.

Unlike pyrene and cypermethrin, feeding inhibition due to chlorothalonil exposure was not sufficiently persistent to be measured during the subsequent post-exposure period. This would suggest that worms were able to rapidly recover from the negative effects of chlorothalonil and resume normal feeding rates. Previous studies of invertebrate feeding behaviour with exposure to chlorothalonil are lacking. There is also a lack of comparable studies on the potential modes of action of such fungicides with aquatic invertebrates. Exposing the channel catfish, *Ictalurus punctatus* to sub-lethal levels of chlorothalonil resulted in acute necrosis of the intestinal epithelial lining after 6 days (Gallagher *et al.* 1992). Similar damage to gut physiology of *L.variegatus* may have resulted in an alteration in feeding behaviour, but it seems unlikely that this effect would be reversible during the relatively short post-exposure period.

From Figure 3.1e it can be seen that there is a relatively weak dose-response pattern with egestion rates during exposure to chlorothalonil, despite the use of a wide range of concentrations. It is suggested that if chlorothalonil caused physiological damage or

poisoning during the exposure period a stronger dose-response pattern might be expected. This could suggest that feeding inhibition was a result of a rapidly reversible mechanism, rather than actual whole body damage or poisoning. Elimination or metabolism of chlorothalonil may also have occurred. A previous study with blue mussel, *Mytilus edulis*, found significant uptake of chlorothalonil during the first 24 hours of exposure but then found rapid elimination from tissues (Ernst *et al.* 1991). An inducible elimination/metabolism mechanism was suggested as a cause for the reduction in chlorothalonil tissue levels. The results from chlorothalonil exposures highlight that the post-exposure feeding response may not be sensitive to all chemicals, due their mode of action and/or toxico-kinetics.

3.5.3 Sediment avoidance behaviour

Sediment avoidance behaviour of *L.variegatus* was not the endpoint of interest in this work, but observations of avoidance behaviour during toxicity tests provided some interesting comparisons to feeding rates. In all cases where sediment avoidance was observed mortality also occurred, which was in contrast to feeding inhibition as an endpoint. Secondly, feeding inhibition was measured at concentrations that did not result in sediment avoidance behaviour. For instance, during exposure to 94mg/kg pyrene egestion rates were reduced to 2% of controls on average, whereas no sediment avoidance was observed. Therefore, the findings in this work with 3 representative contaminants suggest that feeding inhibition of *L.variegatus* is significantly more sensitive as a bioassay endpoint when compared to sediment avoidance behaviour.

The above findings may be significant in light of current recommendations for carrying out bioaccumulation assays with *L.variegatus* (U.S.E.P.A 2000). In those guidelines an initial 4-day toxicity test is recommended prior to bioaccumulation testing. Endpoints for the toxicity test are survival and sediment avoidance behaviour. Avoidance behaviour is included as an endpoint because of the potential for underestimating bio-accumulation potential due to reduced particle ingestion if *L.variegatus* avoids sediments (U.S.E.P.A 2000). Therefore, if reduced particle ingestion is a concern perhaps the guidelines should incorporate the measurement of feeding behaviour, as it is clear ingestion may be almost completely inhibited before concentrations triggering avoidance are achieved. Unfortunately tissue residue data was not available from this work to test this hypothesis more thoroughly.

3.5.4 Metals and feeding behaviour

Neither copper nor cadmium affected worm survival or feeding rates during the exposure and post-exposure periods. There was no avoidance of metal-contaminated sediments, which could have otherwise suggested modification of exposure. The results of tissue analysis for cadmium confirmed that significant quantities of the metal were available for uptake and subsequent accumulation, so the lack of biological effects for cadmium cannot be explained by poor metal bioavailability. The lack of significant mortality in metal exposures compares well with a number of previous studies of acute metal toxicity with oligochaetes e.g (Meller *et al.* 1998), but is in contrast with results for

copper and cadmium-spiked sediments using *L.variegatus* (Chapman *et al.* 1999). In that study significant mortality (LC_{50} not calculated) was found during direct exposure to 16mg/kg and 50mg/kg of cadmium and copper respectively, which is significantly lower than the concentrations used in this work.

The lack of agreement between Chapman *et al's* study (Chapman *et al.* 1999) and the results from the present work may relate to the use of an artificial sediment in the former work. Artificial sediments have previously been found to increase metal bioavailability and toxicity when compared to field-collected sediments (Harrahy and Clements 1997). If the biota:sediment accumulation factors for cadmium from this work (Table 3.7) are compared to those from Chapman *et al's* study they would seem to support the suggestion of increased bioavailability with artificial sediments. For instance worms exposed to 3mg/kg cadmium in this study produced a 6-day BSAF of 1.93, while in Chapman *et al's* study a similar cadmium concentration (3.6mg/kg) produced a 14-day BSAF of 13.1. It is unclear how much of this variability in tissue cadmium is attributable to differences in the sediment or differences in the length of exposure periods, but this does highlight the difficulty in effectively comparing biological effects in the two studies.

The length of exposure period selected in the present work may also have reduced the potential for feeding inhibition with metals. A 6-day exposure was selected for laboratory studies on the basis of what would be deemed practical for subsequent use *in situ*. However, the calculated BSAF for cadmium showed an inverse relationship with sediment concentration (Table 3.7). This would suggest that cadmium did not reach its maximum

concentration in worm tissues during the six-day exposure, particularly in the high concentrations. Thus, for example, Mendez and Baird (2002) found that survival and egestion rates of the polychaete, *Capitella capitata* were not significantly reduced by a 5-day exposure to 140 mg/kg cadmium, but egestion rates were reduced after a longer 10-day exposure at the same concentration, without causing significant mortality. This would highlight the importance of the length of the exposure period in determining the biological effects of metals.

Rather than too short an exposure period, the lack of a response in *L.variegatus* to metals could also be a reflection of a high tolerance of oligochaetes more generally (Klerks and Levinton 1989;Klerks and Bartholomew 1991;Bouche *et al.* 2000;Gillis *et al.* 2002;Gillis *et al.* 2004). A previous study with *L.variegatus* found evidence of a capacity to detoxify accumulated cadmium through the use of metallothionein-like proteins (MTLP's) and metal rich granules (Bauer-Hilty *et al.* 1989). The time frame for induction of this protective mechanism was not determined in the above study, but enhanced synthesis of MTLP's was measured in the tubificid oligochaete, *Tubifex tubifex* after only 8 hours exposure to cadmium-spiked sediments (Gillis *et al.* 2004). This would suggest that oligochaetes such as *L.variegatus* are capable of inducing such a protective mechanism over as short a time frame as that used during these exposures. However, it should be highlighted that short-term protection against the effects of metals in oligochaetes may have an associated energetic "cost", in terms of the resources available for growth and reproduction.

3.6 SUMMARY

The main points to be taken from this section of work are as follows:

- Feeding inhibition with *L.variegatus* has been shown to be sensitive to 3 of the 5 chemicals tested during direct exposure at concentrations that did not cause lethality or sediment avoidance behaviour
- Feeding inhibition during the exposure period persisted during the post-exposure period in clean sediment with 2 of the 3 organic chemicals. This confirmed that feeding inhibition was sufficiently persistent to be used as a post-exposure endpoint
- For some toxicants e.g chlorothalonil feeding inhibition may be caused by a rapidly reversible mechanism that would limit detection during the post-exposure period
- The apparent insensitivity of *L.variegatus* to metals may be due to the relatively short exposure time and/or the ability to cope with significant metal accumulation in body tissues via rapidly inducible detoxification mechanisms

While the bioassay endpoint has not been demonstrated to be sensitive to all of the chemicals tested, adaptation and testing in field situations where organic chemical contamination is a primary concern may provide further useful information on the bioassays sensitivity and applicability (Chapter 4). Additional work relating the longer-term implications of short-term toxicant-induced feeding inhibition for *L.variegatus* populations

could also help to further establish the ecological relevance of the bioassay endpoint (Chapter 5).

4 CHAPTER 4 – ADAPTATION AND TESTING OF THE BIOASSAY IN THE FIELD

4.1 INTRODUCTION

In the previous chapters a method was firstly developed for measuring post-exposure egestion rate of *L.variegatus* under "clean" conditions (Chapter 2). This method was then used to study post-exposure feeding inhibition under controlled toxicant exposures in the laboratory (Chapter 3). The focus of this chapter was the adaptation and testing of the bioassay technique under field (*in situ*) conditions. This involved exposing test organisms to field sites for a 6-day period then subsequently measuring feeding behaviour during a 24-hour post-exposure period using the method developed in Chapter 2. Exposure to a variety of potentially contaminated sites would help to determine if the bioassay was sufficiently sensitive to detect toxicity-induced feeding inhibition.

An overview of the reasoning behind the use of *in situ* bioassay techniques, feeding behaviour as an *in situ* bioassay endpoint, and the adoption of *L.variegatus* feeding behaviour as an *in situ* bioassay endpoint was previously given in Chapter 1. The use of invertebrate feeding behaviour with *in situ* bioassays was developed from early work using the "scope for growth" (SFG) of transplanted marine mussels e.g (Donkin and Widdows 1986). An organism's SFG predicts the energy available for growth and reproduction from physiological-based energy budgets using the amount of energy ingested, egested, assimilated, metabolised and stored. Negative SFG under contaminant stress may occur due

to the energetic demands of detoxification and excretion processes (i.e “demand side responses”), or through a reduction in the energy ingested and/or assimilated (i.e “supply side responses”)(Barber *et al.* 1990).

Negative SFG in mussels was reported with exposure to a variety of contaminant stressors e.g (Lack and Johnson 1985;Widdows and Page 1993;Widdows *et al.* 1995). The SFG approach has since been adopted for freshwater macro-invertebrates such as the amphipod, *Gammarus pulex* (Naylor *et al.* 1989;Maltby *et al.* 1990b;Maltby *et al.* 1990a). As energy intake is of fundamental importance to the SFG equation, reductions in energy intake are often the main cause of a reduction in SFG (Maltby 1999). Recognition of the importance of feeding inhibition in changes to SFG has led to the development of a more straightforward measurement of *G.pulex* feeding rate under field conditions (Maltby *et al.* 1990a) (Maltby 1992).

In order to measure *in situ* feeding rate of *G.pulex*, animals are exposed to field sites in small chambers that contain a defined quantity of food (usually pre-wetted leaf discs). Feeding rates are then quantified by measuring the consumption of the leaf discs. This technique has been used to study the effects of a variety of contaminants/stressors e.g (Crane and Maltby 1991;Crane *et al.* 1995;Maltby *et al.* 2000;Maltby *et al.* 2002). The leaf disc technique has also been extended for use with other amphipod species (Hatch and Burton 1999). However, not all invertebrate species possess feeding habits that are amenable to measurement directly *in situ* (Section 1.4.4). This has led to the development of post-exposure feeding inhibition as an *in situ* bioassay response with *D.magna* and

L.peregra respectively (Mcwilliam and Baird 2002b;Crichton 2003). Extension of the post-exposure feeding approach to a true deposit-feeding organism such as *L.variegatus* could provide an ecologically relevant and sensitive endpoint for assessing contaminated sediments *in situ*.

4.1.1 Study design considerations

Techniques for exposing macro-invertebrates to *in situ* conditions vary considerably according to the demands of the study design. Single-species bioassays usually involve transplanting animals from laboratory cultures, or the collection of individuals from natural populations at unimpacted sites. Exposure to field conditions involves holding animals in some form of chamber, and then measuring the desired endpoint during or at the end of the exposure period (Chappie and Burton 2000). By manipulating the design of the holding chambers test organisms may be exposed to the water column and/or the underlying substrate separately, and thus identify the major source(s) of contamination (Hatch and Burton 1999;Crane *et al.* 2000). This approach may be particularly useful in studies where overlying waters are relatively uncontaminated but sediments may still contain a legacy of historical pollution problems.

Although the separation of environmental media may improve the discriminatory power of *in situ* sediment bioassays, it may also reduce the realism of any exposure as animals in the natural environment are usually exposed to a variety of contaminant uptake routes at the same time. Separation of water column and sediment may also cause undesirable alterations

to water quality that could interfere with the bioassay endpoint e.g (Crane *et al.* 2000). The primary aim of the current work was to improve the realism of any bioassay exposure, rather than determine the importance of individual uptake routes. Chamber design considerations were therefore based on trying to create as realistic an exposure scenario as possible. This required that worms were exposed to all forms of potential uptake routes i.e water column and sediment. Therefore, worms should have free access to the sediment, and water should exchange freely between the bioassay chamber and the surrounding environment. Further, since disruption of the sediment layer may influence contaminant bioavailability and toxicity (Section 1.2.3), bioassay chambers should also minimise sediment disruption during deployment. Additional considerations included how effective the chambers were in containing animals and their ease of use in a variety of field situations.

4.2 AIMS

The aims of this chapter were:

- To successfully deploy *L.variegatus* in bioassay chambers at a variety of field sites
- To determine if the bioassay was sufficiently robust for use in field situations i.e. if sufficient numbers of live, actively feeding worms were recovered from uncontaminated sites to carry out the post-exposure feeding bioassay

- To adapt the laboratory technique for measuring post-exposure egestion rate with *L.variegatus* for *in-situ* use
- To determine if the bioassay was sufficiently sensitive to a variety of *in-situ* contamination problems. On the basis of results from Chapter 3 it was hypothesised that post-exposure egestion rate of *L.variegatus* will be reduced at contaminated sites when compared to upstream sites.

4.3 MATERIALS AND METHODS

4.3.1 Field study sites

Bioassay deployments were carried out during the summer of 2003 at field sites in 3 areas of New Brunswick, Eastern Canada (Figure 4.1 and Table 4.1). All field sites were selected with the help of research staff based at the University of New Brunswick (Fredericton and St John) and the National Water Research Institute (Fredericton). Sites were selected to represent a variety of river systems that were exposed to point-source and diffuse pollution sources.

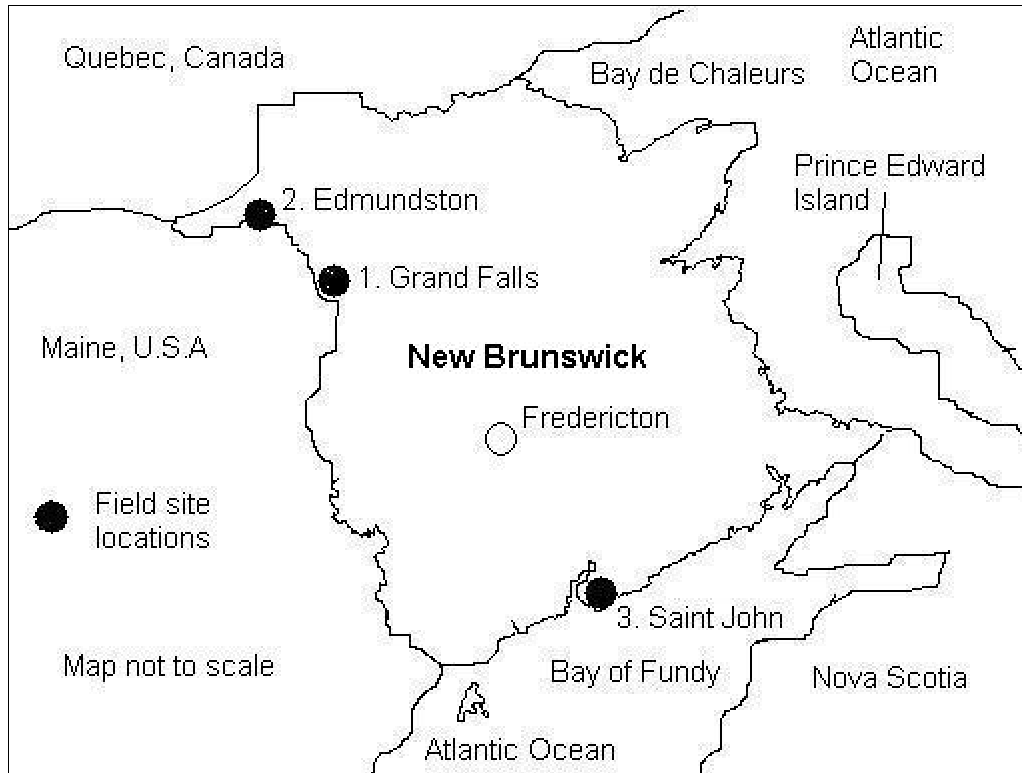


Figure 4.1 The location of the three field site locations within New Brunswick, Canada

Table 4.1 Overview of individual deployment sites within each of the study areas, US = upstream, DS = downstream

Study area	Deployment Date(s)	Site name	Site description	Potential impact(s)	Grid reference
Little River near Grand Falls	1st - 03/07/03 2nd - 05/08/03 3rd - 19/08/03	LR1	Confluence of Ten Mile Brook with the Little River	US ("control")	47 ° 10.22'N 67 ° 40.59'W
"	" " "	LR2	Black Brook, US of confluence with Dead Brook	Agriculture	47 ° 04.86'N 67 ° 44.63'W
"	" " "	LR3	"Bled" – US of confluence with the Little River	Agriculture	47 ° 05.03'N 67 ° 43.90'W
"	" " "	LR4	Little River, DS of confluence with "Bled"	Agriculture	47 ° 04.91'N 67 ° 42.84'W
Saint John River near Edmundston	19/07/03	Ed1	Approx 50km US of Edmundston	US ("control")	47 ° 11.26'N 68 ° 52.94'W
"	"	Ed2	Edmundston, DS of paper mill effluent and sewage	Pulp and paper mill effluent,sewage	47 ° 21.64'N 68 ° 19.57'W
"	"	Ed3	4km DS Edmundston, downstream of pulp mill effluent	Pulp and paper mill effluent, sewage	47 ° 21.48'N 68 ° 16.23'W
Little River near Saint John	10/09/03	St J1	800m US of effluent pipe	US ("control")	45°17.20'N 66°01.16'W
"	"	St J2	500m US of effluent pipe	US ("control")	45°17.23'N 66°01.17'W
"	"	St J3	200m US of current effluent pipe, site of old effluent pipe	Mixed industrial effluent e.g hydrocarbons, metals	45°17.29'N 66°01.14'W
"	"	St J4	Vicinity of effluent pipe	Mixed industrial effluent e.g hydrocarbons, metals	45°17.25'N 66°01.13'W

4.3.1.1 Little River near Grand Falls

The Little River is a fourth-order stream that eventually joins with the Saint John River at the town of Grand Falls. In its upper portion the river largely drains forestry plantation areas. Further down the catchment intensive agriculture increasingly dominates land use pattern, with potato as the most common crop. The dashed line in Figure 4.2 represents the transition area from largely forested land in the north and east, to that dominated by agriculture in the south and west. Potatoes are usually grown in rows that are regularly ploughed throughout the growing season. During the frequent heavy rainfall events that occur during the summer, surface water flow is concentrated in these channels and considerable soil erosion can take place. This results in large inputs of sediment into the streams surrounding agricultural areas, which causes a very visible change to the physical habitat of the streambed when compared to upstream sites. Physical alteration of aquatic habitats via increased sedimentation is well recognised as a major impact of intensive agricultural practices (Merrington *et al* 2002). Soil erosion may also promote the eutrophication of surrounding streams, via the release of nutrients such as phosphorous from the soil aggregate. Eutrophication may then be further enhanced through the application of fertilisers during the production cycle.

Along with alterations to physical habitat, concern has also been raised regarding the potential for contamination of surface and groundwater bodies via the intensive use of pesticides (Milburn *et al.* 1991;Oneill *et al.* 1989). A variety of insecticides, herbicides and fungicides are used in an attempt to control the pest species of potato production (Caux *et*

al. 1996). Applied pesticides may be transferred to surrounding water bodies in solution via surface or groundwater run-off (Merrington *et al* 2002). Many pesticides also show a tendency to bind to soil particles shortly after application, so the risk of contamination in surrounding streams may be significantly increased where soil erosion is also accelerated (Matthiessen *et al.* 1992).

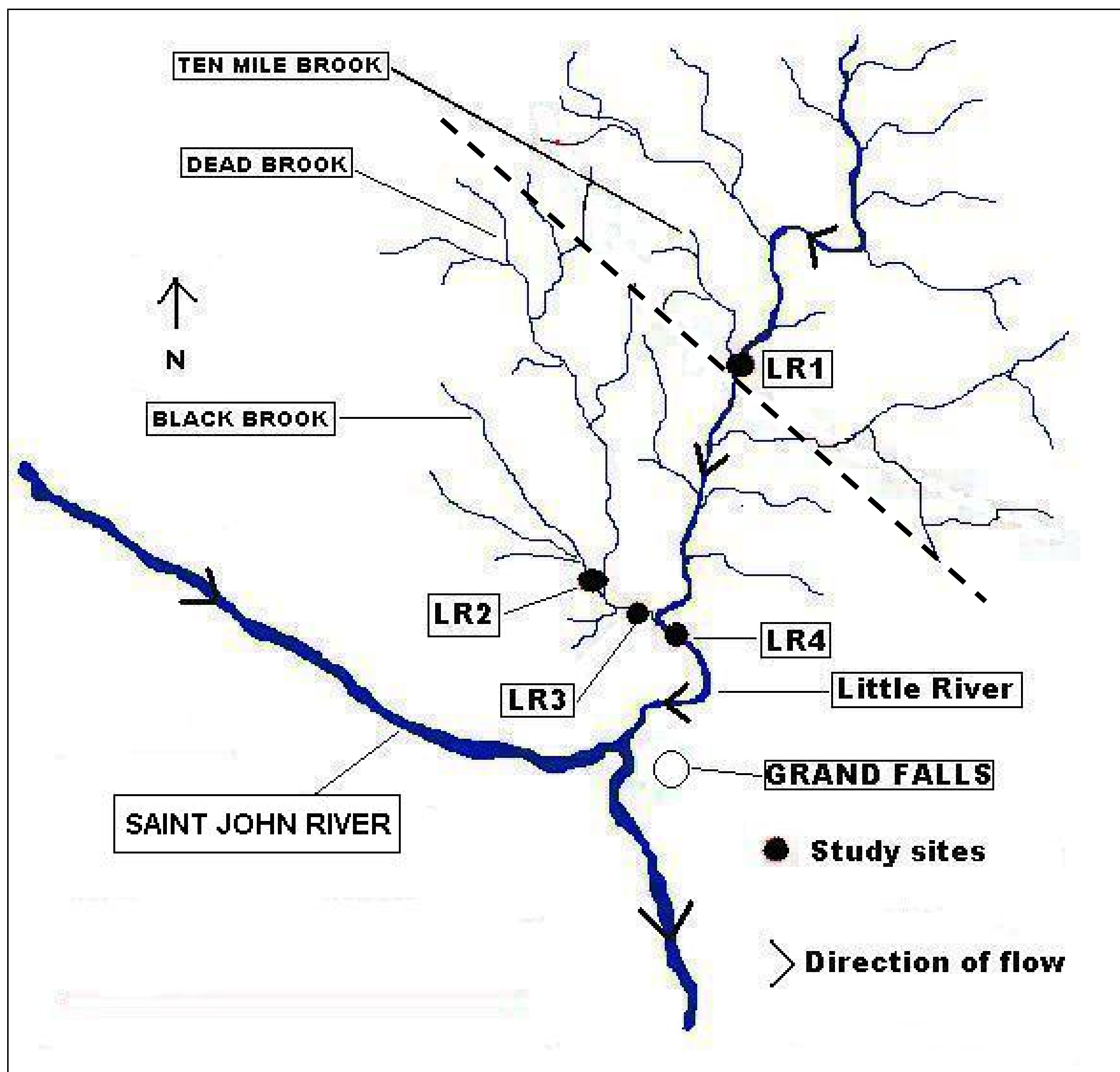


Figure 4.2 Location of the four sites within the Little River watershed near Grand Falls, the dashed line marks the approximate boundary from largely forested land upstream to that dominated by agriculture downstream (map not to scale)

Within the Little River catchment the most intensive area of agricultural land use is in the Black and Dead Brook watersheds (M.Gray, *pers comm*). For this reason two of the three "impacted" sites were chosen from this area. It was also decided to carry out repeated deployments over the relatively short (May-September) summer growing season. Many fungicides and herbicides are regularly applied on a prophylactic basis during this period, whereas insecticides are more commonly applied in response to a problem infestation. So although no data was available for environmental levels of commonly used pesticides, it was thought that there could be significant differences in potential exposure during the summer. Periodic heavy rainfall events over the summer months will also likely play a role in pesticide exposure, as surface water run-off and soil erosion are very much increased at this time. Three deployments of the bioassay were therefore carried out on the 3rd July, and the 5th and 19th August 2003.

Site LR1 marks the approximate transition between mostly forested areas upstream and land increasingly dominated by potato production downstream (Figure 4.2). Bioassay chambers were placed in an area of soft substrate, at the site of the confluence of Ten-Mile Brook with the main Little River channel. At this point flow rate was 0.04m/second, channel width was 6.5m and depth was 0.6m. The substrate was largely sand and gravel with a thin overlying layer of finer particles and coarse organic debris (leaves etc).

Site LR2 was situated at Black Brook, which is a major tributary stream within the Little River catchment. The Black Brook watershed covers approximately 1400 hectares of

land north of the town of Grand Falls, approximately 65% of which is intensively farmed on a regular basis (S.Brasfield, *pers comm*). The site was approximately 50 metres upstream of where Black Brook joins with Dead Brook. Bioassay chambers were placed in a pool area formed by a small man-made weir. Flow rate was less than 0.02m/second and channel width and depth were 3.8m and 0.4m respectively. Substrate was dominated by a thick accumulation (approximately 10cm) of fine silt and clay particles.

Site LR3 represented a point downstream of where the Black and Dead Brooks join together (hence "Bled"), but upstream of the junction with the Little River main channel (Figure 4.2). Bioassay chambers were placed in a slow-flowing pool area. Channel width, depth and flow rate at this point was 5.0m, 0.55m and 0.03m/second respectively. The underlying substrate was dominated by sand and gravel, which was covered with a thin layer of silt and coarse organic debris.

Site LR4 was situated in a large pool area within the Little River main channel, approximately 100 metres downstream of where the "Bled" joins with the Little River. Chambers were placed in a side pool area where average flow rates were 0.01m/second, although strength and direction of flow varied considerably according to flow in the middle channel. Substrate was mainly composed of sand and gravel, with a thin overlying layer of silt.

4.3.1.2 Saint John River near Edmundston

The Saint John River originates from an isolated area of northern Maine in the United States. It then flows between Maine and the far north-west portion of New Brunswick, forming a natural border between the US and Canada around the town of Edmundston. Upstream of Edmundston the catchment is relatively sparsely populated, with limited industrial and urban development. In the immediate vicinity of the town effluents from a paper mill, a pulp mill and three municipal sewage plants are discharged into the river (Figure 4.3b). The paper mill is situated on the United States (south) side of the river. Primary-treated effluent is discharged from a series of lagoons into an area of the river directly opposite the town. On the Canadian (north) side, two sewage treatment plants discharge effluent into the Madawaska River, just upstream of where it joins with the Saint John River. There is also a third sewage treatment plant that discharges effluent in the immediate vicinity of the junction of the Madawaska and the Saint John Rivers. The pulp mill at Edmundston transfers its waste effluent by pipeline to a series of treatment lagoons situated approximately 4 km downstream of the town, on the north side of the river. The lagoons discharge approximately 75,000 m^3 of secondary-treated effluent into the river each day (Galloway *et al.* 2003)

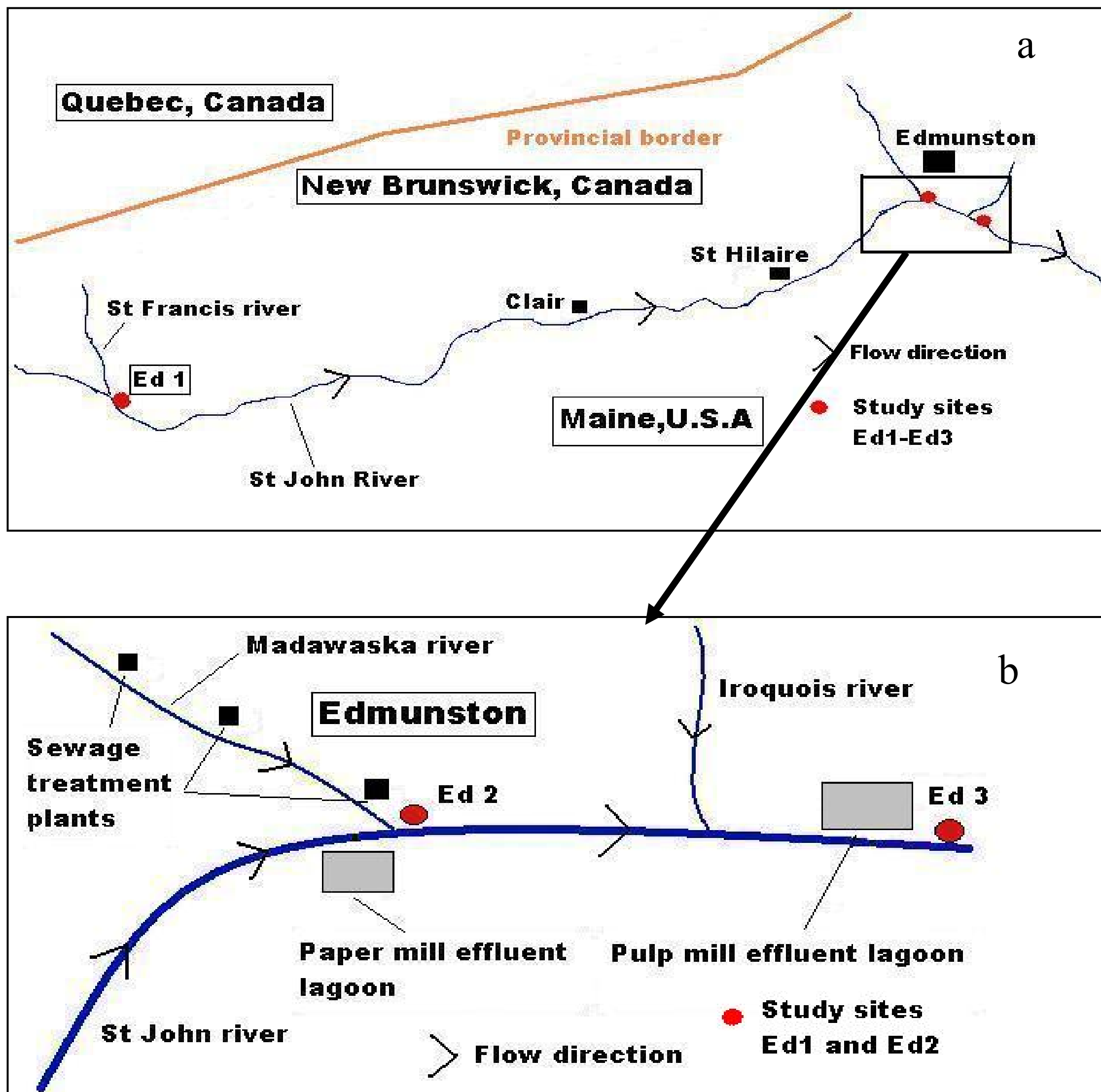


Figure 4.3 (a) Location of the three sites on the Saint John River near Edmundston (b) Detail of the two impacted sites closer to Edmundston (maps not to scale)

Three sites were selected to deploy the bioassay on the 19th July 2003. Site Ed1 was approximately 50km above the town of Edmundston (Figure 4.3a). This was selected as an upstream site for comparison with impacted sites in the Edmundston area. Chambers were placed in the margins of the river channel in water 0.45m deep with a flow rate of 0.1m/second. Substrate was largely composed of sands, gravels and pebbles. Two sites

close to the town of Edmundston were designated as impacted. Site Ed2 was situated approximately 100 metres downstream of the third sewage outfall pipe marked on Figure 4.3b. Chambers were placed in water with a depth of 0.58m and a flow rate of 0.05m/second. Site Ed3 was situated approximately 200 metres downstream of the pulp mill lagoon marked in Figure 4.3b. Chambers were placed in water 0.49m deep with a flow rate of 0.06m/second. Substrate at both Ed2 and Ed3 were very similar and composed of sand, gravel and pebbles, which was covered with a thin layer of finer silts and organic material. Channel width was not measured at any of the sites due to the size of the river (>100 metres).

4.3.1.3 Little River near Saint John

The Little River is a relatively short (<20km), small river that originates from a series of lakes and marshland on the northeast edge of the city of Saint John. Prior to flowing into the outer Saint John harbour, the river passes a series of large industrial facilities. Four sites were selected within a 1km stretch of river (Figure 4.4). Waste effluent was discharged from a large facility at the location marked on Figure 4.4. Although the composition of the effluent is not available, it is likely to consist of a complex mixture of hydrocarbons, additional organic chemicals and metals. Prior to release the effluent is subject to settlement and biological oxidation. The rivers flow in the area downstream of the weir marked on Figure 4.4 is also influenced by the rise and fall of the tides in Saint John harbour.

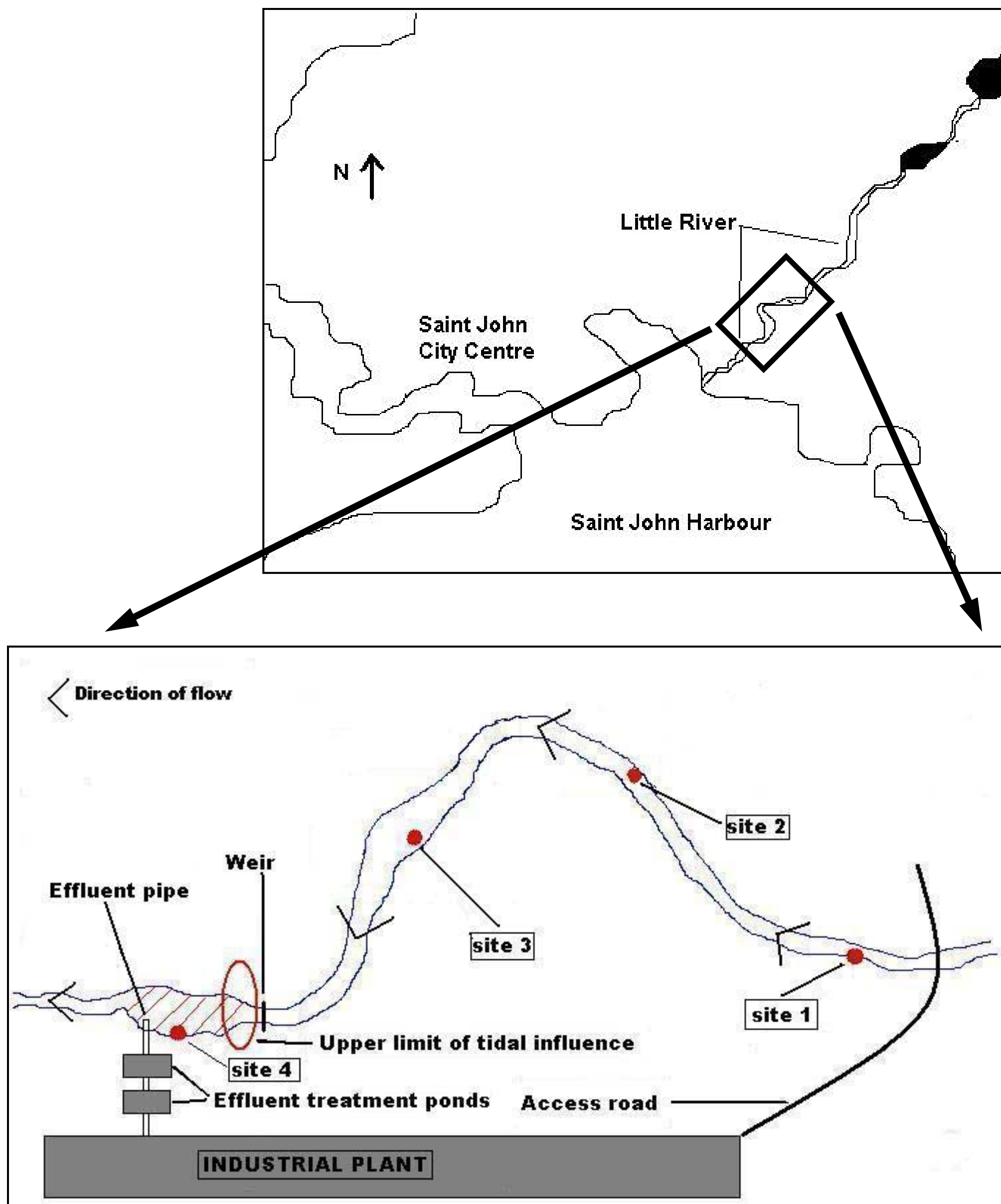


Figure 4.4 Location of the four sites on the Little River near Saint John, New Brunswick (maps not to scale)

The bioassay was deployed on the Little River near Saint John on the 10th September 2003. Site 1 was chosen as an upstream site, approximately 800 metres above the effluent pipe. Chambers were placed into a soft, muddy substrate that was rich in decaying plant

material. The river channel at this point was 4.1m wide and 0.54m deep, with a flow rate of <0.02m/sec. Site 2 was originally selected as a second upstream site, approximately 500 metres upstream of the outfall. Channel width, depth and flow rate at this point were 5.2m, 0.65m and <0.02m/sec respectively. Substrate was very similar to that at Site 1.

Site 3 was chosen as an impacted site as there used to be an effluent pipe in the area that discharged waste from the nearby industrial plant (G.Vallieres,*pers com*). The site was located on the edge of a deep pool area, approximately 200 metres upstream of the current effluent pipe and above the weir marked on Figure 4.4. Chambers were placed in an area with a flow rate of <0.02m/sec and a depth of 0.5m. Underlying substrate was a soft mud, which was covered with a thick "mat" of organic debris. Site 4 was situated in a wide pool area, approximately 25 metres upstream from the site of the outfall. Chambers were placed in 0.45m of water with a flow rate of <0.01m/sec. Substrate was composed of a very thick layer (approx 0.4m) of loosely compacted organic material. The flow of the river below the weir marked on Figure 4.4 was tidally influenced, therefore there was concern regarding the use of a freshwater oligochaete. However, measurements made on a preliminary visit at high tide suggested that salinity did not increase significantly (0.1ppt or lower) in the area where chambers were placed

4.3.2 Bioassay chamber design and deployment

The simple chamber design used to deploy *L.variegatus* is shown in Figure 4.5. Chambers were constructed from a 30 cm piece of clear PVC tubing (internal diameter

7cm). Clear tubing was used to account for the potential for photo-induced toxicity of polycyclic aromatic hydrocarbons (Monson *et al.* 1995). Two 5 cm diameter circular holes were cut into opposing sides of the chamber to aid water exchange. The holes were covered with a 125µm nylon mesh, which was fixed to the chambers using a silicon-based sealant suitable for aquarium use. The top of the chamber was covered by a removable polyethylene endcap (Consolidated Plastics Inc, Twinsburg, Ohio), into which two 1-metre long pieces of clear aquarium tube were fixed (4mm internal dia). One of these tubes was used to add worms to chambers at the start of the exposure period. The second tube was used at the end of the exposure period to extract a water sample from inside the chamber. A small piece of 125µm nylon mesh covered the end of the sample tube in order to prevent any blockage while taking a water sample. Both tubes were sealed with a plastic clamp when not in use (Fisher, Canada). The bottom end of the chamber was left open and the outer edge was ground to an angle of 45° to facilitate pushing the chambers vertically into the substrate.

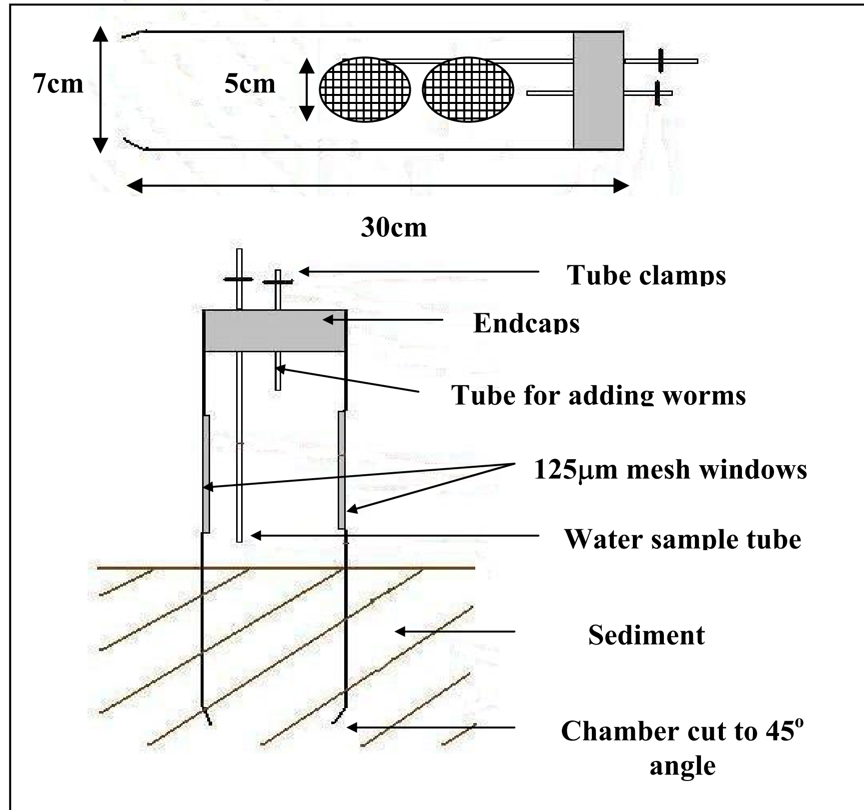


Figure 4.5 The *in situ* bioassay chamber design used throughout bioassay deployments

Five replicate chambers were used at each site. At the start of each deployment chambers were pushed into the substrate to a depth of approximately 10cm. They were fixed into position using a single strip of plastic garden netting (3m x 0.15m,“anti-predator” netting, 1cm mesh, Figure 4.6). The netting was stretched over each chamber and held down in place with rocks or large tent pegs, depending on substrate type at each site.

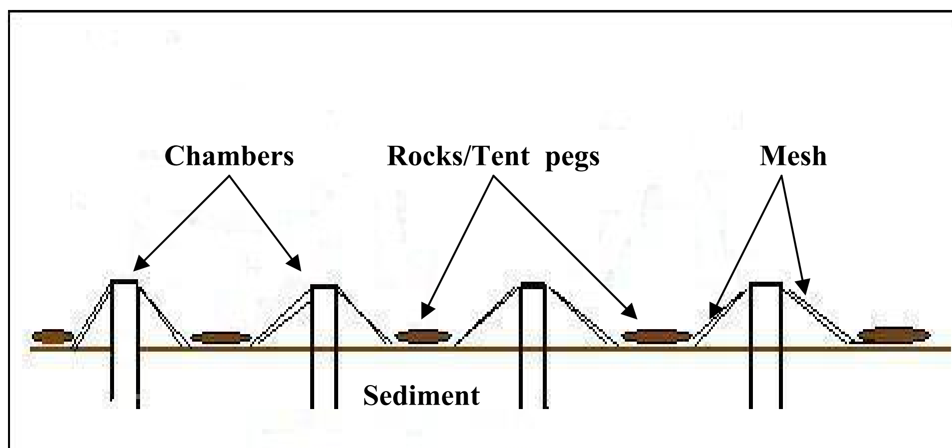


Figure 4.6 Positioning of the bioassay chambers in the sediment substrate

4.3.3 Deployment of *L.variegatus in-situ*

Twenty-four hours prior to deployment of the bioassay individual worms were selected from cultures held at the University of New Brunswick. The procedures for identifying feeding and non-feeding worms were then used (Section 2.6.1.1). The next day worms with full guts and fully formed head and tail segments were transported to field sites using portable aeration (approximately 700 worms). Once bioassay chambers were fixed in position 30 worms were added to each of the five replicates using the delivery tube and a 3ml plastic pipette. The chambers were then left undisturbed for the entire six-day exposure period.

4.3.4 Retrieval of *L.variegatus*

Prior to retrieval of the worms from bioassay chambers on Day 6, water samples were taken for analysis from one of three randomly selected chambers. All chambers were then carefully removed from the sediment with a gentle twisting action. During removal a plastic cup was slipped under the bottom of the chamber to hold the sediment core intact. The contents were then rinsed through a 250μm sieve to remove worms. The number of live and dead worms recovered from each chamber was recorded, and any other indigenous macro-invertebrate species were collected and preserved using 70% ethanol.

4.3.5 Measurement of post-exposure egestion rate *in-situ*

Field sites on the Little River near Grand Falls and the Saint John River near Edmundston were a considerable distance away from the laboratory in Fredericton (3 hour drive). Transport in clean media prior to starting the post-exposure feeding period in Fredericton might have allowed for a significant recovery in feeding rates. Therefore, the twenty-four hour post-exposure feeding period was carried out on-site for these two locations.

Post-exposure feeding beakers were set-up as follows. In the laboratory on Day 5 of the exposure period 20g portions of Little Magaguadavic Lake sediment (Section 2.3.3.3) were distributed to 5 x 60 ml tall glass beakers (Fisher,Canada). Beakers were then filled with water and left to settle overnight at 20°C. The next day (Day 6) before leaving for field sites

the overlying water in all beakers was exchanged. The water used throughout experiments was from the same supply used to culture worms (Section 2.3.1.2).

Upon arrival at each site the beakers were placed in polystyrene coolers, which were filled with river water to just below the lip of the beakers. As portable controlled temperature equipment could not be used (owing to availability/the risk of theft) polystyrene coolers were used in an attempt to maintain a steady ambient temperature throughout the feeding period in the field. For deployments where temperatures would be significantly lower than 15°C (based on temperatures at the start of deployments) beakers were acclimatised to that temperature for 24 hours prior to arrival at field sites.

Once all of the worms had been recovered from bioassay chambers (Section 4.3.4) five worms with full guts and fully formed head and tail fragments were added to a corresponding post-exposure feeding beaker. Post-exposure egestion rates were then measured over 24 hours using the method described previously (Section 2.6.1.2). Prior to leaving each site the temperature of overlying water in each beaker was taken. The polystyrene cooler was then hidden in a sheltered area out of direct sunlight. Twenty-four hours later the temperature of each beaker was taken again before collecting faecal pellets. Faecal pellets were stored on ice during transport to the laboratory in Fredericton, prior to processing and calculation of egestion rates (Section 2.6.1.2).

A different approach to measuring post-exposure egestion rate was taken with worms exposed to sites on the Little River near Saint John. During the period where post-exposure

feeding rates were due to be measured (Days 6-7), access to the sites was restricted due to the operations of the nearby industrial plant (safety-related issues). Therefore, instead of leaving beakers on-site, it was decided to measure post-exposure egestion rates in the laboratory at the University of New Brunswick in Saint John. The lab was situated close to the refinery (<15 minute drive), so the maximum time period the worms were in clean water (after retrieval from *in-situ* sediment) was approximately 2 hours. Post-exposure egestion rates were measured at a controlled temperature of $20^{o}C$ using the method described previously (Section 2.6.1.2).

4.3.6 Laboratory controls

During each separate deployment of the bioassay laboratory controls (LC's) were used. These consisted of exposing worms to uncontaminated Little Magaguadavic Lake sediment (Section 2.3.3.3) for a six-day exposure period, followed by a twenty-four hour post-exposure feeding period, both of which were carried out in the laboratory. The reasoning behind LC's in this study was two-fold. Firstly, they were used to identify any variation in the bioassay endpoint that was due to variation in the physiological condition of worms at the start of the deployments. Secondly, LC worms were selected from the worms that were taken into the field in order to deploy the bioassay *in situ*. Therefore, any effects of transport and handling-related stress could be identified.

The worms that would be used for LC's were brought back to the laboratory after each separate deployment of the bioassay in the field. After being held overnight in clean water

the next day worms were added to beakers containing 20g of Little Magaguadavic Lake sediment. LC's were started one day later than *in-situ* exposures, as the LC post-exposure feeding period had to be started upon return to the laboratory, after completion of the bioassay in the field. LC's were held in the laboratory for six days at 20°C, water was exchanged on Days 2 and 4. On Day 6 worms were transferred to new beakers containing 20g of fresh Little Magaguadavic Lake sediment. Post-exposure egestion rates were then measured over 24 hours at 20°C $\pm$0.5^{o}C using the method previously described in Section 2.6.1.2.

4.3.7 Water physico-chemistry

Flow rates, channel widths and depths were measured for each deployment where bioassay chambers were positioned. Flow rates were measured with an Ott 5 current meter (Ott, Kempten, Germany). Dissolved oxygen, pH, temperature and conductivity were measured where bioassay chambers were positioned at the start and end of the exposure period using a YSI 340 multiprobe portable meter (YSI Inc,Yellowsprings, Ohio).

Dissolved oxygen, pH, temperature and conductivity were also measured inside the bioassay chambers at the end of the exposure period. A 200ml sample was withdrawn from triplicate bioassay chambers with the aid of a large syringe and the sample tube shown in Figure 4.5. Samples were then immediately transferred to 250ml high-density polyethylene bottles where measurements were made. The water sample was filtered on-site (MF200, 47mm glass fibre) and transported to the laboratory on ice. Samples were stored frozen for

a maximum of 1 week, prior to ammonia analysis using the method described previously in Section 2.3.2.2.

4.3.8 Carbon and nitrogen content of sediments

Samples of sediment were taken from all sites at the start of deployments in order to determine organic carbon and nitrogen content. For sites on the Little River near Grand Falls samples were taken during the last of the three deployments. For all sites sediments were collected with a hand-held Ekman sediment grab. In the laboratory sediment was passed through a 2mm sieve and settled out for 48 hours. Three replicate sub-samples were taken and dried at 110°C for 48 hours. Dried samples were taken back to Stirling in October 2003 where carbon and nitrogen content was determined from triplicate sub-samples using a Perkin Elmer series II 2400 CHNS/O elemental analyser. In order to correct for inorganic carbon sub-samples were combusted at 550°C for four hours prior to analysis.

4.3.9 Statistical analysis

Post-exposure egestion rates from each separate deployment and laboratory controls were compared with analysis of variance. Fishers least significant difference multiple comparisons and student's t-tests were used to identify individual sites that were significantly different from one another. All statistical analysis was carried out using Minitab version13.

4.4 RESULTS

4.4.1 Water physico-chemistry

Measurements from the outside and inside of chambers at the end of the exposure period from all of the bioassay deployments are shown in Table 4.2. Conditions inside of chambers closely reflected those of the surrounding river. Ammonia levels from inside of chambers were usually very low. Notable exceptions were chambers at Sites 3 and 4 on the Little River near Saint John, where ammonia levels were significantly higher when compared to Site 1. At Site 4 dissolved oxygen was significantly lower and conductivity was significantly higher both inside and outside of chambers when compared to the other 3 sites. Salinity at Site 4 at the start and end of deployments (both measured at high tide) was less than 0.1ppt, which confirmed that the influence of seawater was minimal. For sites on the Little River near Grand Falls temperature and pH tended to increase moving down through the catchment.

Table 4.2 Water physico-chemistry measurements from inside (in) and outside (out) of chambers for all bioassay deployments, *n* = 3 for all data

Site	Date	Temp (^oC)		Dissolved oxygen (%)		p.H		Conductivity (uS)		Ammonia	
										Total (mg/l^{-1})	Unionised (mg/l^{-1})
		In	Out	In	Out	In	Out	In	Out		
LR 1	09/07/03	17.6	17.3	85.6	89.4	7.1	7.0	190	181	0.002	1^{-04}
LR 2	"	21.1	20.4	73.3	76.0	7.3	7.3	198	193	0.310	0.002
LR 3	"	-	20.6	-	78.1	-	7.3	-	179	-	-
LR 4	"	21.6	21.5	75.8	76.4	7.2	7.4			0.03	4^{-04}
Ed 1	-	-	-	-	-	-	-	-	-	-	-
Ed 2	25/07/03	18.7	18.5	58.3	64.6	7.3	7.3	175	179	0.100	0.001
Ed 3	"	17.2	17.4	56.6	60.1	6.6	6.7	372	332	0.510	0.006
LR 1	18/08/03	13.4	13.0	81.6	82.4	6.9	6.9	167	159	0.001	6^{-04}
LR 2	"	16.3	16.1	74.2	74.9	7.4	7.3	195	192	0.092	0.004
LR 3	"	16.0	16.0	72.7	74.1	7.5	7.6	201	197	0.050	0.001
LR 4	"	16.1	16.0	78.3	76.3	7.4	7.4	175	162	0.130	0.002
LR 1	25/08/03	10.5	10.5	85.7	82.3	6.9	7.0	191	197	0.01	3^{-04}
LR 2	"	11.7	11.5	73.3	73.9	7.3	7.3	198	201	0.11	0.001
LR 3	"	12.2	12.1	83.1	83.0	7.5	7.4	194	191	0.08	0.002
LR 4	"	12.6	12.5	81.0	81.9	7.6	7.6	160	156	0.07	0.001
St J1	10/09/03	19.2	18.7	64.9	64.0	7.0	6.8	184	181	0.1	0.002
St J2	"	18.4	18.0	71.8	72.0	7.2	6.8	187	176	0.6	0.004
St J3	"	18.2	17.1	64.5	64.4	6.8	7.0	210	205	2.5	0.006
St J4	"	19.1	18.3	48.9	52.2	6.8	6.8	287	312	14.1	0.035

Water quality data from the inside of chambers for the first deployment at Site LR3 is missing as a result of the theft of the chambers. There is also no data available for Site Ed1 as a result of the loss of chambers due to very high river levels. Chambers were deployed on the 19th July; by the 24th the Saint John River in the area around Edmundston had risen by 0.5m (U.S geological survey data, http://waterdata.usgs.gov). It was decided to try and retrieve chambers after a 5-day exposure as river levels were still rising. Chambers at both

impacted sites (Ed2 and Ed3) were successfully recovered, but those at Ed1 were not accessible. By the 26th July river levels were 2 metres above those at the start of the deployment and remained high for a prolonged period. When river levels were eventually low enough to safely access Ed1 (5th August) none of the chambers were found.

4.4.2 Sediment organic carbon and nitrogen content

Organic carbon and nitrogen content of sediments from field sites are shown in Table 4.3. Both carbon and nitrogen were increased at agricultural sites (LR2-4) in the Little River watershed near Grand Falls when compared to the upstream (LR1) forested site. Similarly, organic carbon and nitrogen contents were elevated at the two impacted sites (Ed2 and 3) on the Saint John River near Edmundston when compared to the upstream area (Ed1). Sediments from all of the sites on the Little River near Saint John (St J1-4) contained relatively large quantities of carbon and nitrogen, but levels were slightly higher close to the effluent pipe (Site 4).

Table 4.3 Organic carbon and nitrogen content (as % of dry weight) of sediments from all field sites, samples from the Little River near Grand Falls were taken after the third deployment, brackets denote standard deviations from triplicate samples

Site	Organic carbon (%)	Nitrogen (%)
LR 1	1.3 (0.10)	0.08 (0.01)
LR 2	5.2 (0.22)	0.32 (0.03)
LR 3	4.4 (0.34)	0.15 (0.02)
LR 4	2.0 (0.40)	0.35 (0.04)
Ed 1	0.8 (0.14)	0.05 (0.01)
Ed 2	2.6 (0.08)	0.53 (0.08)
Ed 3	2.4 (0.16)	0.41 (0.04)
StJ 1	6.7 (0.27)	0.25 (0.06)
StJ 2	7.3 (0.56)	0.39 (0.05)
StJ 3	7.5 (0.90)	0.36 (0.03)
StJ 4	8.2 (0.75)	0.43 (0.03)

4.4.3 Post-exposure egestion rates from laboratory controls

Figure 4.7 shows the post-exposure egestion rates of laboratory control (LC) animals from each individual deployment. There was no significant difference between LC data for each of the five deployments of the bioassay (ANOVA, p=0.399). When all of the LC data was pooled together the mean post-exposure egestion rate was 0.39mg dry faeces/individual worm/hour (represented by the dashed line in Figure 4.7). The average coefficient of variation was 16.9% (20.9% max, 14.1% min) across all five LC data sets. This confirmed that it was possible to obtain a consistent bioassay response under controlled laboratory conditions, which in turn meant that the results of the *in situ* bioassay could be interpreted with greater confidence.

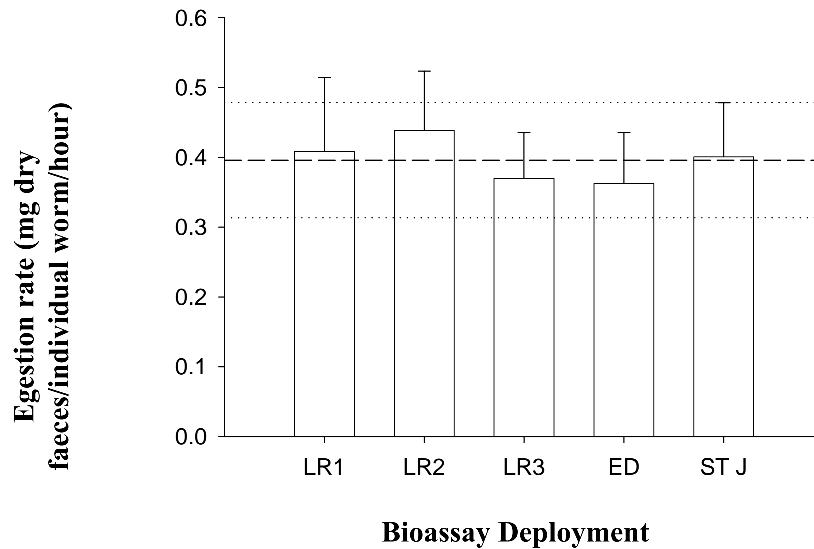


Figure 4.7 Post-exposure egestion rates of laboratory controls from the five separate deployments of the bioassay, LR1-LR3 = 1st - 3rd deployments on the Little River near Grand Falls, ED = Saint John River near Edmundston, ST J = Little River near Saint John, individual error bars represent 95% confidence intervals, dashed line represents mean of the pooled data, dotted lines represent upper and lower 95% confidence intervals of the pooled mean

4.4.4 Survival and recovery of worms

The recovery of live worms was above 95% in most deployments, which enabled successful completion of the bioassay. Recovery sometimes exceeded 100%, due to reproductive fragmentation. There was no clear pattern as to when or where fragmentation occurred, but there were always sufficient numbers of intact worms with full guts to use in subsequent feeding tests. The only occasion where recovery was less than 95% was at Site 4 on the Little River near Saint John. Here only 45% of worms added to chambers were found alive after six days. Worms that were recovered alive were very thin and transparent-

looking. This was also the only site where dead worms were found in bioassay chambers at the end of the exposure period, which accounted for on average 9% of the 30 worms originally added to each chamber.

Indigenous macro-invertebrates were often found within bioassay chambers. The total number of individuals for all species never exceeded 20. Indigenous organisms were dominated by small chironomids, with smaller numbers of Trichoptera (caddisflies) and small Ephemeroptera (mayflies). No larger predatory species were recorded throughout all of the deployments, which when combined with the consistently high recovery of oligochaetes from chambers at upstream sites would suggest that predation of worms was not a significant problem in the current work.

4.4.5 Post-exposure egestion rates from *in-situ* deployments

4.4.5.1 Little River near Grand Falls

Post-exposure egestion rates from repeated deployments on the Little River near Grand Falls are shown in Figures 4.8a-c. During the first deployment chambers at LR3 were vandalised/stolen, therefore no feeding data was available. During the first deployment egestion rates were significantly increased at LR4 (agriculture) when compared to LR1 (upstream "control", t-test, p=0.022). This was contrary to the original hypothesis i.e that pesticide contamination might cause feeding inhibition at agricultural sites. After obtaining egestion rates from the first deployment, where worm weights were not taken, it was

decided to measure worm dry weight for all subsequent deployments of the bioassay. Consequently egestion rates in Figures 4.8b and c are normalised to worm dry weight, whereas those in Figure 4.8a are not.

During the second deployment on the Little River a prolonged period of heavy rainfall meant that the chambers could not be safely retrieved until 13 days after the start of deployments. By this time worms at LR3 and LR4 (agriculture) were significantly larger than those from site LR1 (upstream) (t-test, LR3 vs LR1, $p=0.011$, LR4 vs LR1, $p=0.016$). Before normalising to worm dry weight, post-exposure egestion rates from LR3 were significantly higher than LR1 (t-test, $p=0.009$). However, after correcting for dry weight (i.e. mg dry faeces/mg dry worm/hr), no significant difference was found between any of the four sites (ANOVA, $p=0.406$, Figure 4.8b). For the third deployment all of the chambers were successfully recovered after the planned six-day exposure. Post-exposure egestion rates, worm dry weights and weight-corrected egestion rates were not significantly different between all four sites (ANOVA, $p=0.493$, $p=0.230$ and $p=0.318$ respectively).

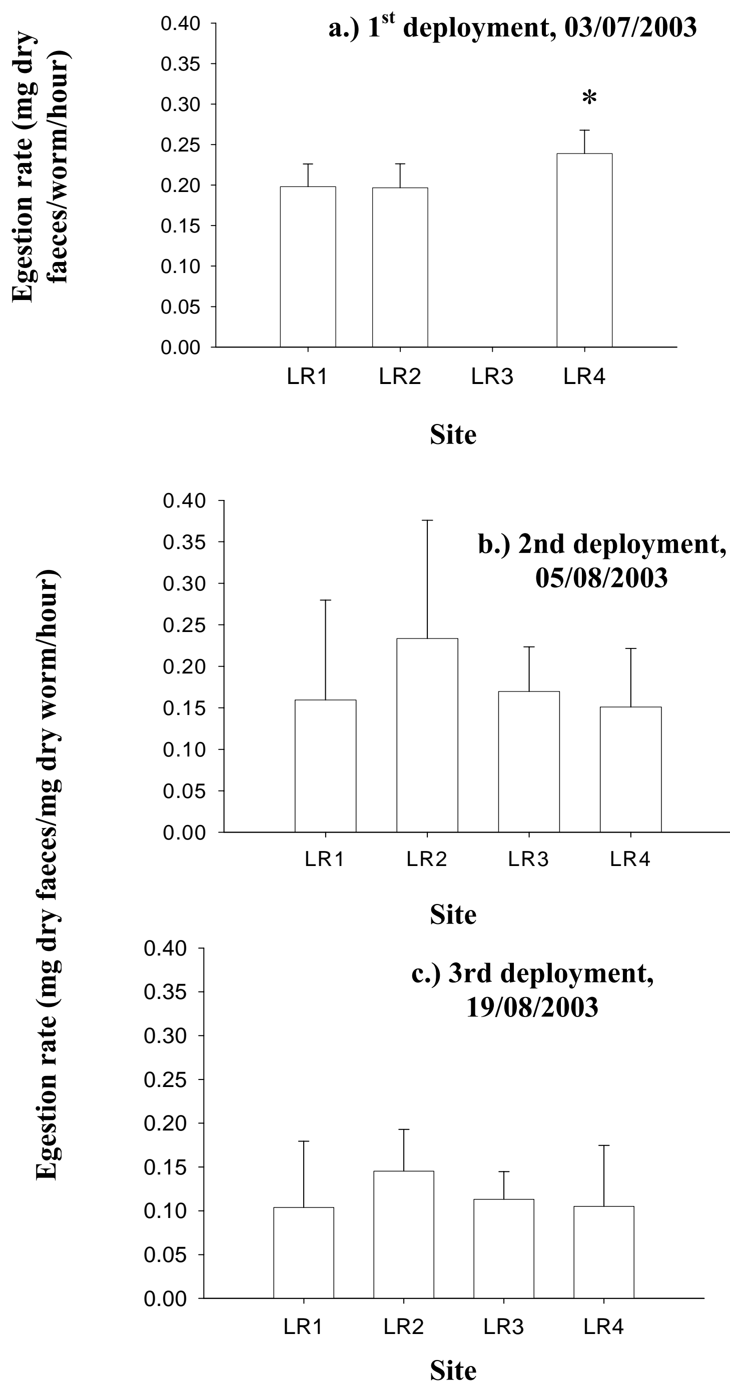


Figure 4.8 (a-c) Post-exposure egestion rate of *L.variegatus* after exposure to sites within the Little River watershed near Grand Falls, LR1=upstream, LR2-4=agriculture, data for the first deployment at LR3 is missing, all data for the second deployment is for a 13-day exposure period (see text for explanations), * denotes where egestion rates were significantly different from LR1, all error bars represent 95% confidence intervals

4.4.5.2 Saint John River near Edmundston

Heavy rainfall caused problems during the deployment of the bioassay on the Saint John River near Edmundston. High water levels meant that *in situ* bioassay data from the upstream site (Ed1) was lost and the data for the two impacted sites (Ed2 and Ed3) was from a shorter five-day exposure.

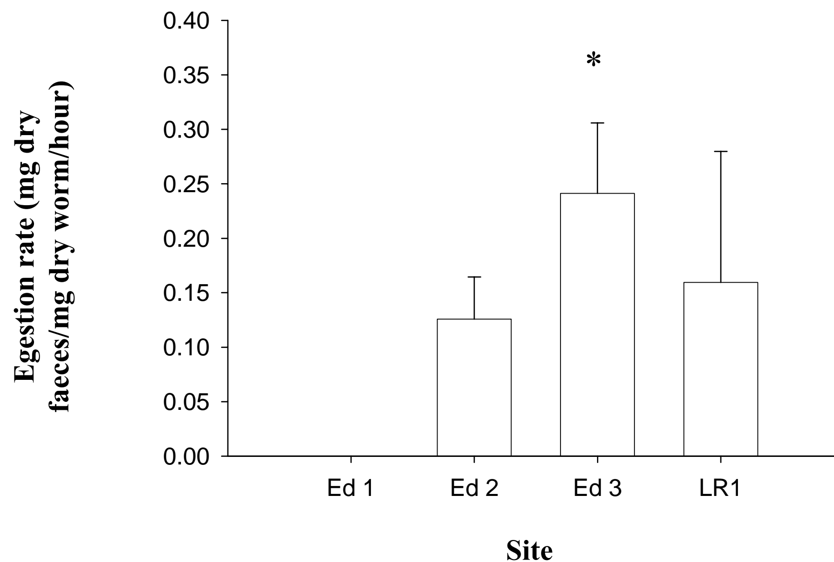


Figure 4.9 Post-exposure egestion rates of *L.variegatus* after exposure to 3 sites on the Saint John River near Edmundston, Ed1=upstream,Ed2 and 3=impacted, data for Ed1 is missing (see text) so data from the second deployment at LR1 is included for comparison,*denotes where egestion rates are significantly higher than Ed2, error bars represent 95% confidence intervals

Worms at Ed3 (pulp mill lagoon) were significantly larger in terms of dry weight when compared to those exposed to Ed2 (sewage and paper mill) (t-test, p=0.01). Despite dry weight-correction, post-exposure egestion rates were higher at Ed3 when compared to Ed2 (t-test, p=0.005, Figure 4.9). Due to the chamber losses at the upstream site Figure 4.9 also

includes bioassay data from the second deployment at the upstream site (LR1) on the Little River near Grand Falls. Post-exposure egestion rates at both Ed2 and Ed3 were not significantly different from LR1 (t-tests, p=0.148 and 0.498 respectively).

4.4.5.3 Little River near Saint John

Post-exposure egestion rates from the Little River near Saint John are shown in Figure 4.10. Egestion rates at Site 1 (upstream site) were significantly higher than all of the sites downstream (ANOVA, p=0.001). There was no significant difference in post-exposure egestion rates between Sites 2,3 and 4 (ANOVA, p=0.505). This finding was surprising as Site 2 was originally selected as an additional upstream (control) site. Worm dry weights were not significantly different from one another at Sites 1 and 2 (p=0.42), but were both significantly heavier than worms exposed to Sites 3 and 4 (p=0.007).

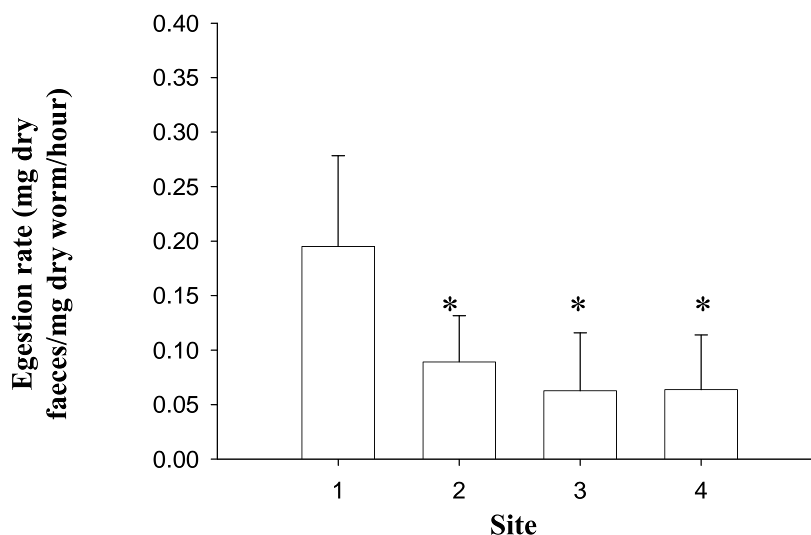


Figure 4.10 Post-exposure egestion rates of *L.variegatus* after exposure to 4 sites on the Little River near Saint John, Sites 1 and 2 (upstream), Sites 3 and 4 (impacted), *denotes where egestion rates were significantly lower than Site 1 (upstream), error bars represent 95% confidence intervals

4.5 DISCUSSION

The results from repeated deployments of the *in situ* bioassay suggest that the technique meets a number of important requirements. Firstly, recovery of live worms from upstream (control) sites was consistently high, which enabled the successful completion of the post-exposure feeding period. High recovery also confirms there were minimal losses due to predation and escapees using the open-ended chambers, and the environmental conditions within chambers were of sufficiently good quality. Reproduction did occur during some of the deployments, but there were always sufficient numbers of actively feeding worms to carry out the bioassay. The egestion rates of laboratory controls were also consistent across all deployment periods. This confirms that the worms used for each separate deployment were of a similar physiological condition at the start of deployments. Therefore, variation in the *in situ* bioassay response due to culture conditions and/or handling and transport-related stress was suggested to be minimal.

4.5.1 Little River near Grand Falls

No evidence of feeding inhibition was found over the course of three deployments of the bioassay in agricultural areas. In fact the exact opposite i.e. an increase in post-exposure egestion rates in agricultural areas was found after the first deployment (Figure 4.9a). During the second deployment it was found that including worm dry weight into the egestion rate calculation reduced prior differences in uncorrected post-exposure egestion rates to statistically insignificant levels. Unfortunately worm dry weights were not taken

during the first deployment, so it is unclear if the increase in the bioassay response at LR4 (agriculture) is genuine, or simply a reflection of larger average worm sizes. This problem highlighted the need to measure worm dry weights at the end of the bioassay. The original decision to not measure worm dry weights was based on previous laboratory studies, where only very small changes in weight were found over six-days. However, it appears that worms grow at more variable rates in the field over the same period, as a result of site-specific conditions.

The lack of response at agricultural sites may be due to a number of factors. Firstly, it may be a reflection of low levels of contamination in these areas. When compared to the previous summer there was an obvious increase in the proportion of land left fallow (i.e not intensively farmed) in the areas surrounding sites LR2, 3 and 4. Fallowed sites would very likely result in reduced pesticide inputs into surrounding streams. Unfortunately data on pesticide usage in the area or on levels in the aquatic environment are not available.

Alternatively, if it is assumed that there was significant pesticide contamination in the agricultural areas, these results would suggest that the bioassay may not have been sufficiently sensitive. Previous laboratory work in Chapter 2 was limited to 2 pesticides (cypermethrin and chlorothalonil). Only cypermethrin was found to cause persistent feeding inhibition during the post-exposure period. As no other published work is available on the response of *L.variegatus* to pesticide contamination, it is very difficult to discuss the relative sensitivity of the species further. Further laboratory work with a wider range of pesticides would be very useful.

Poor sensitivity could also be a result of confounding variables such as temperature and nutrient enrichment, which may have reduced the strength of any potential bioassay signal. Post-exposure feeding beakers had to be held on-site as a result of the distance to the campground used for accommodation. The use of portable controlled temperature equipment at each site was ruled out due to the risk of theft. As a consequence beakers were held in polystyrene coolers filled with river water at ambient temperature. This was far from ideal but at least maintained a constant temperature throughout the feeding period. However, because ambient temperatures between upstream and downstream sites varied by up to 4°C during one of the deployments (Table 4.2), it is not possible to rule out the effects of temperature variation on the bioassay signal.

Comparisons of the carbon and nitrogen content of sediments in the Little River catchment (Table 4.4) seemed to confirm nutrient enrichment as a result of agricultural land use (Merrington *et al* 2002). A significant increase in food availability and/or nutritional quality may have buffered any negative effects of toxicity-induced stress at agricultural sites, as better fed animals often exhibit enhanced resistance to toxic chemicals e.g (Taylor *et al.* 1998) (McWilliam 2001). Previous work on fish populations in the area has identified a productivity gradient moving downstream from forested to agricultural areas. For instance the average individual size of the slimy sculpin, *Cottus cognatus* was significantly increased in agricultural areas when compared to upstream forested sites (Gray *et al.* 2002). Therefore, it is possible that the dominant environmental "signal" from agricultural land

use is nutrient enrichment, rather than the pesticide toxicity that was originally hypothesised.

More information would have been gained from the Little River deployments by using at least two upstream sites. Multiple upstream sites could be used to determine if downstream agricultural areas were within a range of upstream ("control") values. Only one upstream site was chosen as a result of the limited vehicle access further up into the catchment. As defined feeding periods were used for the bioassay, travel time between sites was an important consideration in the study design. However, a previous study found no difference in the life history characteristics of the slimy sculpin, *C.cognatus*, at Site LR1 when compared to a number of sites further upstream (Gray *et al.* 2002). This would suggest that at least in terms of fish populations, LR1 is representative of a general upstream condition for the watershed.

4.5.2 Saint John River near Edmundston

The loss of chambers at the upstream site caused problems with the interpretation of bioassay data from impacted sites. This type of risk is implicit with any *in situ* bioassay approach in rivers. This problem also highlights the value of using multiple upstream sites for bioassay deployments. However, a number of points can be taken. Worms exposed to Ed3 were larger than those at Ed2 by the end of the exposure period. Dry weight-corrected egestion rates at Ed3 were also increased when compared to those from Ed2. These two points suggest that conditions at Ed3 were more favourable. In a previous study artificial

streams were used to determine the ecological impact of the pulp mill effluent released from the lagoons at Ed3 (Culp *et al.* 2003). Rather than toxicity, the main effect of the effluent was suggested to be nutrient enrichment. This could explain why worms exposed to Ed3 were larger than those exposed to Ed2. Unfortunately with no upstream data for comparison it is difficult to ascertain whether the difference in the bioassay response at sites Ed2 and Ed3 is a result of nutrient status at Ed3 or toxicity-induced feeding inhibition at Ed2.

4.5.3 Little River near Saint John

Deployments of the bioassay on the Little River near Saint John managed to detect post-exposure feeding inhibition at suspected contaminated sites (Figure 4.10). These results were in line with the findings of recent fish population studies in the same area (G.Vallieres, *pers comm*). For instance, a reduction in diversity and abundance of individuals within a species was found downstream of the outfall pipe (Site 4) when compared to an area close to Site 1 (upstream) in this study.

Comparisons of feeding data, survival rates and worm dry weights are suggested to highlight the sensitivity of the bioassay endpoint. For instance, survival rates at Sites 3 (impacted) were not significantly different from Site 1 (upstream), whereas post-exposure egestion rates were significantly reduced. Also, post-exposure feeding inhibition was found at Site 2 (originally upstream site), yet worm dry weights were not significantly different from Site 1. Although worm growth rate was not followed exactly (as worm size was not

taken at the start of exposures), worms were selected from a small size range in an attempt to control for reproduction. Therefore, any differences in worm size are largely attributable to differences in exposure conditions. Based on the above, *L.variegatus* post-exposure feeding inhibition appears to be a sensitive response to sediment toxicity.

Feeding inhibition at Site 2 was somewhat surprising as this was originally selected as an additional upstream (control) site, some distance away from any obvious sources of contamination. The site certainly appeared to be relatively un-impacted (i.e good water quality, abundant aquatic life). However, it is possible that there may have been an effluent outfall in the area some time ago (i.e >20 years, G.Vallieres, pers comm). Unfortunately it was not possible to measure sediment contamination levels, but it is likely that the bioassay was able to detect evidence of a historical contamination problem. Historical contamination of sediments was very likely the cause of the feeding inhibition at Site 3. There was no active outfall in the area and the site was approximately 200 metres upstream of Site 4 (active outfall). However, there was old pipework in the vicinity of the site and oil slicks formed on the water surface when sediments were disturbed.

Site 4 was the only occasion where worm recovery was less than 95%. Additional evidence suggested that this was more likely a product of reduced survival, rather than escapees or predation. For instance, this was the only occasion where dead worms were found within bioassay chambers. Further, no oligochaete predators were found at the end of the exposure period. Like many invertebrates, oligochaetes rapidly decompose after death (Chapman 2001), which would account for the missing worms. The cause of the mortalities

may have been a combination of contaminant toxicity and poor water quality. Low dissolved oxygen levels and elevated ammonia levels in chambers at Site 4 (Table 4.3) would have increased the physiological stress of the worms. For example, the average concentration of total ammonia in bioassay chambers was 14mg/l. Previously a 10-day LC50 of 75mg/l was calculated for total ammonia with *L.variegatus* at a similar pH (Schubauer-berigan *et al.* 1995). With the above evidence in mind it is suggested that the poor recovery of worms at Site 4 was a result of mortalities caused by a combination of environmental stressors.

4.5.4 Practical considerations with the bioassay

Deployments of the *in situ* bioassay highlighted a number of important practical issues. Water quality analysis confirmed that the chamber design provided adequate water exchange. The bioassay chamber was relatively simple and inexpensive to construct (approx $6 U.S/chamber), which meant that the all too frequent losses due to flooding/vandalism were easily replaced. Chambers were also simple to deploy at a variety of sites, although coarse substrates provided a greater challenge when pushing chambers into place.

The open-ended bioassay chamber created a very realistic exposure, as the sediment was not significantly disrupted during deployment. However, there are concerns associated with escapees and indigenous organisms if using an open-ended chamber. As an alternative, the bioassay chamber could have simply been closed off at both ends with an

endcap. This would have entailed filling chambers with sediment from the riverbed, and then subsequently burying the chamber into the substrate before adding worms. However, this would have resulted in complete disruption to the sediment layer, which is likely to alter the bio-availability of any sediment-associated contaminants (Section 1.2.3). Since reducing sediment sampling-related artefacts was one of the key points for developing the *in-situ* bioassay technique, it was decided to use the open-ended design.

Preliminary trials of the bioassay chamber attempted to control for escapees by using a one-way closure system (plastic collar), similar to that developed in a previous study (Sibley *et al.* 1999). However, like the previous authors it was found that the sediment showed signs of considerable disruption, so the idea was abandoned. Sibley et al (Sibley *et al.* 1999) suggested that *L.variegatus* escapees were effectively controlled by simply pushing bioassay chambers into the substrate for a sufficient depth. In this work the distance was not standardised (approximately 10cm), as it was usually determined by the nature of the substrate. However, it should be emphasised that worm recovery was consistently high at sites with a variety of fine and coarse substrates.

Indigenous organisms might directly impact on bioassay organisms via predation (Chappie and Burton 1997), and may also cause more subtle effects on growth, reproduction and behaviour (Reynoldson *et al.* 1994). Sibley et al used a similar open-ended chamber design to expose *C.riparius* and *L.variegatus in situ* (Sibley *et al.* 1999). They recommended that the type and number of organisms found in each chamber be documented at the end of the exposure period. The presence of known predatory species

and/or high densities of inter-specific competitors could suggest that the results from individual chambers need to be interpreted with caution. Regression techniques could also be used to normalize bioassay data to the density of indigenous invertebrates, if it was thought that this might affect the bioassay response. In the current work predation was not found to be a problem, as recovery of worms was consistently high and no predatory species were found in chambers. Non-predatory macro-invertebrate species were found in bioassay chambers (predominantly small chironomids), but they were always present at low densities (<20 total individuals for all species).

Finally, an issue that is common to most *in situ* sediment bioassays is the potential for misidentification of bioassay organisms. In the current work indigenous oligochaetes were relatively few in number and were easily distinguished from bioassay organisms due to marked differences in colouration. However, some form of marking technique may be required in situations where oligochaetes are more plentiful and/or similar in colour. Misidentification of bioassay chironomids has previously been addressed by marking individual *C.riparius* with model paint prior to deployments (Crane *et al.* 2000). This was not deemed appropriate for *L.variegatus*, as it is difficult to blot dry a segment of the worm (in order to apply the paint) without inflicting damage. One alternative could be the use of a non-toxic dye or marker that is incorporated into the worm via food or water prior to deployment. This would require further work to develop a protocol and ensure that the dye does not have any confounding effects on the bioassay endpoint.

4.6 SUMMARY

The results of field deployments of the bioassay firstly demonstrated that the technique could be adapted for *in-situ* use. The bioassay chamber design allowed for the maintenance of good water quality and consistently high recovery of live, actively feeding worms, which in turn enabled completion of the subsequent post-exposure feeding period. Post-exposure feeding inhibition was detected at a number of sites on the Little River near Saint John, at levels of contamination that did not significantly affect worm survival or growth. This confirmed that the bioassay was sensitive to sediment-associated contamination *in situ*.

Deployments of the bioassay on the Little River near Grand Falls could suggest that a.) pesticide levels and/or bioassay sensitivity was insufficient to elicit a response or b.) the bioassay could have been subject to a number confounding variables that reduced the strength of any potential toxicity "signal". When combined with the loss of reference site data for the Saint John River, this last point highlights the value of using multiple upstream or reference sites to compare with potentially impacted sites, so that a baseline bioassay response in the field can be established. This issue and other points that have been highlighted in this preliminary trial are discussed further in Chapter 6.

5 CHAPTER 5 – THE EFFECTS OF FEEDING INHIBITION AT THE POPULATION LEVEL

5.1 INTRODUCTION

Previous work in Chapters 3 and 4 had demonstrated that the post-exposure feeding rate of *L. variegatus* was significantly inhibited after exposure to contaminated sediments in the laboratory and field. As previously highlighted in Section 1.4.2, the inhibition of *L.variegatus* feeding may be of relevance to both populations, through changes to individual “vital” rates (i.e survival, growth, reproduction, (Maltby *et al.* 2001)), and to the wider community as a result of changes to important ecological functions associated with oligochaete feeding behaviour.

A major challenge in using individual-level bioassay endpoints such as feeding inhibition is in translating their effects on whole populations (Stark and Banks 2003). For example, very small, statistically insignificant changes to feeding rate could result in larger effects at the population-level. Alternatively, compensation processes at the population level might buffer the effects of changes to individual endpoints. Therefore, the aim of this chapter was to study the relationship between short-term measures of feeding inhibition and longer-term population level responses. According to the available literature this type of study has not been carried out previously with *L.variegatus*, so this would hopefully improve the interpretative value of *L.variegatus* feeding inhibition as a bioassay endpoint.

5.1.1 Extrapolating the effects of feeding inhibition

The population-level effects of feeding inhibition can be studied by direct comparison during longer-term experiments, or by using bioenergetics-based modelling techniques (Calow and Sibly 1990). In bioenergetics-based modelling measured reductions in energy acquisition (via feeding inhibition) are used to predict effects on individual growth and reproductive performance. Predicted changes to growth and fecundity are then incorporated into population models in order to predict the effects of feeding inhibition on population dynamics (Maltby 1999). Predictions from population models can be compared with observations of field populations that have been exposed to similar levels of contamination in the field. For example, Klok and De Roos (1996) used a combination of energetics-based and population dynamics models to predict the concentration of copper in soils that would result in a decline in the numbers of the terrestrial oliogochaete, *Lumbricus rubellus*. The results of the model output were correlated with an observed decline in *L.rubellus* numbers at similar levels of copper contamination in the field. In this way a bioenergetics-based approach can provide a powerful tool for mechanistically linking the effects of feeding inhibition to consequences at the population level in the field.

The principle limitation with energetics-based modelling is that the dynamics of natural populations are not only influenced by changes to individual energetic budgets (Calow and Sibly 1990;Maltby 1999). Population abundance will also be determined by a variety of intrinsic and extrinsic factors, such as immigration, emigration, intra- and inter-specific competition, predation, disease and variation in the physical and chemical environment

(Begon *et al* 1996). In this way toxicants can be considered as just one of a large number of potential "stressors" that will determine overall population "fitness" (Van Straalen 2003).

As an alternative to an energetics-based approach, direct comparisons of feeding behaviour and population-level effects could be carried out using longer-term laboratory experiments. An often-cited criticism with long-term laboratory studies is that they tend to be labour-intensive. However, experimental designs can be manipulated to reduce the amount of time involved in carrying out an experiment (Sibly 1999). Laboratory experiments cannot replicate natural systems. However, experimental studies can be designed that include some of those factors that naturally determine the abundance of populations mentioned above. In this way at least some of the complexity of natural systems might be incorporated into a long-term experiment, thereby providing a more realistic toxicant exposure scenario with which to compare feeding and population effects.

5.1.2 Population density and toxicant exposure

Most natural populations experience some form of density-dependent growth via the competitive interactions of individuals (Begon *et al* 1996). At low densities populations may grow at an exponential rate, but as population size increases resources (e.g food, space) become limiting and growth rates decline, until populations begin to fluctuate around carrying capacity in a state of dynamic equilibrium. This negative feedback is considered to be a fundamental part of natural population regulation (Sibly and Hone 2002). Most ecotoxicology tests are carried out under conditions of low density and high

food availability e.g (U.S.E.P.A 2000). There is concern that the results from these tests may significantly over- or under-estimate the effects of toxicants on natural populations, as a result of density-dependent mechanisms that interact with the effects of toxicants (Sibly and Hone 2002). Therefore, in recent years increasing attention has been paid to the interactions between toxicant and density-dependent effects on populations.

Both density and toxicant exposure might be predicted to cause negative effects on populations, but a recent review of the subject area highlights the complexity of interactions that are possible (Forbes *et al.* 2001). The first scenario predicts that both factors are independent of one another, so they are described as additive in nature. A second scenario predicts that increasing population density impairs the physiological condition of the entire population, thereby increasing population sensitivity for a given toxicant concentration. In this case the effects of density heighten the negative effects of a toxicant, and so would be described as more than additive. Alternatively, a third scenario predicts that toxicant-induced reductions in growth or survival are compensated for by an increase in the availability of limited resources to those individuals more able to cope with toxicant effects. In other words the negative effects of a toxicant might be buffered by a reduction in the intensity of density-dependence, and in this case the interaction would be described as less than additive. It was concluded that all types of interaction may be possible in experimental studies, largely as a result of the details of the study design and concentration of a given toxicant (Forbes *et al.* 2001).

For the current work incorporation of density-dependence into the experimental design was an attempt to compare the effects of toxicants on individual feeding behaviour with effects on a defined population under both toxicant and density-dependent control. While it was recognised that laboratory exposures would not allow for many other important factors that determine the abundance of natural populations (e.g immigration, emigration, inter-specific competition, predation, disease), inclusion of density-driven population regulation could arguably provide a toxicant exposure scenario that is more reflective of natural situations. Along with comparisons of feeding and population-level effects, such an experiment would also provide potentially useful information regarding toxicant and density interactions with *L.variegatus*, as this has not been studied previously.

5.1.3 Population-level measures of toxicant stress

In order to compare feeding inhibition with population-level effects in a laboratory setting, a useful population endpoint was required. There are a wide variety of population measures of toxicant stress (Maltby *et al.* 2001). These include measures of population size (e.g biomass, number of individuals, equilibrium abundance), population persistence (e.g extinction probability, minimum viable population) and population structure (e.g size and age structure, genetic diversity). Population growth rate is widely used in ecotoxicological studies, as it integrates the combined effects of toxicants on individual life history traits (e.g birth, growth, and mortality rate), thereby providing a useful summary of population "fitness" (Maltby *et al.* 2001;Sibly and Hone 2002;Stark and Banks 2003).

Estimates of population growth rate can be derived from detailed life table information, partial life tables, and more simply by the instantaneous rate of population increase (Walthall and Stark 1997). Demographic analysis based on life table response experiments (LTRE's) provides a very detailed picture of the mechanistic basis of the population-level effect of toxicants, but it is also very time consuming and may not be suitable for species with relatively long reproductive periods (Sibly 1999). The instantaneous rate of increase, usually denoted as (r_i) is calculated from the change in numbers or biomass of a population over time (Walthall and Stark 1997). Juveniles are not removed from the experiment so the population may eventually experience density-dependent growth. Because complete life table information is not required, the technique is also less demanding in terms of time and labour. However, the relative simplicity of calculating r_i means that it does not provide information on the mechanistic basis of any changes in population growth rates i.e the relative sensitivity of individual life-history traits to toxicant exposure and the relative sensitivity of population growth rate to changes in those life-history traits (Maltby *et al.* 2001).

What was required for this work was a useful population endpoint that could be compared to the feeding bioassay response during a long-term laboratory experiment. On this basis it was decided to use the instantaneous rate of increase (r_i), as it provided a relatively simple to measure endpoint that could also incorporate density-dependent population regulation into the experimental design (Forbes *et al.* 2003).

5.2 AIMS

The aims of this chapter were:

- To carry out a longer-term laboratory experiment to determine the effects of a representative toxicant and population density on the dynamics of *L.variegatus* populations
- To carry out short-term measurements of *L.variegatus* feeding behaviour at the same time as the long-term experiment, in order to compare the effects of toxicity-induced feeding inhibition with effects on population-level endpoints

5.3 MATERIALS AND METHODS - PRELIMINARY EXPERIMENT

It was anticipated that the sediment used in the main experiment would be spiked with a known toxicant on a single occasion. This would avoid using separate spiking periods throughout the experiment, which might otherwise create significant differences in toxicant exposure (Northcott and Jones 2000). However, spiking this quantity of sediment would involve a good deal of time, equipment and chemicals. Therefore, prior to spiking it was important to ensure that the sediment was suitable for use in the experiment. The sediment should be able to support the growth of *L.variegatus* populations, as this would be the primary endpoint in the long-term experiment.

Previous studies with *L.variegatus* using natural, field-collected sediments similar to the one used here had found poor individual growth over 7-10 days when no additional food was added (Ankley *et al.* 1994;Leppanen and Kukkonen 1998a). Therefore, a preliminary experiment was conducted to compare the growth of worm populations exposed to test sediment with varying levels of an additional food source.

5.3.1 Preliminary experiment set-up

A two-factor experiment was designed using the number of worms per beaker (2 and 5 individuals/beaker or 2083 and 5208 individuals m^2) and food level (0,10 and 30mg per 20g wet sediment in each beaker) as factors. *L.variegatus* were supplied from cultures held at Stirling University during 2003/2004 (Section 2.3.1.3). Test sediment was collected from the Goodie River on the 22nd November 2003 and prepared according to the methods previously described in Section 2.3.3.2. The experiment was started by adding 20g of wet sediment to five replicate 60ml tall glass beakers for each treatment group (Day –1). The appropriate quantity of additional food was then added to beakers in a solution of water from the culture supply. The food source consisted of a 50:50 mixture of ground Tetramin fish flakes and Hikari algae wafers (both Animal House, Batley, U.K). Beakers were then topped up with water from the culture supply, shaken for 30 seconds to mix the sediment and food, and then left to settle for 24 hours.

On Day –1 the procedure for identifying feeding and non-feeding worms was also started (Section 2.6.1.1). The next day (Day 0) overlying water was exchanged in

experimental beakers. Worms with full guts were identified and those estimated to be below 9mg wet-weight were selected for experiments. A six-hour purge period was then used to empty worm guts prior to measuring worm wet weight (Mount *et al.* 1999). Wet weights were taken by carefully blotting dry the small plastic weigh pan containing worms, rather than directly blotting dry worms themselves. Immediately after weighing, individual groups of worms were randomly assigned to experimental beakers and the experiment was started.

Beakers were held at 20°C $\pm$0.5 in complete darkness within a constant temperature room for 14 days. No additional food was added over the course of the experiment. The preliminary experiment was ran for a 2-week period, as this was the time period between sampling points that was selected for the main experiment. Greater information might have been gained from sampling at more regular intervals (e.g weekly), however, Forbes et al (2003) had previously highlighted that sampling at more regular time intervals may impact on organism growth rate.

5.3.2 Water quality analysis

During the preliminary experiment overlying water in beakers was exchanged on Days 3,6,9 and 12. Prior to each exchange triplicate samples from each treatment group were analysed for dissolved oxygen, temperature and pH using a WTW Multiprobe 340i meter (WTW, Weilheim, Germany). On the first and last water exchange (Days 3 and 12) a 50ml

sample of overlying water was taken for ammonia analysis. Total ammonia was analysed using the method described previously in Section 2.3.2.1.

5.3.3 Determination of end biomass

The preliminary experiment was ended on Day 14 by passing the sediment in each beaker through a 250µm sieve and collecting the retained worms. The number of individuals per beaker was noted. A six-hour gut-purging period was then used, prior to measuring the total wet weight of worms from each beaker.

In order to estimate worm dry weight from wet weight at the start and the end of the experiment, 50 worms were randomly selected from cultures at the start of the experiment. Wet weights were taken and worms were subsequently dried at 110°C for 24 hours. The average wet:dry ratio of 0.13 (sd=0.015) was then used to estimate the dry biomass of experimental worms (i.e wet weight x 0.13). The change in dry biomass or number of worms in each beaker during the experiment was expressed as either a percentage increase or decrease using the Equation 5.1 below:

Equation 5.1 (end value – start value) / start value *100

5.3.4 Statistical analysis

The effects of food level and starting worm density on worm biomass and number of individuals at the end of the experiment were examined with analysis of variance. Post-hoc Fishers least significant difference multiple comparison test and one-way t-tests were used to examine differences between individual treatments. All statistical analysis was carried out with Minitab version.13.

5.4 RESULTS – PRELIMINARY EXPERIMENT

5.4.1 Water quality analysis

Throughout the experiment dissolved oxygen was always above 70% saturation and temperature and pH were always deemed to be within an acceptable range (Temp 20°C $\pm$0.5^{o}C, pH 6.9-7.5). The highest single observations for total and unionised ammonia were 0.83 and 0.0064mg/L respectively and came from the 30mg food group. Based on previous studies it is unlikely that these levels of ammonia would have caused any toxic effects with *L.variegatus* (Schubauer-berigan *et al.* 1995).

5.4.2 Worm biomass and numbers

Worm biomass at the start of the experiment was not significantly different between all food levels (ANOVA, 2 worms, p=0.785, 5 worms, p=0.994). This confirmed that biomass

at the start of the experiment was equal among food level treatments. However, biomass at the end of the experiment was significantly different between food levels for both density treatments (ANOVA, 2 worms, p=0.004, 5 worms, p=0.006). The changes in worm biomass over 2 weeks (expressed as a percentage increase or decrease) with different food levels for both worm densities are given in Figures 5.1a and b. When no extra food was added to sediments worm biomass decreased in both density treatments. The decrease was more pronounced in 5 individuals per jar (-5.5%) when compared to 2 individuals per jar (-2.0%). Increasing the quantity of additional food resulted in an increase in worm biomass. The addition of 10mg of food caused a 16% increase in biomass with 2 worms per beaker and there was a slightly smaller increase of 13% with 5 worms per beaker. The largest increase was seen with 30mg food per beaker. There was a 32% and 22% increase with 2 and 5 worms per beaker respectively.

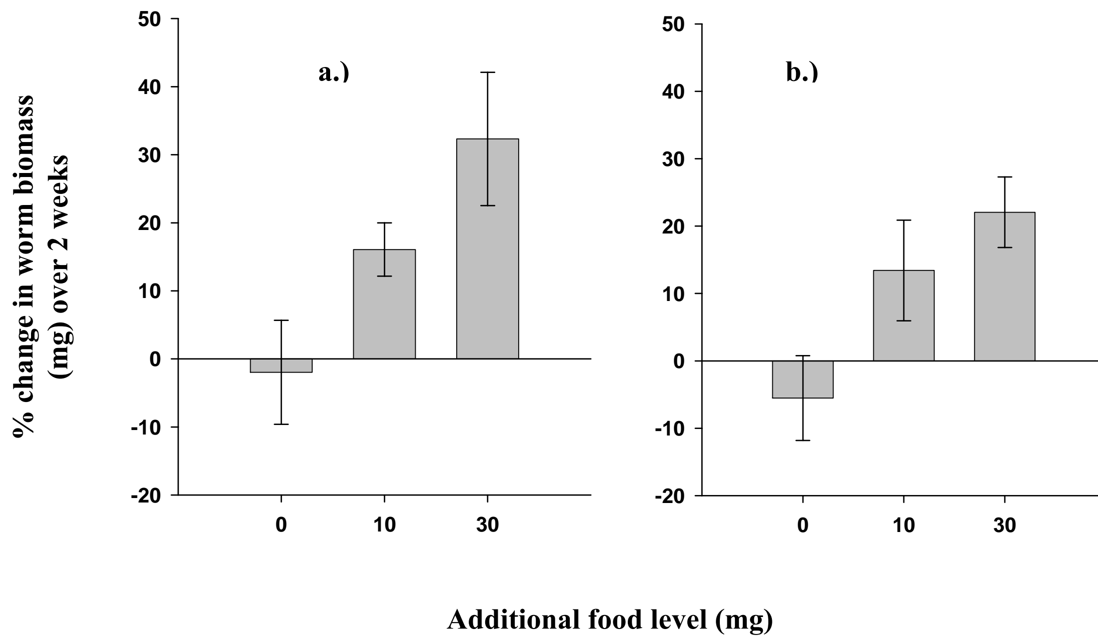


Figure 5.1a and b The % change in worm biomass over 2 weeks in response to the addition of 3 food levels to Goodie River sediments; a.) = 2 individual worms/beaker, b.) = 5 individual worms/beaker; error bars = standard deviations

When the effects of adding food are expressed as a change in the number of individuals quite different results are produced (Figures 5.2a and b). Food level appeared to have no effect on the number of individuals at the end of the experiment in beakers initiated with 2 worms at the start of the experiment (ANOVA, p=0.740). For beakers started with 5 worms there was a significant difference between food levels (ANOVA, p=0.017), but it was the middle food level (10mg) that produced the highest numbers of individuals.

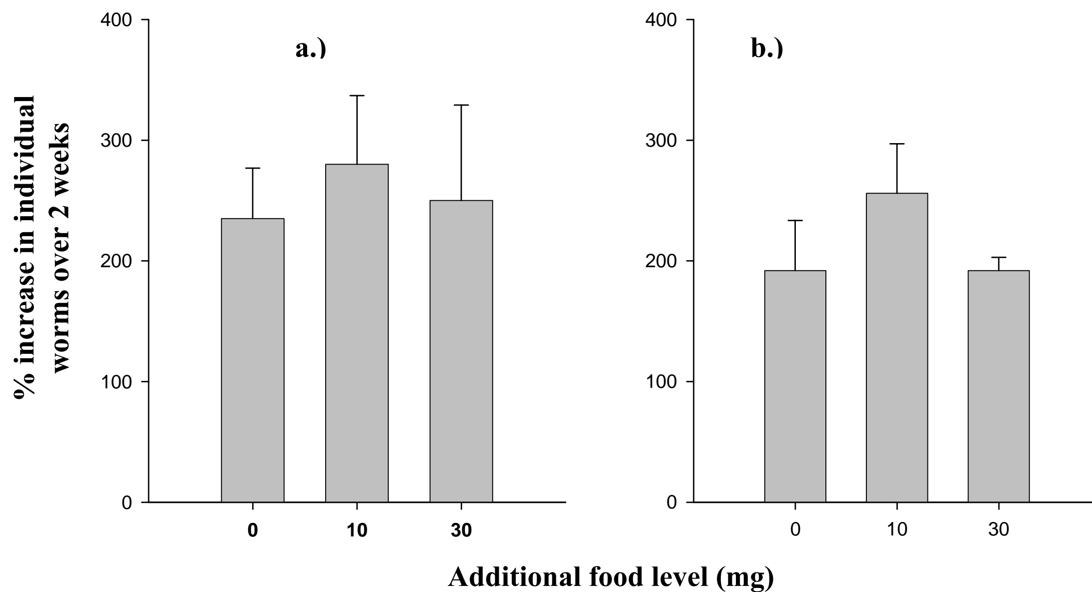


Figure 5.2a and b The % increase in the number of individual worms over 2 weeks in response to the addition of 3 food levels to Goodie River sediments; a.) = 2 individual worms/beaker, b.) = 5 individual worms/beaker; error bars = standard deviations

5.5 DISCUSSION – PRELIMINARY EXPERIMENT

The preliminary experiment highlighted a number of points that were significant to the set-up of the main experiment.

Worms that relied upon the sediment alone to provide their nutritional requirements lost weight over the course of 2 weeks. By the end of 2 weeks the sediment in each beaker had been passed through worms and largely consisted of faecal pellets. Similar losses in weight using uncontaminated, field-collected sediments have previously been found in laboratory microcosm studies with *L.variegatus*, and were suggested to be a result of the rapid depletion of food supplies (Ankley *et al.* 1994;Leppanen and Kukkonen 1998a). As

highlighted previously, significant worm growth would be required in the main experiment. On this basis it was decided to supplement sediment in the main experiment with 30mg of additional food for each 2-week period. This quantity of additional food did not significantly impact on water quality.

At some point during the long-term experiment control population growth rate (pgr) would ideally reach equilibrium or carrying capacity (i.e where pgr approximates zero), as this can provide a useful summary of the effects of toxicants (Grant 1998). Starting experiments with 5 worms per beaker as opposed to 2 appeared to reduce the positive effects of adding food on worm biomass, which is likely to reflect increased competition for food. Therefore, it was decided to start the main experiment with five worms per beaker, so that carrying capacity might be reached in a shorter space of time.

In contrast to the results when expressed on a biomass basis, food level appeared to have varied and inconsistent effects when population growth was expressed on a per capita basis (i.e number of individuals/beaker). Describing *L.variegatus* population size in this way will give undue weight to small, recently fragmented individuals, and thus potentially mask any effects of additional food. This problem has been noted in previous studies with a variety of aquatic organisms and describing population size in terms of biomass was suggested to be a more accurate measure of population growth (Barlow 1992).

5.6 MATERIALS AND METHODS – MAIN EXPERIMENT

5.6.1 Contaminant selection

Pyrene is a member of the polycyclic aromatic hydrocarbons (PAH's). As a group the PAH's are ubiquitous environmental contaminants that are often found in aquatic sediments at elevated concentrations (Van Metre *et al.* 2000). Although sensitive to ultra-violet light, PAH's are considered to be persistent in the environment as a result of their resistance to bio-degradation (Walker *et al* 2001). As previously described above, it was likely that the chosen toxicant would be spiked into sediments on a single occasion. The sediment would then be stored frozen prior to use in experiments. The stability of the test chemical was therefore an important consideration. Previous analytical work in Chapter 3 highlighted the stability of pyrene during sediment spiking procedures and subsequent short-term feeding experiments. A reliable and sensitive method for extracting and analysing pyrene was also developed.

Previous work in Chapter 3 also demonstrated that pyrene causes significant feeding inhibition of *L.variegatus* at sub-lethal levels of contamination. The feeding EC_{50} for pyrene was calculated as 62.1mg/kg (95%CI = 45.7-78.5), whereas pyrene concentrations as high as 300 mg/kg only reduced survival to 75% of controls, so no LC_{50} could be calculated. This means that it should be possible to reliably compare the effects of pyrene-induced feeding inhibition with any potential effects on *L.variegatus* populations.

5.6.2 Sediment spiking

Test sediment was from the same supply as that used in the preliminary experiment (Section 2.3.3.2). As discussed previously, pyrene was added to sediment on one single occasion, and then stored frozen for later use in experiments. Prior to spiking sub-samples of sediment were taken to measure moisture content and organic carbon content. Moisture content was used to calculate nominal target concentrations of pyrene on a dry weight basis i.e mg pyrene/kg dry sediment. Target concentrations for pyrene were 0 (control), 10,30 and 90mg/kg.

Pyrene was added to sediments using the same sand layer methodology described previously in Section 3.3.3.1, as this technique was previously found to produce low variation (<20%) in pyrene concentrations (Section 3.4.2). The desired quantity of pyrene (98% purity, Sigma-Aldrich, Germany) was added to 20ml of HPLC-grade acetone (Fisher Chemicals, UK). The exact quantity of pyrene required for each spike was calculated by weighing defined portions of wet sediment and approximately 2300g of sediment was required per treatment group. The solvent carrier was then added to a stainless steel mixing bowl (Hobart, U.K) that contained 9g of silica sand (<100um grain size, Fisher, U.K). The mixing bowl was rotated until all the solvent had evaporated and the pyrene was left coated onto the sand surface. Sediment was then added and the bowl contents were mixed for 30 minutes at approximately 500 rpm using a Hobart food mixer. The same procedure (i.e solvent and sand addition, mixing) was also carried out for the controls.

Immediately after spiking triplicate samples were taken from each treatment group for chemical analysis of pyrene concentration. Test sediments were then transferred to 2L Pyrex glass beakers and stored at 4°C in complete darkness for 14 days. This period allowed for the equilibrium of spiked pyrene between dissolved and solid phases (Conrad *et al.* 2002;Leppanen and Kukkonen 2000). After 14 days storage individual portions of sediment (20g wet weight) were then distributed to 60ml tall glass beakers (Fisher, U.K) that were used in experiments. All beakers were then stored frozen at -20°C until required over the course of the experiment.

5.6.3 Experiment set-up and maintenance

Experiments were started by defrosting 5 replicate beakers from each pyrene treatment overnight at 20°C in complete darkness (Day –2). The next day (Day –1) beakers were topped up with water and 30mg of additional food was added as previously described in Section 5.3.1). Beakers were then shaken for 30 seconds and left to settle out for a further 24 hours.

Worms used in the main experiment were from the culture previously described in Section 2.3.1.3. On Day –1 the procedures for identifying feeding worms and subsequently taking group wet weights prior to the start of the experiment were carried out (Section 5.3.1). Overlying water in experimental beakers was exchanged on Day 0, prior to the addition of worms.

Once 5 actively feeding worms were identified and their wet weights taken they were added to individual beakers and held in complete darkness at $20^{o}C\underline{+}0.5$ for a 2-week period. Populations in each beaker were then sampled at the end of each 2-week period for the duration of the experiment (14 weeks). Population size, as total live wet weight, dry weight and number of individuals, was determined every 2 weeks using the methodology previously described in Section 5.3.3 above.

Prior to sampling populations, fresh beakers were prepared for use in the next two-week period. At the end of each sampling point worms were placed in new beakers containing fresh sediment at the corresponding pyrene level. Water exchange and water quality analysis were carried out as described previously above (Section 5.3.2), except that samples for ammonia analysis were only taken prior to the last water exchange on Day 12 of each 2-week period.

5.6.4 Analysis of sediment pyrene concentration

Sediment samples from all four pyrene concentrations (0,10,30 and 90mg/kg) were taken for analysis immediately after spiking, in order to check for the degree of mixing during spiking. At the time of spiking analytical beakers identical to those used in the experiment were made up and stored frozen at $-20^{o}C$. Triplicate beakers for each concentration were defrosted at the same time as experimental beakers at the start of each two-week period. Pyrene was then extracted and subsequently analysed by high performance liquid chromatography (HPLC) using the same method as described

previously in Section 3.3.8.1. Triplicate procedural blanks (no sediment) were also used throughout the extraction and analytical process.

5.6.5 Feeding behaviour during the main experiment

Post-exposure egestion rates of *L.variegatus* were measured during Weeks 2 and 14 of the main experiment. Feeding behaviour was not measured on a more frequent basis, as it was thought that the associated disturbance might affect worm growth rates. Post-exposure egestion rates were measured in clean sediment (same as that used in the main experiment) using five worms that were sub-sampled from each of the replicate populations for each pyrene treatment in the main experiment. Worms for the feeding tests were separated immediately after the first and last population sampling periods. Only worms with fully-formed heads and tails and full guts were selected.

Post-exposure egestion rates were measured over a 24-hour period using the methodology described previously (Section 2.6.1.2). Egestion rates are expressed as mg dry faeces/mg dry worm. Total wet weight of the worms used in feeding tests was determined at the start of the feeding period i.e at the same time as sampling population biomass in the main experiment. Total dry weight was calculated as the total wet weight of the 5 worms in each beaker x 0.13. Worms that were not selected for use in the first feeding period (Week 2) were placed in their respective beakers containing fresh sediment for the next 2-week period of the main experiment. The worms that were used in feeding tests were returned to their corresponding beakers 24 hours later at the end of the feeding period.

5.6.6 Calculations and statistics

The effects of pyrene on population biomass and egestion rates for individual time periods was examined with t-tests, one-way ANOVA and Fishers least significant difference multiple comparisons. Biomass and feeding data were x^2 transformed prior to ANOVA if the assumptions of normally distributed data were not met. Population growth rate (pgr) between each sampling period was calculated using Equation 5.2 below:

Equation 5.2

$$\frac{1}{\Delta} \ln \frac{\text{population biomass at time } t + \Delta}{\text{population biomass at time } t}$$

Time is measured in weeks and Δ refers to the two-week census period (after (Forbes *et al.* 2003)). A general linear model (GLM) was used to examine the effects of pyrene concentration, biomass and time on pgr. Prior to GLM Levene's test was used to check for the equality of variance across treatment groups and biomass data were transformed to a log10 scale in order to improve the fit of data.

The following GLM model was specified: **pyrene + replicate (pyrene) + time + pyrene*time**

The brackets denote where replicates were nested within pyrene treatment group. The use of nesting in the model accounts for the non-independent (repeated) measurements of pgr from the same replicate population over 8 time periods. Replicate was used as a random factor and biomass was inserted into the model as a covariate of pgr. Bonferroni's pairwise comparisons were used to compare pgr's in response to different pyrene concentration and

time combinations. In order to run pairwise comparisons replicate was omitted from the random effects term and instead was inserted into the model as a fixed factor. Residuals and fitted values were checked for normality of distribution. All statistical analysis was carried out using Minitab version 13.

5.7 RESULTS – MAIN EXPERIMENT

5.7.1 Water quality and sediment pyrene concentration

Throughout the experiment dissolved oxygen was always above 70% saturation and temperature and pH were always deemed to be within an acceptable range (Temp 20°C $\pm$0.5^{o}C, pH 6.9-7.5). The highest single observation for total and unionised ammonia was 0.61 and 0.0058 mg/L respectively. Based on previous studies it was unlikely that these levels of ammonia would have caused any toxic effects with *L.variegatus* (Schubauer-berigan *et al.* 1995).

Analysis of sediment pyrene concentrations found less than a 20% coefficient of variation between replicates for each treatment group at the time of spiking, which was deemed to be an acceptable degree of variation in pyrene concentration (Northcott and Jones 2000). Pyrene concentrations were always within 10% of nominal target concentrations throughout the experiment. There was a very slight decrease in pyrene concentration between the start and end of the experiment for all treatments, but the difference was never significant. This confirms that there was no significant degradation of

pyrene or change in extraction efficiency as a result of the relatively long (up to 4 months) storage period. Based on the above all subsequent pyrene concentrations refer to nominal concentrations.

5.7.2 Population-level responses to pyrene

5.7.2.1 Change in biomass

Figures 5.3a and b show the change in *L.variegatus* populations over the course of the experiment. Population sizes are expressed as both biomass density (mg/m^2) and individual density (individual worms/m^2). As in the preliminary experiment using the number of individuals seemed to give undue weight to small, recently fragmented worms. For instance in 90-PYR group the number of individuals increased by 24% between Weeks 0 and 2, whereas corresponding biomass was reduced by 27%. Therefore, unless otherwise stated all further analysis and discussion of population size and growth rates is based solely on changes in worm biomass.

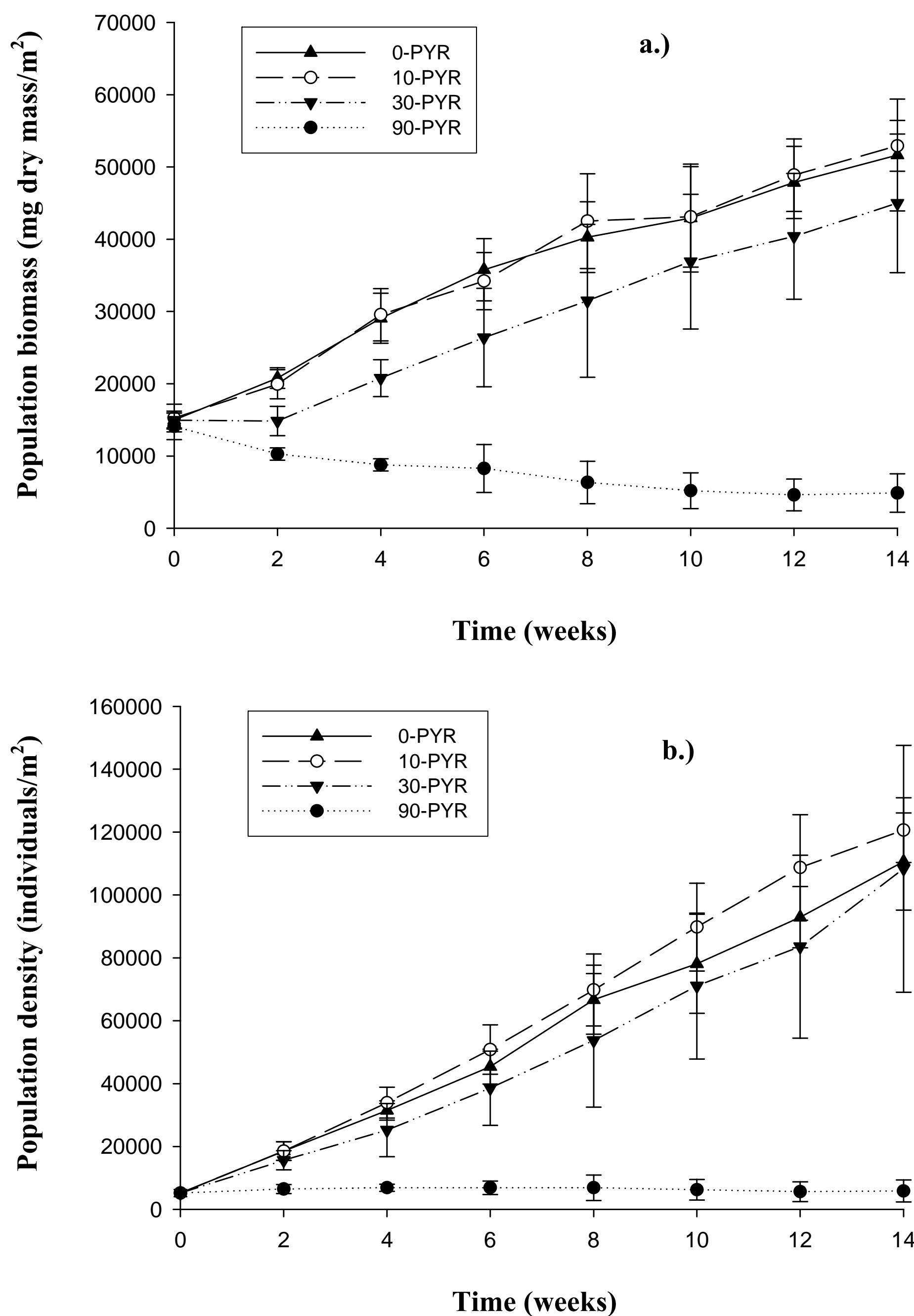


Figure 5.3 Effects of 0,10,30 and 90mg/kg pyrene on population size of *L.variegatus* over a 14-week period, population sizes are expressed as a.) total worm biomass and b.) individual worm density; error bars represent 95% confidence intervals

The biomass of worms at the start of the experiment was not significantly different across treatment groups (ANOVA, p=0.536), which confirms that starting biomass was approximately equal for all treatment groups. Table 5.1 summarises the proportional increase or decrease in worm biomass between the start and the end of the experiment. Populations of worms exposed to 0-PYR (control) persisted and biomass increased throughout the experiment. The pattern of increase in biomass for the 0 and 10-PYR treatments was remarkably similar throughout the entire experiment (Figure 5.3a).

The biomass of worms exposed to 30-PYR was significantly lower than controls after 2 weeks (t-test, t=6.78, p=<0.0001). This pattern continued until week 8 of the experiment, where the difference between 0 and 30-PYR was no longer significant (t-test, t=1.87, p=0.11). By the end of the experiment biomass in the 30-PYR group was still slightly reduced when compared to controls, but the difference was not statistically significant (t-test t = 1.51, p=0.170).

The only mortalities were found in the highest pyrene (PYR) group (90 mg/kg), but the average number of individuals in 90-PYR was the same at the start and end of the experiment. Figure 5.3a highlights the very obvious effects of the highest pyrene treatment (90-PYR) on population biomass. In contrast to all other treatments starting biomass was gradually reduced over the course of 14 weeks, resulting in an overall decrease of 65% by the end of the experiment (Table 5.1).

Table 5.1 Mean population biomass at the start and end of the experiment and the proportional change in mean population biomass expressed as a percentage over the course of 14 weeks, coefficient of variation in brackets, n=5

Pyrene level (mg/kg)	Dry biomass (mg/m^2)		Mean biomass change over 14 weeks
	Week 0	Week 14	
0 (control)	14968 (6.7)	51653 (12.1)	245 % increase
10	15242 (10.1)	52915 (5.3)	247 % increase
30	14922 (5.6)	44960 (17.2)	201 % increase
90	14096 (10.6)	4872 (44.0)	65 % decrease

5.7.2.2 Population growth rates

Figures 5.4a-d show the population growth rates (pgr's) of *L.variegatus* over the course of the experiment. During the first two weeks control and 10-PYR populations grew by 0.16 and 0.13/week respectively. However, from Week 4 onwards there was a relatively consistent decline in pgr's for both control and 10-PYR populations, which suggested that the effects of density-driven regulation were increasingly evident (Figure 5.4a and b). By week 14 the average population growth rate of controls had not quite reached carrying capacity (i.e pgr= zero, Figure 5.4a). Unfortunately, due to logistical constraints the experiment had to be stopped at this point. Therefore, the biomass that marked carrying capacity under the experimental conditions used here could not be estimated.

Population growth rates for 30-PYR were slightly negative after 2 weeks (-0.004/week). However, there was a marked turnaround during Weeks 2-4 (Figure 5.4c), where population biomass grew by 0.17/week. Growth rates of the 30-PYR treatment then followed a similar decline to that seen in the control and 10-PYR groups for the rest of the

experiment. Population growth rates of *L.variegatus* exposed to 90-PYR were reduced throughout the experiment when compared to the controls (Figure 5.4d). For example, after 2 weeks the average pgr was -0.16/week. For the rest of the experiment pgr's were usually negative, although there was some fluctuation above the dotted line marked in Figure 5.4d.

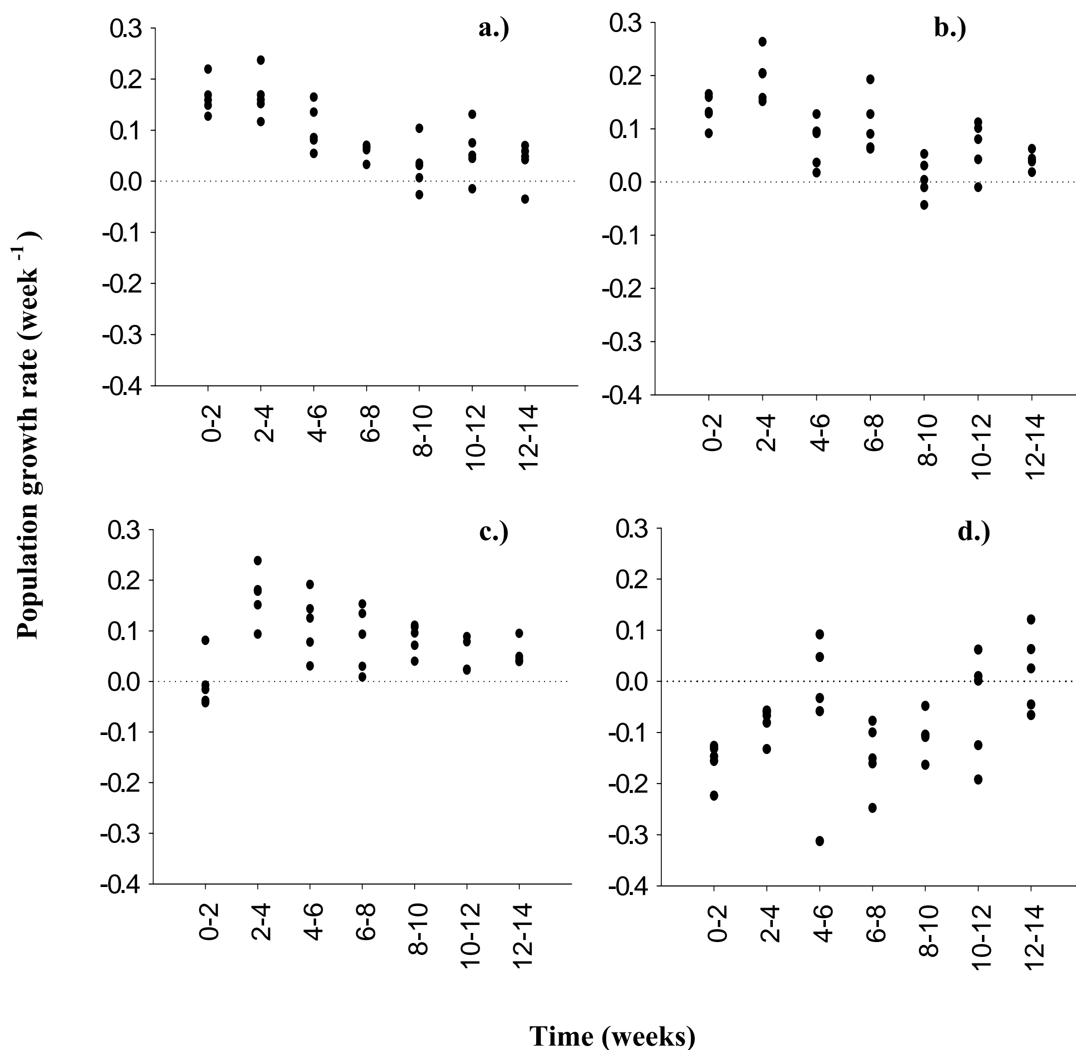


Figure 5.4a-d Population growth rate (pgr) of *L.variegatus* during exposure to four pyrene (PYR) concentrations; a.) 0mg/kg-(control), b.) 10mg/kg, c.) 30mg/kg, d.) 90mg/kg; pgr is calculated on a weekly basis for each 2-week period (x axis), dotted lines represent the theoretical point of population carrying capacity i.e pgr=0

5.7.2.3 The effects of pyrene and density on population growth rates

The output from the general linear model is summarised in Table 5.2. Pyrene concentration and biomass (density) both had a significant effect on pgr. Pairwise comparisons indicated that pgr's of 0 and 10-PYR were never significantly different from one another throughout the experiment. Population growth rates of the 30-PYR worms were only significantly lower than controls during the first two weeks of the experiment ($p=<0.0001$), whereas pgr's of 90-PYR worms were always significantly lower than the other treatment groups throughout the experiment ($p=<0.0001$).

Table 5.2 General linear model output for the effects of pyrene exposure, time and their interaction on the population growth rate of *L.variegatus*, the regression of population biomass (as a covariate in the model) is also included

Source	df	Seq SS	Adj SS	Adj MS	F	P
Biomass	1	0.190050	0.078355	0.078355	32.21	<0.0001
Pyrene	3	0.730456	0.208119	0.069373	11.29	<0.0001
Replicate (pyrene)	16	0.128159	0.123204	0.007700	3.17	<0.0001
Time	6	0.094543	0.107269	0.017878	7.35	<0.0001
Pyrene*Time	18	0.143620	0.143620	0.007979	3.28	<0.0001
Error	95	0.231114	0.231114	0.002433		
Total	139	1.517940				
Regression	**Coef**	**SE Coef**	**T**	**p**		
Constant	0.9995	0.1686	5.93	<0.0001		
Biomass	-0.439	0.0774	-5.98	<0.0001		

Figures 5.5a-d show the regressions of pgr and $\log_{10}$ biomass for each pyrene treatment. Table 5.3 also shows the associated fitted line equations, *r*-sq and p values for each regression. In the 0 and 10-PYR treatments pgr's declined linearly and significantly with

increasing $\log_{10}$ biomass. This confirms that 0 and10-PYR worms experienced significant density-dependent growth during the experiment. In contrast, the regressions of pgr and $\log_{10}$ biomass for both 30 and 90-PYR were not significant, which suggests that the effects of density on pgr were weakened by the higher toxicant exposures. For 90-PYR there is substantial scatter in the relationship, whereas for 30-PYR the results of the regression appear to be heavily skewed by the effects of data from the first (Week 2) sampling point (points identified as open squares in Figure 5.5c). If this data is removed the modified regression suggests that pgr in 30-PYR worms declines linearly and significantly with an increase in $\log_{10}$ biomass (i.e experienced density-dependent regulation, see Table 5.3 for comparison of regressions).

Table 5.3 Fitted line equations, *r*-sq and p values for each of the regressions in Figures 5.5a-d respectively, 30-PYR also contains results from a modified regression where pgr and $\log_{10}$biomass from the first two weeks were removed

Pyrene concentration	Regression equation	*r*-sq	p value
0-PYR	pgr = 0.8 - 0.302 $\log_{10}$biomass	64.8	<0.0001
10-PYR	pgr = 0.752 - 0.281 $\log_{10}$biomass	52.6	<0.0001
30-PYR			
- Original regression	pgr = 0.266 - 0.0834 $\log_{10}$biomass	4.9	0.203
- 1st 2 weeks removed	pgr = 0.649 - 0.243 $\log_{10}$biomass	45.5	<0.0001
90-PYR	0.152 - 0.135 $\log_{10}$biomass	9.9	0.066

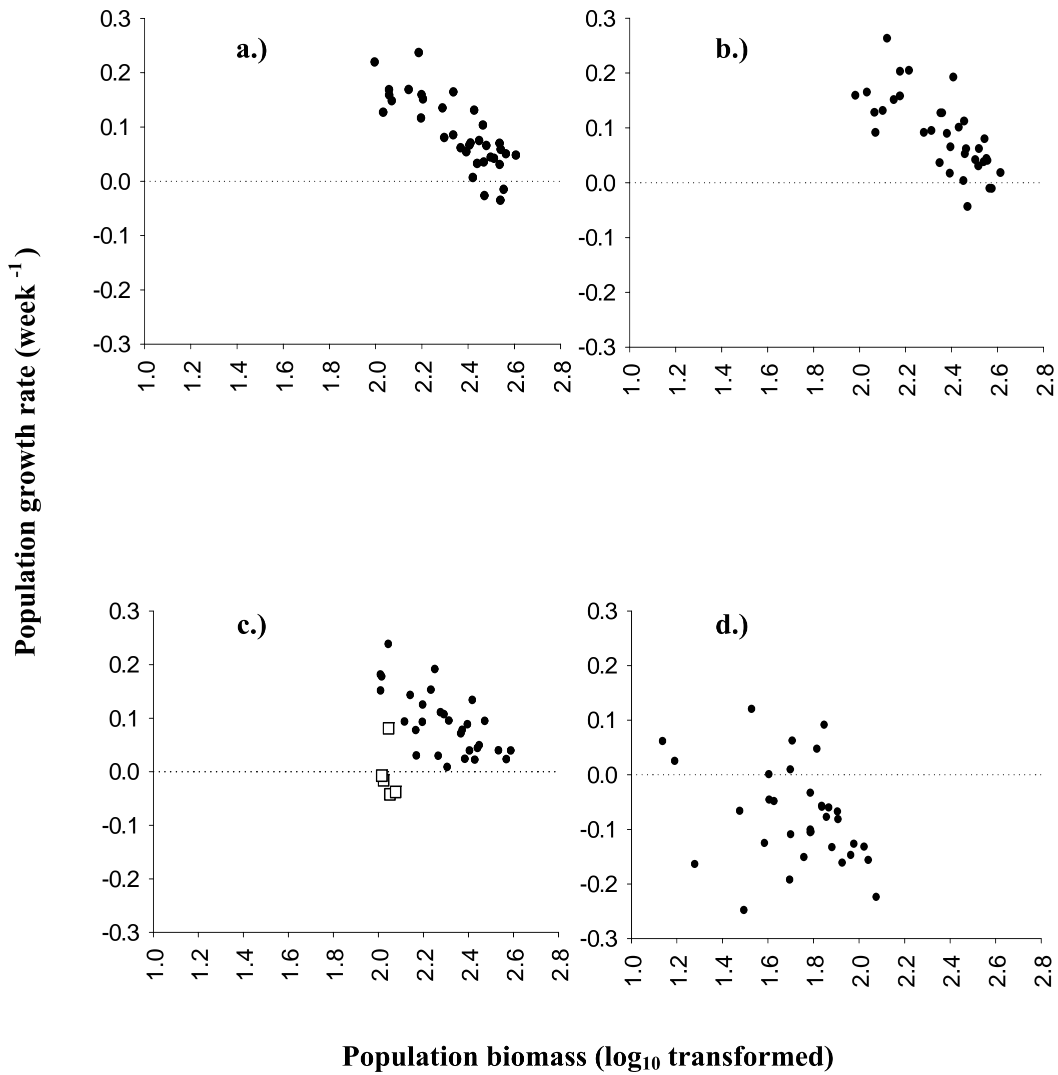


Figure 5.5a-d Population growth rate as a function of $\log_{10}$-transformed biomass of *L.variegatus* exposed to four pyrene (PYR) concentrations; a.) 0mg/kg (control), b.) 10mg/kg, c.) 30mg/kg, d.) 90mg/kg; open squares in c.) represent data from the first two weeks of the experiment; dotted lines represent the theoretical point of population carrying capacity i.e pgr=0

5.7.3 Worm behaviour

All worms in the 0,10 and 30-PYR treatments were fully burrowed into sediments throughout the experiment, and thus showed no evidence of sediment avoidance behaviour. By week 2 onwards all of the sediment in 0 and 10-PYR beakers appeared to have been completely processed (i.e. ingested by worms and egested onto the sediment surface as faecal pellets). When 0 and 10-PYR worms were sampled sediment was always visible within the gut.

There was less evidence of sediment processing by worms during the first 2 weeks exposure to 30-PYR. A considerable proportion (approximately 40%) of worms also had empty guts by week 2. This situation seemed to change from week 2 onwards, as more worms with full guts were found and sediment processing appeared to reach similar levels as controls by weeks 6-8.

Worms exposed to 90-PYR were found on the surface avoiding sediments, although individual worms were sometimes found burrowed into sediment during the last four weeks. Even when worms were burrowed there was no evidence of faecal pellet production or physical disruption of sediment due to feeding activity, as was seen in the other treatment groups. Throughout the experiment 90-PYR worms were thin and transparent-looking with no sediment visible in the gut.

5.7.4 Post-exposure egestion rates

Post-exposure egestion rates for 0,10 and 30-PYR worms from the start and end of the experiment are shown in Figure 5.6. Egestion rates of control worms for both feeding periods were not significantly different from one another (t-test, $t=0.82$, $p=0.441$). This confirmed that the bioassay procedure was reproducible over the course of the 14-week experiment. The 0 and 10-PYR treatments were not significantly different from one another for both week 2 (t-test,$t=1.45$, $p=0.190$) and week 14 (t-test,$t=-0.65$, $p=0.539$) feeding periods. Exposure to 30-PYR caused significant feeding inhibition during both Weeks 2 and 14 (t-test, Week 2, $t=5.4$, $p=0.006$; Week 14, $t=4.89$, $p=0.008$). When compared to the corresponding controls egestion rates in the 30-PYR group were reduced by 71 and 70% for Weeks 2 and 14 respectively. As 90-PYR worms continued to avoid the clean sediments in the post-exposure feeding beakers, post-exposure egestion rates could not be measured.

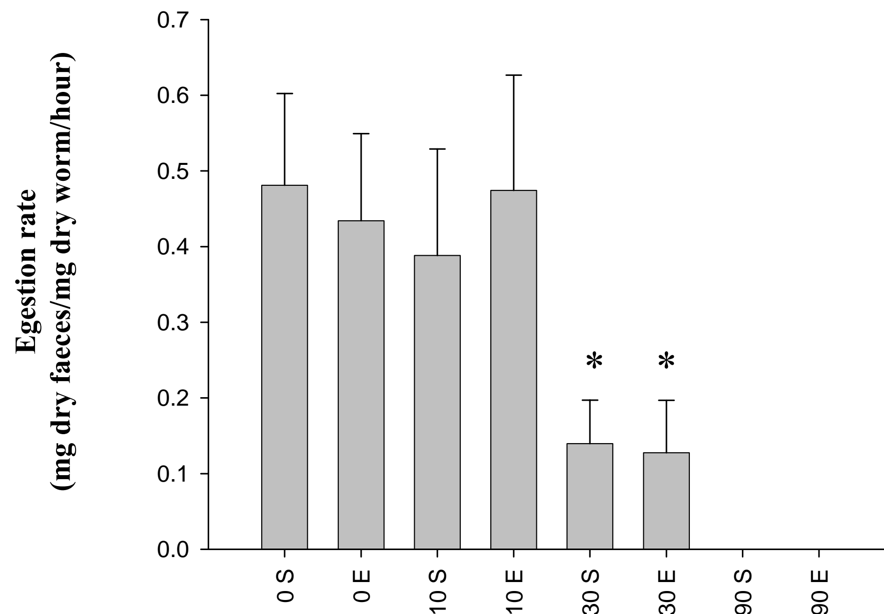


Figure 5.6 Egestion rates of *L.variegatus* populations at the start (S) and end (E) of a 14-week exposure to four pyrene concentrations (0, 10 and 30 mg/kg), S = egestion rate between Days 14 and 15, E = egestion rate between Days 98 and 99, *denotes treatments that are significantly different from the corresponding control, egestion rates for 90mg/kg not measured due to sediment avoidance behaviour, error bars represent 95% confidence intervals

5.8 DISCUSSION – MAIN EXPERIMENT

Long–term (14 weeks) exposures to pyrene caused the inhibition of both *L.variegatus* feeding behaviour and population growth rates. As population biomass increased during the experiment, population growth rates were also subject to the regulatory effects of density-dependence. Previous studies related to density-dependent processes with *L.variegatus* populations are lacking. In a study with the tubificid oligochaete, *T.tubifex*, strong density-dependent population growth was a result of a delay in the maturation period as a result of a

reduction in individual growth rates (Bonacina *et al.* 1989). High densities of the Lumbriculid oligochaete, *S.heringianus*, resulted in the complete reworking of sediment in the top layer of beakers (White *et al.* 1987). Similar exhaustion of sediment also occurred in control beakers during the current experiment, therefore it is likely that density-dependence was at least partly driven via increasing intra-specific competition for scarce food resources.

5.8.1 10 and 90mg/kg pyrene

The lowest pyrene treatment (10mg/kg) had no effect on population biomass, population growth rates and post-exposure egestion rates throughout the experiment. Therefore, the results from the post-exposure feeding bioassay were in good agreement with population-level responses for this concentration of pyrene. The highest pyrene concentration (90mg/kg) had a marked negative effect on *L.variegatus* populations. Animals did not reproduce and those that started the experiment steadily lost weight over the course of 14 weeks. As a result population growth rates were reduced throughout the experiment. The loss in weight of 90-PYR worms is suggested to be a result of feeding inhibition. Worms avoided 90-PYR sediments and had empty guts throughout the experiment, which confirmed that sediment ingestion was stopped. Therefore, feeding inhibition as demonstrated with the feeding bioassay was in good agreement with negative effects observed at the population-level for 90mg/kg pyrene.

The ability to relate the effects of individual-level bioassay endpoints such as feeding inhibition to effects at higher levels of organisation will improve the ecological relevance of the bioassay (Calow 1989). Significant inhibition of oligochaete egestion rate has been correlated with reduced individual growth and reproduction previously (Keilty *et al.* 1988b;Lotufo and Fleeger 1996). One surprising point with 90-PYR worms was that populations starved for 14 weeks did not completely die off. It was expected that worms would not have sufficient energetic reserves to survive over this length of time without feeding. Unfortunately there are no previous studies available with which to compare long-term survival rates of starved oligochaetes.

5.8.2 30mg/kg pyrene

The relationship between the feeding bioassay endpoint and population-level effects for the 30mg/kg pyrene treatment varied over time. Post-exposure egestion rates and population growth rates (pgr's) were both significantly reduced during the first two weeks of the experiment. Therefore, the feeding bioassay endpoint was predictive of the population-level effects of 30mg/kg pyrene at this point in time. However, from Week 2 onwards pgr's increased and were not significantly different from the controls for the rest of the experiment. As a result, by the end of the experiment the population biomass of 30-PYR worms was not significantly different from the controls, despite the fact that post-exposure egestion rates were reduced by 70% at the end of the experiment. These results suggested that the feeding bioassay over-estimated the potential longer-term population-

level effects of 30mg/kg pyrene. Possible mechanisms behind these results are discussed below.

5.8.2.1 Compensation at the population level

The potential negative effects of the 30-PYR treatment may have been compensated at the population level by density-dependence in the control populations. Growth rates of controls were reduced as population density increased during the experiment, until populations appeared to be close to carrying capacity (i.e pgr=0) by week 14 (Figure 5.4a). When the control and 30-PYR populations were compared at the end of the experiment, density-driven regulation in the controls might have buffered any negative effects of 30-PYR. The negative effects of density in toxicant-exposed populations might also be weakened if the increase in density reduces toxicant exposure per individual, particularly if a major uptake route is via the body surface (Forbes *et al.* 2003). This would imply that the interaction between density-dependence and 30-PYR was less-than additive in nature. Similar less than additive interactions between the effects of density and toxicants have previously been found with a variety of invertebrates and toxicants (Sibly *et al.* 2000;Barata *et al.* 2002;Moe *et al.* 2002;Liess 2002;Forbes *et al.* 2003).

5.8.2.2 Compensation at the individual level

While it is possible that the effects of density regulation may have buffered the negative effects of 30-PYR at the population level, density-dependence does not adequately explain

how growth rates in 30-PYR worms rapidly recovered to control rates from week 2 onwards (Figure 5.4c), despite the fact that egestion rates were reduced by approximately 70% at the same time. A number of previous studies with deposit feeders (including *L.variegatus*) have also failed to relate toxicant-induced feeding inhibition to effects on growth and/or reproduction (Keilty *et al.* 1988b;Jensen *et al.* 2001;Landrum *et al.* 2004b). Unfortunately no potential explanations for these results were offered in these studies.

In a previous study with *L.variegatus*, significant feeding inhibition had no effect on the rate of carbon assimilation (Landrum *et al.* 2004b). This suggests that assimilation efficiency increased in response to increased gut passage time (caused by a reduction in feeding rate). Similar inverse relationships between assimilation efficiency and feeding rate have also been found in a variety of freshwater and marine deposit feeders (Klump *et al.* 1987;Lopez and Levinton 1987). Therefore, the effects of feeding inhibition in the 30-PYR treatment may have been compensated by improvements to nutrient assimilation efficiency. However, the above does not explain why population growth rates in the 30-PYR group were initially inhibited during Weeks 0-2, only to recover to control rates for the rest of the experiment. If the quantity of nutrients remained unchanged despite significant feeding inhibition, why were pgr's reduced for the first two weeks? An alternative explanation could relate to the toxic mode of action of pyrene.

It was assumed that pyrene would act on *L.variegatus* via a general narcotic response that would interfere with normal feeding behaviour (Vanwezel and Opperhuizen 1995). However, if the pyrene that was associated with ingested sediment particles interfered with

normal digestive processes, this could have led to a reduction in nutrient assimilation during the first two-weeks. Exposure to sediment-bound copper caused significant inhibition of a digestive protease in the gut fluids of the lugworm, *Arenicola marina* (Chen and Mayer 1998). Unfortunately similar studies with deposit feeders and organic contaminants are lacking.

Following on from above, if worms were able to adapt to the negative effects of pyrene on digestive processes (via some form of inducible detoxification mechanism) this could have improved nutrient assimilation (and hence growth) for the rest of the experiment. A previous study isolated cytochrome P-450 mixed function oxidase, which is involved in the metabolism/detoxification of many organic chemicals, from the gut of the terrestrial oligochaete, *L.terrestris* (Berghout *et al.* 1991). Therefore, although evidence is tenuous, it is possible that *L.variegatus* possesses a similar metabolism/detoxification mechanism that enabled adaptation to the otherwise harmful effects of 30-PYR exposure (Lee 1998).

5.8.3 Population status at the time of toxicant exposure

Density-dependence was chosen as an important mechanism by which populations are regulated, which may also interact with the effects of toxicants (Section 5.1.2). The results from the 30-PYR treatment could suggest that the effects of density-driven regulation in control populations might buffer the negative effects of 30-PYR exposure. However, it is important to recognise that population abundance will be determined by a large number of intrinsic and extrinsic factors, that may be dependent or independent of population density

(Begon *et al* 1996). For example, a population recovering from a major environmental perturbation (e.g extreme flood event) will experience very little density-driven regulation, as population biomass will be low. However, a population in a relatively stable environment might experience strong density-driven regulation via intra-specific competition for scarce resources. Therefore, if we are concerned with the potential interaction between the effects of density and toxicants, defining the status of a population at the time of toxicant exposure (i.e the strength of any density-driven regulation) will be crucial.

Following on from above, the effect of population status may be reflected in the results from the 30-PYR group. In the first two weeks of the experiment feeding inhibition was related to a significant reduction in population growth rate (pgr). At this point control populations did not experience significant density-dependent growth (Figure 5.4a). However, by the end of the experiment the strong density-dependent growth rates in controls may have indirectly compensated for the negative effects of feeding inhibition with 30-PYR. This implies that the effects of feeding inhibition with the 30-PYR treatment may not result in a net population change under conditions of strong density-dependence (as experienced at the end of the experiment), but they may become more significant when density-dependence is weakened e.g increased mortality due to predation/disease, recovery after a environmental perturbation (Forbes *et al.* 2001).

5.8.4 The importance of indirect effects

One of the principle limitations with the current experiment design was that it did not consider the potential for indirect effects of *L.variegatus* feeding inhibition on populations and communities (DeAngelis 1996). Indirect effects are defined as “changes in the abundance of a population resulting not directly from the action of a causal agent (such as a toxicant) but indirectly through the effects of the casual agent on other species” (DeAngelis 1996). A good example of the indirect effects of toxicant-induced feeding inhibition is an increase in phytoplankton abundance as a result of reduced zooplankton grazing (Slijkerman *et al.* 2004).

Previous studies that have looked at the indirect effects of oligochaete feeding inhibition as a result of toxicants are lacking. However, the feeding behaviour of oligochaetes in uncontaminated conditions has previously been correlated with changes to the structure and productivity of the sediment bacteria community (Wavre and Brinkhurst 1971), which is of fundamental importance to the cycling of nutrients in aquatic systems (Storey *et al.* 1999). Therefore, the inhibition of oligochaete feeding may have important indirect effects on microbial community dynamics and nutrient cycling. With interest in the use of *L.variegatus* feeding behaviour as a bioassay endpoint growing e.g (Landrum *et al.* 2002;Landrum *et al.* 2004a;Landrum *et al.* 2004b), determining the potential for indirect effects of feeding inhibition could be a very interesting area for future research.

5.9 SUMMARY

The results from this section of work confirmed that short-term measures of feeding inhibition with *L.variegatus* could be related to longer-term effects on population biomass and growth rates. This fulfilled an important requirement for the wider relevance of the bioassay. Additional studies using a wider variety of toxicants with different modes of action would further highlight the relationship between feeding inhibition and population-level effects. Also, the potential for important indirect effects of *L.variegatus* feeding inhibition should not be overlooked, and this is suggested to represent a promising area for further study. Although further work is required, the results for the 30mg/kg pyrene treatment suggested that, under certain circumstances, the effects of feeding inhibition may be subject to a number of possible compensatory mechanisms acting at the population (i.e density-dependence) and/or individual levels (i.e assimilation efficiency/toxicant tolerance). However, in the case of density-driven compensation, the status of a population during toxicant exposure (i.e density-dependent or density-independent?) will be crucial in determining the strength of any interaction.

6 CHAPTER 6 – GENERAL DISCUSSION

The purpose of this thesis was to develop and test an *in situ* sediment bioassay using the post-exposure feeding behaviour of *L.variegatus*, which met the requirements highlighted in Section 1.5. The main requirements of the bioassay were that it produced ecologically relevant and reliable results in a cost-effective manner. Owing to the problems of directly measuring *L.variegatus* feeding rates in the field, the bioassay consisted of a contaminant exposure period and a subsequent post-exposure period where feeding rates were measured under clean conditions. This method was dependent on the persistent inhibition of *L.variegatus* feeding rate, which has not been demonstrated in either the laboratory or field previously.

The following discussion is divided into three sections:

- Laboratory bioassay studies
- Field development of the bioassay
- Overall conclusions

6.1 LABORATORY STUDIES

6.1.1 Bioassay relevance

To be ecologically relevant the selected species should be sensitive to contaminants, or representative of an important trophic group that contributes to the functional properties of the system that is being studied. It should also be possible to extrapolate the effects of the

bioassay endpoint to higher levels of organisation e.g populations, communities (Calow 1989). *L.variegatus* is suggested to be an ecologically relevant tests species for sediment bioassays. It is representative of an important functional group in freshwater ecosystems, which contribute to the transfer of energy from detritus-based food webs to higher trophic levels. Through their sediment reworking behaviour, oligochaetes have also been demonstrated to significantly alter the biological, chemical and physical characteristics of the benthic environment.

As a group oligochaetes are often viewed as pollution tolerant, which has raised doubts over their use in toxicity bioassays (Chapman 2001). Undoubtedly the lethal tolerances of many species are relatively high, but tolerances are toxicant and species-specific (Chapman and Brinkhurst 1984). Further, sub-lethal endpoints such as feeding behaviour, growth and reproduction have often been shown to be very sensitive measures of toxicant stress with oligochaetes (Chapman 2001). In a previous study comparing the sensitivity of *L.variegatus* with other sediment bioassay organisms (amphipod, *Hyalella azteca*, chironmid, *Chironomus tentans*), no single species was found to be the "most sensitive" to a range of ten toxicants (West *et al.* 1993). Therefore, selection of *L.variegatus* was primarily based on the important functional role that oligochaetes play in wider ecosystem function (Section 1.4.2).

The need for an *in situ* sediment bioassay using *L.variegatus* may also be questioned, as *in situ* bioassays using the deposit feeding chironomid, *C. riparius* have already been developed (Conrad *et al.* 1999;Sibley *et al.* 1999;Crane *et al.* 2000;Olsen *et al.* 2001;Castro

et al. 2003). However, a recent European Commission technical guidance document recommended the development of both *C.riparius* and *L.variegatus* toxicity bioassays, as each organism was suggested to represent distinct feeding niches in benthic systems (European Commission 2003).

Traditionally oligochaetes and chironomids have been classified within the same functional feeding group (i.e collector-gatherers) (Cummins and Klug 1979). However, important non-trophic effects may also be used to classify benthic species (Chapin *et al.* 1992). For example, mode of sediment reworking has been used to classify benthic species in lakes, as it incorporates the role of both feeding and feeding-related effects (e.g bioturbation) on sediment processes (Van De bund *et al.* 1994). Oligochaetes are considered upward, “conveyor belt” deposit feeders (Robbins 1982), who continuously mix the top layer of sediment, whereas chironomids are usually considered micro-gatherers of surface sediment (McCall and Tevesz 1982). In a recent study that compared the roles of the chironomid, *Prodiamesa* sp., and the oligochaete, *T.tubifex*, each species was found to have quite different effects on key ecological processes in sediments such as microbial activity and nutrient transport (Mermillod-Blondin *et al.* 2002). Therefore, it is argued that the role of chironomids and oligochaetes in benthic ecosystems are sufficiently distinct from one another that both will be of value in sediment assessments.

Along with the functional feeding niche of an organism, information on habitat preference and life cycle will also improve the ecological relevance of risk assessment tools (Baird *et al.* 2001). For example, because chironomids have an adult winged form they

were used to study the accumulation of contaminants from aquatic sediments to pond bats (Reinhold *et al.* 1999).

The work in Chapter 5 compared the short-term effects of pyrene on the feeding behaviour of *L.variegatus* with longer-term changes to population biomass. The results of the post-exposure feeding bioassay developed in previous chapters were predictive of population-level effects with two of the three concentrations tested. This confirmed that short-term observations of feeding behaviour could be directly related to longer-term responses at higher levels of organisation, which was one of the main requirements for the bioassay (Section 1.5.1).

The experimental design in Chapter 5 did not consider the potential indirect effects of *L.variegatus* feeding inhibition on ecosystem structure and function. Further empirical studies with more complex model ecosystems could provide a very interesting area for future research. In addition, only one type of toxicant was used in Chapter 5, so additional experiments with toxicants that possess different modes of action could provide further useful information on the relationship between *L.variegatus* feeding inhibition and population-level effects.

For one of the pyrene concentrations (30mg/kg) tested in Chapter 5, significant feeding inhibition could not be related to any effects on population biomass at the end of the experiment. Possible mechanisms for this result include density-dependent compensation at the population level, and improvements to nutrient assimilation efficiency and/or a rapidly

inducible detoxification/repair mechanism at the individual level. In the case of density-dependent compensation, it is important to recognise that the status of a population at the time of toxicant exposure (i.e is the population experiencing significant density-dependent regulation or not?) will be crucial in determining the strength of any density-driven compensation. Further work using a variety of population starting densities, radio-labelled tracers and toxicants with different modes of action might help to further identify the mechanisms behind these results.

6.1.2 Bioassay reliability

The culture techniques used throughout this work produced a regular supply of worms. Survival of worms in control treatment groups was consistently high throughout both short (six-day) and long-term (14 weeks) laboratory and *in situ* exposures. The work in Chapter 2 aimed to develop a basic method for the bioassay that minimised the variation in post-exposure egestion rates under controlled laboratory conditions. The bioassay methodology developed in Chapter 2 produced a coefficient of variation of 17%. Subsequent use of the bioassay in uncontaminated laboratory conditions in Chapters 3,4 and 5 produced a very consistent baseline egestion rate between 0.35 and 0.45mg dry faeces/individual worm/hour (Figure 6.1). The average coefficient of variation for the 12 individual control egestion rates in Figure 6.1 was 16.5% (sd=3.7).

The bioassay methodology produced reliable results with different culture supplies and sediments possessing different physio-chemical characteristics (as a result of working in

both the U.K and Canada). The method is therefore easily transferable to different laboratories. Consistent baseline (control) responses also enabled toxicity-induced feeding inhibition to be detected in the laboratory (Chapters 3 and 5). Reproducibility in toxicant responses was also found with repeated exposures to pyrene. For example after previous exposure to 30mg/kg of pyrene post-exposure egestion rates were reduced by 62% in Chapter 3 and 70% in Chapter 5. Therefore, it was concluded that the basic bioassay technique developed in the laboratory was both reliable and repeatable.

Chapter 2 also examined the number of replicates required to produce a statistical power of 80%, as this is traditionally defined as the desired power of a statistical test (Zar 1999).When a minimum detectable difference of 20% was specified the number of replicates required to produce 80% power was 11, which was deemed impractical owing to the time and resources required to carry out the bioassay with this number of replicates. A statistical power of 0.53 was calculated with 5 replicates and 20% minimum detectable difference, which was subsequently able to reliably detect post-exposure feeding inhibition in the laboratory.

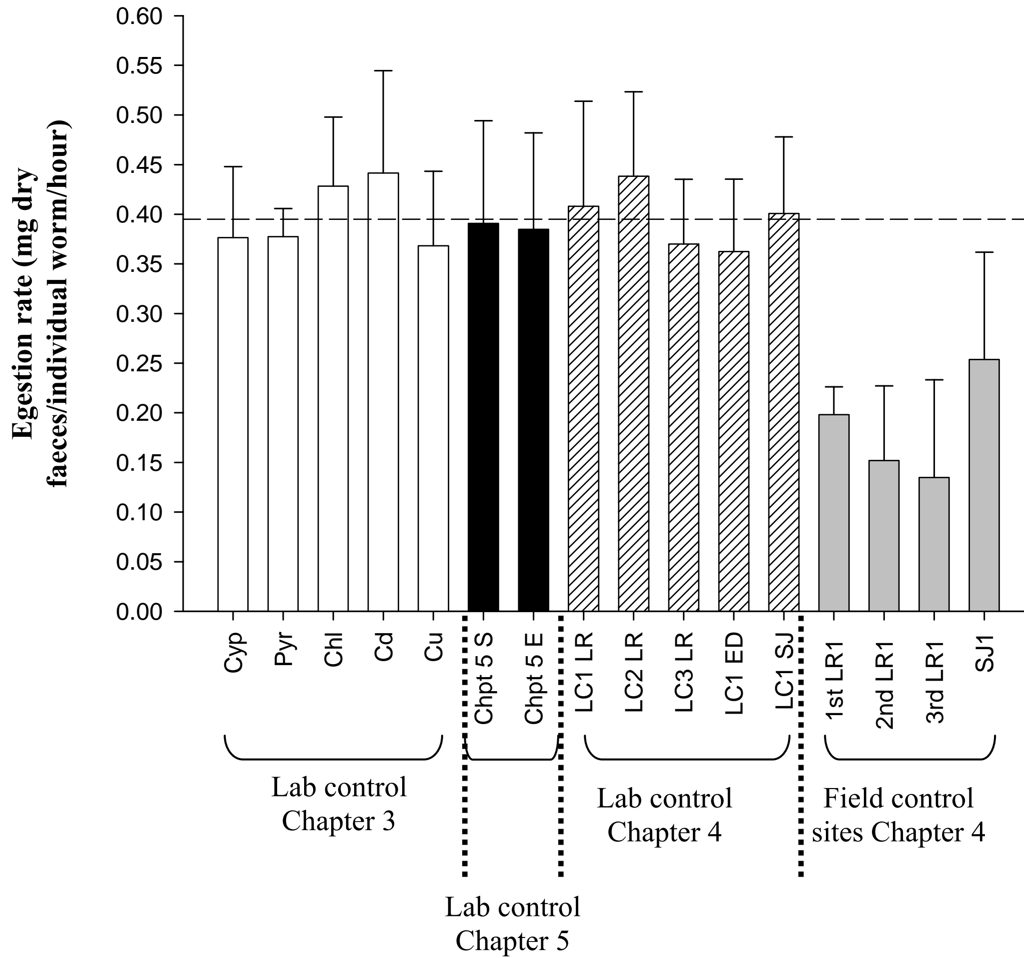


Figure 6.1 Post-exposure egestion rates of *L.variegatus* after exposure to "clean" (control) sediments in both the laboratory and field, the dashed line represents the mean post-exposure egestion rate from all laboratory exposures

6.1.3 Endpoint sensitivity

Three out of the five chemicals tested in Chapter 3 caused significant feeding inhibition at sub-lethal toxicant concentrations. Two recent studies with *L.variegatus* have found significant feeding inhibition at sub-lethal concentrations during exposure to 3,4,3',4'-tetrachlorobiphenyl (Landrum *et al.* 2004a;Landrum *et al.* 2004b). For a given chemical concentration, the mode of action will determine if feeding or lethality is the most dominant

effect. However, in the current work *L.variegatus* feeding was always inhibited at concentrations significantly below those that caused lethality. Further work could determine if a similar relationship exists with a wider variety of chemicals with different modes of action.

Current bioaccumulation test guidelines that use *L.variegatus* specify that a preliminary 4-day toxicity test should be carried out to determine if sediment toxicity will affect estimates of bioaccumulation (U.S.E.P.A 2000). Worm survival and sediment avoidance behaviour are observed. Sediment avoidance behaviour is measured as worms avoiding contaminated sediments may significantly modify their exposure as a result of reduced burrowing and particle ingestion (West and Ankley 1998). Toxicant exposures in Chapters 3 and 5 highlighted that for some common, persistent contaminants e.g pyrene, feeding inhibition will occur at lower concentrations than those that cause sediment avoidance behaviour. Therefore, if there is concern regarding the under-estimation of contaminant exposure due to reduced particle ingestion, the toxicity-screening test that is used prior to bioaccumulation testing with *L.variegatus* should also measure egestion rates.

As post-exposure feeding inhibition of *L.variegatus* is a novel endpoint, it should be sensitive to a wide range of chemicals with different modes of action. The effects of cypermethrin and pyrene were sufficiently persistent to be detected during the post-exposure feeding period. Feeding inhibition occurred during the direct exposure period to chlorothalonil, but egestion rates recovered to control levels during the post-exposure

feeding period. This may have been the result of a rapidly reversible mechanism that could not be identified using published literature.

Cadmium and copper did not have any effects on both exposure and post-exposure egestion rates and organism survival. This may have been a result of a rapidly inducible detoxification mechanism e.g metallothionein-like protein. Further testing with additional metals would be useful, but these results would suggest that the bioassay would be of little use in situations where metal-contaminated sediments are the primary concern e.g mining effluents. In this situation complimentary *in situ* bioassays using *C.riparius* could be used for toxicity evaluations. The significant accumulation of cadmium in worm tissue does however highlight the risks associated with trophic transfer of metals via oligochaetes (Wallace and Lopez 1996;Wallace and Lopez 1997;Wallace *et al.* 1998).

6.1.4 Cost and timing

Along with minimising variation in the bioassay response, the work described in Chapter 2 also aimed to develop a method for measuring the post-exposure egestion rate of *L.variegatus* that kept cost and timing to a minimum. Both culture and bioassay techniques were simple and inexpensive to use, and no specialised equipment or knowledge was required. The bioassay methodology was practicable in terms of time and cost associated with its use in the laboratory. Additionally, statistical analysis of feeding rate data was simple. Six days was selected for the exposure period in an attempt to ensure that worms had sufficient time to accumulate and/or respond to contaminants, and twenty-four hours

was selected for the post-exposure feeding period. A time period of six hours or less for the post-exposure period produced unacceptable (>30%) levels of variation, and time periods of between six and twenty-four hours were likely to be impractical during subsequent use in the field.

6.2 *IN SITU* BIOASSAY

The post-exposure feeding bioassay was successfully adapted for *in situ* use at a number of reference and potentially contaminated sites in New Brunswick, Canada (Chapter 4). Significant feeding inhibition was found at a number of sites on the Little River near Saint John. Chemical analysis of sediments and overlying water at the affected sites was not carried out, but it is likely that sediments contained a complex mixture of polycyclic aromatic hydrocarbons released from a nearby industrial plant. From the results of a concurrent study, fish populations in the area downstream of the effluent pipe were also impacted when compared to upstream populations.

Deployments of the bioassay on the Saint John River near Edmundston were subject to equipment losses as a result of high river levels, so the interpretation of data from impacted sites is limited. Deployments of the bioassay on the Little River near Grand Falls did not find clear evidence of feeding inhibition at suspected contaminated sites. This may have been a result of a lack of chemical contamination, insufficient sensitivity of the bioassay, or the result of confounding factors masking any potential bioassay signal. The development

and testing of the bioassay for *in situ* use highlighted a number of important issues that are considered below.

6.2.1 Bioassay chamber design

The principle advantage of using an open-ended chamber design was that it reduced the disruption to the sediment layer during deployment, which may otherwise alter the bioavailability of sediment-associated contaminants. However, an open-ended chamber design also raises concerns over the potential for escapees and the effects of indigenous organisms (Reynoldson *et al.* 1994). The results from repeated deployments in a variety of substrate types confirmed that escapees were not a significant problem with the current chamber design. However, oligochaetes decompose quickly after death, so reduced recovery of animals from any bioassay chamber that uses an open-ended design e.g (Sibley *et al.* 1999) cannot be definitively attributed to either reduced survival or escapees.

The problems that are potentially associated with the presence of indigenous organisms in bioassay chambers (i.e predatory and non-predatory interactions) are not confined to the bioassay chamber used here; any *in situ* deployment that includes field sediments in the bioassay chamber will need to consider this issue. The potential for adverse effects of the indigenous community could be better controlled through post-exposure observations of the contents of each chamber. As found in this work, such evaluations need not be taxonomically demanding, a cursory evaluation will identify those chambers where known predators and high numbers of inter-specific competitors are present (Sibley *et al.* 1999).

The presence of similar indigenous species could also present difficulties in correctly identifying bioassay organisms. Misidentification of bioassay worms was not found to be a problem in this work, but it could be more significant at sites where indigenous oligochaetes are more abundant and/or are less easily distinguished from bioassay organisms. The use of a non-toxic marking technique e.g (Crane *et al.* 2000) might circumvent this problem and further work in this area would be very useful. Despite the above concerns, it is suggested that the benefits of including natural, undisturbed sediment within the overall exposure scenario will outweigh any difficulties with the open-ended chamber design.

6.2.2 Non-toxicant confounding factors

Figure 6.1 contains bioassay data from Chapter 4 for both laboratory controls and *in situ* deployments at upstream (control) sites. Post-exposure egestion rates from upstream field sites were consistently lower than the baseline rates obtained from laboratory controls. A reduction in the baseline response at upstream field sites is very significant, as the statistical power of the bioassay to detect contaminant-induced feeding inhibition at suspected contaminated sites would be reduced.

As discussed in Section 4.5.2, results from deployments on the Little River near Grand Falls were confounded by differences in temperature during the post-exposure feeding period. The use of portable incubators could solve this problem at secure field sites, but as

was the case here the risk of theft might preclude their use. Alternatively the portable incubators could be held at a single secure location e.g accommodation. However, it may not be possible to repeatedly drive from each site to the nearest accommodation area owing to the distances/time involved. If controlling temperature during the post-exposure feeding period is not possible post-exposure egestion rates could be normalised to temperature. This technique has previously been used to normalise the *in situ* feeding rate of *G.pulex* to ambient temperatures (Maltby *et al.* 2002), but this would require a more substantial data set than the one that is available at present.

As post-exposure egestion rate was measured under controlled temperature conditions in a nearby laboratory, bioassay data for sites on the Little River near Saint John were not subject to the confounding effects of temperature. However, post-exposure egestion rates of worms previously exposed to the field control site were still significantly lower than the baseline for laboratory controls (Figure 6.1). The upstream site was a significant distance from any obvious sources of pollution, but without sediment chemistry it is difficult to categorically state that the site was relatively "clean". This highlights the value of chemical analysis as an additional line of evidence. However, as was the case here, sediment chemical analysis is often prohibitively expensive to carry out.

If the upstream site on the Little River near Saint John was uncontaminated, this would suggest that the bioassay response was confounded by additional non-contaminant factors. The results of Experiment 3 in Chapter 2 (2.7.3) highlighted that an exposure temperature of $5^{o}C$ reduced the post-exposure egestion rate of *L.variegatus* at $20^{o}C$. However,

temperature differences of 15°C between the exposure and post-exposure periods were not experienced during control deployments on the Little River. It is not known if additional site-specific characteristics (e.g pH, hardness, sediment particle size and organic carbon) had a sufficiently persistent effect on worms during the exposure period for them to be felt during the post-exposure feeding period.

One way in which the effects of substrate variability have been controlled for previously is via the use of standardised sediment (field-collected or artificial) that is placed inside the bioassay chamber during *in situ* deployments e.g (Sibley *et al.* 1999;Castro *et al.* 2003). Comparisons between natural (*in situ*) and standardised sediment (artificial or field-collected, Section 2.1) at uncontaminated sites would highlight the effects of substrate on the bioassay endpoint, and could also control for the effects of indigenous organisms in the bioassay chambers. However, even if differences are found in the bioassay response with the use of field control sediment, this does not solve the problem of how to compare clean and polluted field sites with varying substrate characteristics. Alternatively, the effects of substrate variability on *in situ* macro-invertebrate communities has previously been addressed by using a multivariate statistical approach which incorporates 32 individual substrate characteristics (Reynoldson *et al.* 1995). However, this type of approach for *in situ* bioassays would require considerably more time and statistical expertise to carry out, which is likely to significantly increase the costs of such techniques.

Establishing a baseline for the *in situ* bioassay with exposure to a range of uncontaminated field sites may be the best approach for dealing with the potential effects of

confounding factors. This would require repeated deployments of the bioassay at uncontaminated sites to establish a range of response for the bioassay. Deployments at known or suspected contaminated sites could then be compared in order to determine if the bioassay response is sufficiently powerful to detect significant deviations from the baseline range. Site-specific characteristics could be measured and evaluated for their effects on the bioassay response at "clean" sites. If the bioassay response is significantly confounded by site-specific variables (i.e water quality or sediment variables) regression-normalisation techniques could be employed. This relatively simple technique has been successfully used to normalise the *in situ* growth rates of the juvenile clam, *Mercenaria Mercenaria* to site-specific variations in salinity, dissolved oxygen and pH (Ringwood and Keppler 2002).

6.2.3 Time and costs

The bioassay chambers were simple and inexpensive to construct (approx $6 U.S each) and easily replaced when losses occurred. The chambers were also simple to deploy in a variety of substrate types, although more coarse sediments proved a greater challenge when it came to pushing the chambers into the substrate. The six-day exposure period increases the risk of equipment loss due to flooding and vandalism/theft, particularly in systems that are naturally "flashy" i.e regular, sharp rises in river levels. However, shortening the exposure period might reduce the sensitivity of the bioassay, so there is likely to be some form of trade-off between the risk of equipment loss/vandalism and sensitivity.

Similarly, the use of a 24-hour post-exposure feeding period was based on a trade off between reducing baseline variation in the bioassay response, and what would be practical for field deployments. A short (4-6 hours) post-exposure feeding period would have been desirable for field deployments, as this would have enabled completion of the post-exposure period in a single day. However, variability in the bioassay response for these time periods was unacceptable, so a 24-hour period was used instead. This meant that for any deployments a considerable distance from the laboratory (>2 hours), beakers had to be held overnight at field sites, as it was thought that feeding rates could otherwise significantly recover during the transport period. This in turn meant that temperatures during the post-exposure had to be controlled in some way, which caused significant problems at some of the sites used in this work (Section 6.2.2).

6.3 CONCLUSIONS

The post-exposure feeding bioassay is suggested to be a cost effective and reliable technique when carried out under laboratory conditions. Additional work with a wider range of toxicants on the relative sensitivity of the bioassay and the direct and indirect effects of feeding inhibition at higher levels of organisation would be very useful. The apparent insensitivity of the bioassay to metals means that complimentary *in situ* sediment bioassays with the chironomid, *C.riparius* e.g (Sibley *et al.* 1999;Crane *et al.* 2000;Castro *et al.* 2003) would be used in situations where metal contamination was the primary concern. This last point highlights the value of a suite of *in situ* bioassays using

representative organisms from different trophic levels and functional groups (Chappie and Burton 2000).

The bioassay was successfully adapted for *in situ* use, where it was able to detect the effects of sediment contamination. It is suggested that the potential problems associated with confounding factors may be resolved with further work on establishing a baseline range for the bioassay at “clean” field sites. Rather than controlling environmental variability, a principle challenge with *in situ* techniques is in developing methods that reflect and integrate natural variability, whilst maintaining the ability to detect the effects of toxicants. The effort that is required to do this will be balanced with a greater understanding that is gained from conducting bioassay exposures under more realistic *in situ* conditions.

Once further refined and tested, the bioassay technique may prove to be a very useful tool as part of an integrated approach to assessing sediment quality (Section 1.2.4). The development of multi-tiered risk assessment frameworks for sediment quality has been on-going for some time (Chapman 2000;Burton *et al.* 2002). Traditionally these frameworks have focused on combinations of sediment chemistry, *in situ* biological community analysis and laboratory toxicity bioassays (Chapman 2000). Extension of the bioassay approach to *in situ* techniques like the one described in this work should provide a valuable additional line of evidence within such a framework.

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