THE IMPORTANCE OF DIETARY EXPOSURE IN THE DETERMINATION OF BIOAVAILABILITY OF SEDIMENT-SORBED ORGANIC COMPOUNDS TO BENTHIC MACROINVERTEBRATES

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"Even a blind pig finds an acorn now and then"

H. S. Thompson

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ABSTRACT

Hydrophobic organic compounds released into the environment have the potential to persist within aquatic sediments for many decades. The hydrophobic nature of such compounds results in sediment contamination that is orders of magnitude greater than the concentrations within the overlying water column. However, simple chemical measures of bulk amounts of organic contaminants within sediment cannot accurately predict uptake and assimilation of sediment contaminants into aquatic biota. In particular, there is uncertainty related to the potential for uptake of compounds that are sorbed to sediment particles. It has been suggested that the sole fraction of a contaminant that is available for uptake is the proportion found as aqueous solute within the interstitial spaces (termed "pore water") of sediment.

This thesis tests the hypothesis that uptake of particle-sorbed contaminants is a viable assimilation route: the relative importance of which may vary in response to both chemical partitioning and organism biology.

In addressing this hypothesis, four species of benthic macroinvertebrate: *Chironomus riparius* larvae, *Lumbriculus variegatus*, *Asellus aquaticus* and *Gammarus pulex*, were exposed to a compound sorbed strongly to sediment particles and insoluble in water. [^{14}C]-Dioctadecyldimethylammoniumchloride (DODMAC) was used as the model compound for these exposures in both artificial and field-collected sediments. Tissue loadings measured within whole organisms were verified by autoradiography of histological tissue sections and comparison of tissue loadings in feeding and non-feeding individuals as well as moulted and non-moulted individuals. In response to measured variation in DODMAC uptake, organism feeding selectivity, biotransformation, the proportion of lipid and water within tissues and gut retention time were assessed as potential mechanisms of uptake variation. In addition, literature-derived factors were also evaluated as potential mechanisms. Subsequently, the variation in proportional contribution of aqueous and particulate uptake routes was examined in three polyaromatic hydrocarbon compounds (pyrene, fluorene and naphthalene). Novel apparatus was used to measure the proportion of uptake due to pore water relative to uptake in whole sediment. The influence of compound K_{ow} upon route of uptake in *G. pulex* was measured using pyrene, fluorene and naphthalene. Additionally, the effect of the presence of gills upon naphthalene uptake was assessed using the four species previously exposed to DODMAC.

All four species achieved genuine assimilation of DODMAC. The dietary assimilation of this compound varied between the species and between artificial and field-collected sediment. *Chironomus riparius* consistently accumulated higher tissue loadings of DODMAC than the remaining species. The variation in DODMAC assimilation could not be explained by the experimentally-measured factors. Instead, the potential importance of gut-fluid surfactancy, solubilisation of sediment particles and periodic increases in lipid assimilation was highlighted through existing literature.

Polyaromatic hydrocarbon compounds were found to be assimilated from both aqueous and particulate sources. Uptake via sediment ingestion assumed greater importance as compound K_{ow} increased. Conversely, the presence of gills appeared to increase the proportion of aqueous assimilation.

Sediment-sorbed compounds were concluded to be bioavailable to benthic macroinvertebrates via dietary uptake. Such bioavailability appears to vary amongst species. Further research into gut-fluid properties and changes in tissue morphology in relation to contaminant assimilation was prompted. For compounds present in both particulate and aqueous solute fractions, the proportion of dietary uptake increases with K_{ow}. Conversely, ventilatory appendages can increase the proportion of aqueous uptake. These conclusions support the general hypothesis addressed in this thesis.

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1.1 Introduction

1.1.1 Background

Hydrophobic organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organic pesticides, that are discharged into aquatic environments accumulate within sediments (e.g. Connell *et al.* 1988, Landrum and Robbins 1990, Lee 1992, van Leeuwen and Hermens 1995, Means and McElroy 1997). Once incorporated into sedimentary material, such compounds have the potential to persist within this environmental compartment (e.g. van Leeuwen and Hermens 1995, Means and McElroy 1997). The persistence of hydrophobic organic compounds within sediments may endure for many decades (e.g. Beurskens *et al.* 1993, Harkey *et al.* 1995). Thus, it is possible for sediments to act as a long-term repository of potential toxicants.

Contaminants stored within sediments may also act as the principle source of contamination to the food chain (Landrum and Robbins 1990). For example, contaminant-assimilation and toxic responses have been measured in laboratory exposures of benthic invertebrates to contaminated sediment (e.g. Klump *et al.* 1987, Kukkonen and Landrum 1994, Kane-Driscoll *et al.* 1997b, Sheedy *et al.* 1998). Contaminants that are assimilated by benthic invertebrates can also be transferred to higher trophic levels. For instance, organic compounds obtained from sediment by benthic invertebrates can be assimilated by fish species by predation (e.g. Rubinstein *et al.* 1984, Clements *et al.* 1994). In fact, subsequent trophic-transfer of contaminants derived from sediment can potentially result in exposure of top predators including birds, marine mammals and humans (Lee 1992). This prompts consideration of the question "How do hydrophobic organic contaminants transfer from sediments into benthic invertebrates?".

In practice, the process of contaminant transfer from sediment into benthic invertebrates is unpredictable and poorly understood (O'Connor and Paul 2000).

Chemical measures of contaminant concentration in the sediment do not reliably indicate the toxicity of sediment-associated contaminants (e.g. Landrum 1989, Landrum *et al.* 1989, Landrum and Robbins 1990). O'Connor and Paul (2000) for example, found that analysis of the mass of toxicant per unit mass of sediment was unable to predict toxicity in amphipod crustaceans. This conclusion was reached following the analysis of 13,000 contaminated sediment samples. The unpredictable nature of contaminant-transfer between sediment and benthic invertebrates may be related to the behaviour of compounds within sediment. Individual organic compounds within sediment form particle-sorbed and aqueous solute fractions (e.g. Landrum 1989, Di Toro *et al.* 1991, van Leeuwen and Hermens 1995). The proportion of the total contaminant in the interstitial (or pore) water plus contaminant sorbed to sediment particles that can be accumulated by organisms is termed the "bioavailable fraction" (Landrum and Robbins 1990). In the following paragraphs, the possibility that contaminant transfer from sediments to biota depends upon the availability of the particle-sorbed contaminant fraction is discussed. Attempts to identify factors that control bioavailability are detailed as follows: Equilibrium partitioning and fugacity (Section 1.1.2), toxicity measured in aqueous extracts of sediment (Section 1.1.3), aqueous desorption kinetics (Section 1.1.4) and dietary uptake (Section 1.1.5). Subsequently, factors that influence the proportional uptake from aqueous and particulate routes are considered: Potential for multi-route uptake (Section 1.1.6), example of modelled variation in uptake route contribution with compound distribution (Section 1.1.7), ecological determination of uptake route (Section 1.1.8) and assessment of assimilation within tissues (Section 1.1.9).

1.1.2 Equilibrium partitioning theory and fugacity

Di Toro *et al.* (1991) made a significant attempt to establish a basis for variable bioavailability through the development of equilibrium partitioning (EqP) theory. Based upon the partitioning behaviour of non-polar organic compounds between sediment pore water and organic carbon, EqP predicts the amount of compound that is likely to partition into organism lipid (Di Toro *et al.* 1991). Variation in bioavailability was, therefore, suggested to be related to sediment organic content and organism lipid content. However, even though derived pore water concentrations of a compound may be used to predict organism uptake, it is specifically acknowledged that EqP cannot

assert that organisms solely obtain sediment contaminants from the pore water (Di Toro *et al.* 1991). Di Toro *et al.* (1991) found that pore water concentration of non-polar organic compounds was significantly correlated with biological effects. In contrast, bulk sediment contaminant loadings were not significantly correlated to biological effects. This may suggest that pore water is the route of uptake. However, the same study reports that the biological effects correlate "equally well" to the organic carbon normalised sediment concentration. In isolation, this may suggest that sediment organic carbon is the route of exposure. Neither explanation necessarily follows from the data. A further complementary model is presented in the same report. This is based on the concept of chemical fugacity (after e.g. van Leeuwen and Hermens 1995). This concept hypothesises that the biological activity of a chemical is controlled by its chemical potential (or fugacity). Chemicals are said to move from high to low potentials/fugacities. If the pore water is in equilibrium with the sediment organic carbon then the chemical potential (if not the concentration) that the organism experiences from either route is, by definition, exactly equal. This means that equilibrium experiments cannot distinguish the route of exposure (Di Toro *et al.* 1991). Similarly, the dependence upon specific partitioning characteristics of non-polar organic compounds precludes the use of EqP for ionic compounds.

Using contaminant pore water concentration, rather than bulk sediment loading, was observed to reduce variability associated with sediment toxicity (Di Toro *et al.* 1991). Applications of this approach outside the studies used to develop EqP theory suggest that it may be useful in reducing uncertainty. Hoke *et al.* (1995) report a broad similarity between contaminant residues in field populations of benthic invertebrates from contaminated sediment sites and EqP derived sediment quality criteria. It was also noted that only cases where EqP was overprotective (rather than underprotective) of field populations could be detected in this analysis (Hoke *et al.* 1995). Ankley *et al.* (1994) found that toxicity of the pesticide chlorpyriphos to *Chironomus tentans* at 70 ngl^{-1} in aqeuous phase exposure corresponded to equivalent toxicity in two different sediments where the EqP-predicted pore water concentrations were 40 and 70 ngl^{-1}. This is cited as an example of broad agreement between EqP predicted toxicity and measured toxicity mediated by the aqueous fraction of the contaminant (Ankley *et al.* 1994).

Studies utilising EqP have also noted that species differences can produce substantial departures from EqP-predicted toxicity (e.g. Kane-Driscoll and Landrum 1997, Lee *et al.* 2001). For example, Bremle and Ewald (1995) concluded that EqP theory was only useful for rough predictions of accumulation of hydrophobic compounds by aquatic biota. One reason that EqP can only make broad predictions relates to the oversimplification of factors that are assumed to control contaminant partitioning. The practice of normalising contaminant loading to organism lipid content and sediment organic carbon is central to application of EqP methodology (Di Toro *et al.* 1991). However, variation in the quality of organism lipids and sediment organic carbon influenced the observed partitioning to a degree that made these factors unsafe as absolute normalisation variables (Bremle and Ewald 1995). Similarly, O'Connor and Paul (2000) state that two fundamental assumptions of the sediment quality criteria based upon EqP are that: i.) equilibrium exists and ii.) chemicals insufficiently soluble to exhibit aqueous phase toxicity cannot be toxic in sediment. Regarding the first assumption, changes in ecological systems often proceed too rapidly for equilibrium to become established (Borglin *et al.* 1996, Lick and Rapaka 1996, O'Connor and Paul 2000). Also, the second assumption certainly requires testing before general acceptance can be granted.

Equilibrium partitioning may, therefore, be a useful tool for deriving a broad prediction of potential accumulation of non-polar organic compounds from sediment. Established limitations of this approach exist in assumptions related to the existence of system equilibrium, the robustness of normalisation variables and independence from organism behaviour. Additionally, this approach cannot be adopted where the relevant organic contaminants are polar molecules. Although fundamentally unable to discern uptake of aqueous solute or particle-sorbed contaminant, EqP assumes that compounds sorbed strongly enough to particles to preclude aqueous toxicity are non-toxic in sediment. This questionable assumption requires experimental verification.

1.1.3 Toxicity measured in aqueous extracts of sediment

Attempts have also been made to experimentally compare the effects of aqueous sediment extracts and the effects resulting from exposure to the whole sediment (e.g. Ankley *et al.* 1991, Harkey *et al.* 1994a, Liss and Ahlf 1997). These are attempts to

establish the fraction of aqueous solute as the prime determinant of sediment toxicity. The aqueous extracts used in these examples were the isolated pore water and sediment "elutriates" that were originally designed to simulate suspension of toxic sediments resulting from dredging. Elutriates were prepared by suspending the bulk sediments in water (at volumetric ratios of either 1:2 or 1:4 of sediment:water). Mixtures were maintained in suspension for between 30 minutes and two hours before the removal of undissolved material via centrifugation. In the cited studies the pore water was separated from the whole sediment via centrifugation. This pore water will contain the concentration of contaminant that has desorbed from the sediment particles since contamination. In contrast elutriates are a method of stripping readily water-soluble fractions from the sediment. The duration that each of the aqueous phases spend moving towards equilibrium will, therefore, be much greater for pore water than elutriates. These three exemplar studies by Ankley *et al.* (1991), Harkey *et al.* (1994a) and Liss and Ahlf (1997) used a range of endpoints and study systems (Table 1.1). However, in all three studies pore water bioaccumulation resembled whole-sediment bioaccumulation more closely than elutriates. Where bioaccumulation from elutriates was much less than that from whole-sediment, accumulation from pore water approximated to that from whole-sediment. In each of these three studies it was concluded that neither aqueous extract could accurately predict the toxicity of the bulk sediment and that the choice of test species and the sediment characteristics have strong potential to influence toxicity. These examples, therefore, suggest that aqueous fraction exposure does not fully describe contaminant transfer from sediments into benthic biota. Exposure to whole sediment can result in both greater and lesser toxicity than that exhibited by aqueous fractions.

Table 1.1 Summary of test parameters for three studies comparing pore water, elutriate and whole sediment exposures

Study	Sediment	Test Organism	Endpoint
Ankley *et al.* (1991)	Inherently toxic field sediment	Fish, cladocerans, amphipods and oligochaetes	Presence or absence of >50% mortality
Harkey *et al.* (1994a)	Clean field sediment with laboratory-added contaminants	Three species of benthic macroinvertebrate	Contaminant bioaccumulation ††
Liss and Ahlf (1997)	Artificial sediment with laboratory-added contaminants	Micro-organisms	LC_{50} †

† **LC_{50} (median lethal concentration) is defined as the contaminant concentration which results in 50% mortality of the test organism for a given test duration.**

†† **Bioaccumulation is the ratio of compound within organism tissues to compound within the source environmental phase**

1.1.4 Aqueous desorption kinetics

The observation of a characteristic aqueous desorption pattern in PAHs from sediment has been related to bioavailability from sediment (e.g. Landrum *et al.* 1992). This pattern is characterised by a rapid initial desorption rate followed by a subsequent, far slower, rate of desorption. The proportion of this second, more slowly desorbing, fraction has been observed to increase with increasing contact time between sediment and contaminant (e.g. Landrum *et al.* 1992). The proportional increase in slowly desorbing fraction is suggested to relate to a decrease in the overall bioavailable fraction (e.g. Landrum *et al.* 1992).

Kraaij *et al.* (2001) exposed the amphipod *Corophium volutator* to native sediment with existing PAH contamination and sediment that contained PAHs added in the laboratory. Kraaij *et al.* (2001) experimentally determined rapidly desorbing fractions of PAHs and then compared them to accumulation achieved in amphipods

exposed via sediment. An R^2 value of 0.76 was calculated for rapidly desorbing fraction and accumulation by Kraaij *et al.* (2001). Some deviations from this pattern were also reported. For example, the rapidly desorbing fractions of benzo[k]fluoranthene were near-identical between different sediment treatments. Yet, one sediment treatment resulted in approximately double the benzo[k]fluroanthene bioaccumulation than the alternative sediment treatment (Kraaij *et al.* 2001). Kraaij *et al.* (2001) also state that this approach does not discern whether particle-sorbed contaminant is bioavailable, since the mechanisms of both aqueous ventilation and dietary uptake are consistent with study predictions.

Similarly, Lamoureux and Brownawell (1999) and Schuler and Lydy (2001) found that the fraction of contaminant that desorbed rapidly into pore water from sediment was a good broad prediction of bioavailability. Water extraction, rather than exhaustive chemical extractions of sediment using methanol, octanol, acetonitrile or soxhlet (equal volumes of dichloromethane and acetone), most closely resembled bioaccumulation of benzo[a]pyrene and hexachlorobiphenyl by *Lumbriculus variegatus* (Schuler and Lydy 2001). Whereas, historically-contaminated sediment containing PCBs, PAHs and linear alkylbenzenes (LABs) showed aqueous desorption rates that were 1-4 orders of magnitude lower than predicted by sorption kinetic models (Lamoureux and Brownawell 1999). Lamoureux and Brownawell (1999) postulated that the fractions of contaminants residing within the aged sediment were the remaining resistant fractions left following loss of the rapidly desorbing phase. Forty eight-hour bioaccumulation in *Yoldia limnata* was significantly correlated to aqeuous desorbtion of PAHs and LABs; $r^2>0.78$ (Lamoureux and Brownawell 1999). The lower bioaccumulation of historically-contaminated (resistant phase) contaminant was suggested to be due to accretion of compound within intraparticle spaces (Lamoureux and Brownawell 1999 and e.g. Pignatello 2000). In this particular case, resistance to aqueous desorption was therefore likely to be due to compound residing within the integral structure of the particle, rather than surficial-sorption interactions. Therefore, the studies exemplified by Kraaij *et al* (2001), Schuler and Lydy (2001) and Lamoureux and Brownawell (1999), suggest that aqueous desorption can be a basic indication of bioavailability of sediment contaminants. However, uptake cannot be discerned between aqueous ventilation or gut-desorption processes. Further, even though it is clear that desorption-resistance conferred by intraparticulate accretion is likely to

correspond to a lack of bioavailability, it is not clear whether contaminant sorbed strongly to particle surfaces can be bioavailable.

Interactions between contaminant and the surface of sorbant particles have been investigated in relation to bioavailability. For example, Lawrence *et al.* (2000) used aqueous adsorption behaviour to determine the strength of contaminant/sediment interaction, whilst desorption was used to determine the mechanism of contaminant release into the organism (Lawrence *et al.* 2000). Particle interactions in aqueous media were characterised using plots (isotherms) of the increasing amount of contaminant sorbed to sediment with increasing aqueous loading of contaminant (after Giles *et al.* 1960, cited in Lawrence *et al.* 2000). Pentachlorophenol (PCP) and 2,4 dichlorophenol (DCP) aqueous adsorption/desorption isotherms for three types of artificial particle (used in chemical extraction cartridges), organic-amended clay and a natural sediment were compared to bioaccumulation in *Lumbriculus variegatus*. Lawrence *et al.* (2000) suggest that where particle/contaminant interaction is very strong in aqueous media, bioaccumulation is likely to be low or absent. Conversely, when particle interactions with contaminant are so weak that little contaminant is sorbed to the substrate, little bioaccumulation from sediment can occur (Lawrence *et al.* 2000). Therefore, bioavailability of sorbed contaminant was suggested to occur between these two extremes of interaction intensity. This is in agreement with other studies that compared aqueous adsorption/desorption characteristics (e.g. Davies *et al.* 1999a) or solely adsorption coefficients (e.g. Davies *et al.* 1999b) to bioaccumulation from artificial particles. The greatest accumulation of DCP and PCP in *Lumbriculus variegatus* and *Chironomus riparius*, from strictly standardised artificial sediment particles, occurred at intermediate particle/contaminant interaction strengths (Davies *et al.* 1999a, Davies *et al.* 1999b, Lawrence *et al.* 2000).

The results of isotherm analysis of DCP and PCP suggest that particle interactions ranging from too weak to too strong to support sediment bioaccumulation may bracket the bioavailability of a compound (Davies *et al.* 1999a, Davies *et al.* 1999b, Lawrence *et al.* 2000). Although Lawrence *et al.* (2000) used a general approach of modelling particle interaction as an indication of digestive contaminant desorption, this study could not distinguish uptake route. Additionally, bioaccumulation factors were derived from pore water concentrations of the compound. This suggests that contaminant that is already desorbed into the pore water is the

fraction that is accumulated. However, comparisons between exposure solely to aqueous contaminant and exposure to whole sediment (Davies *et al.* 1999a and Davies *et al.* 1999b) suggest that both uptake of freely dissolved contaminant and dietary desorption of compound may be important. The use of aqueous isotherms assumes that desorption into water is a good model for gut desorption. However, major differences in accumulation of DCP and PCP were observed between *Lumbriculus variegatus* and *Chironomus riparius* despite identical substrate particle/contaminant interaction (Davies *et al.* 1999b). Further, it is assumed that compounds strongly resistant to aqueous desorption are not bioavailable through exposure to the digestive environment. The experimental verification of these assumptions and determination of uptake route is desirable.

Based upon the findings of the aqueous desorption approaches exemplified in this section, it is apparent that freely dissolved aqueous fractions of sediment contaminants are likely to be bioavailable. In the above-cited studies that seek to explain bioavailability in terms of the propensity of compounds to partition into water, the availability of compound that remains sorbed to particles is not always addressed. Where uptake of particle-sorbed contaminant is considered, it is assumed that contaminants that resist desorption into an aqueous medium are non-bioavailable. If the lack of such aqueous desorption is due to intraparticulate binding, there is evidence for a reduction in bioavailability. However, for surficially-sorbed contaminants, to assume that the association between aqueous desorption and dietary uptake is ubiquitous requires further investigation. Differential accumulation achieved in identical contaminant/sorbant systems by two benthic species implies that aqueous desorption may not always be a perfect representation of desorption occurring within the gut.

1.1.5 Potential for dietary uptake

As chemicals partition between particulate sediment matter and pore water, there is potential for chemicals to be assimilated from either of these two phases (Landrum and Robbins 1990, van Leeuwen and Hermens 1995). The bioavailability observed when contaminants are present in the pore water is frequently interpreted as evidence that uptake occurs solely from the pore water (Section 1.1.3). However, as

discussed in Section 1.1.3, the exclusive route of uptake cannot be ascribed simply by contaminant presence in the aqueous phase. Dietary uptake of sediment-bound organic chemicals has been implicated by the dosing of feed substances with organic contaminants (West *et al.* 1997). For example the uptake of tetrachlorodibenzo[*p*]dioxin (TCDD) from spiked diets in *Chironomus tentans* and *Lumbriculus variegatus* was demonstrated to result in maximal tissue concentrations between 6.9 μgg^{-1} lipid and 9.5 μgg^{-1} lipid (West *et al.* 1997). However the desorption of compound into aqueous phases was not quantified, resulting in the possibility for uptake solely attributable to compound that was freely dissolved prior to ingestion.

Other approaches to investigating dietary uptake include several methods of estimating assimilation efficiency of compounds from sediment during gut processing. Here, "assimilation efficiency" is functionally defined as the efficiency with which a substance is removed from ingested material during passage through the gut. These include "dual tracer" studies in which two radiolabelled substances are used to spike the sediment (e.g. Klump *et al.* 1987, Kukkonen and Landrum 1995a, Forbes and Forbes 1997). One of the pair of labelled compounds will be known to pass through the animal without being assimilated (the conservative tracer e.g. ^{51}Cr or polydimethylsiloxane). The other will be the assimilated compound under examination. By comparing the ratios of both compounds in the ingested sediment and in the faecal matter, the proportional removal of compound may be calculated. It is crucial, however, to accurately establish how much of each tracer is ingested. This may not be simply related to the concentration in the bulk sediment. Contaminant association with specific sediment particle fractions, in combination with organism selective feeding upon specific particles, results in ingested substrate that differs considerably from bulk sediment (e.g. Harkey *et al.* 1994b, Kukkonen and Landrum 1995a, Kukkonen and Landrum 1996). In response, studies have employed techniques to obtain accurate measures of contaminant depletion in egested material. Two studies that represent possible approaches to improving the accuracy of estimated assimilation efficiency are Kukkonen and Landrum (1995a) and Forbes and Forbes (1997). Firstly, Kukkonen and Landrum (1995a) compared alternative methods to accurately estimate contaminant depletion in faecal material. In one method, the contaminant loading in bulk sediment and faecal matter was normalised to organic carbon content. This was combined with a measure of the organic carbon enrichment within ingested material compared to the bulk sediment. The organic carbon "selectivity index" could then be used to estimate ingested material contaminant loading. A second approach evaluated

by Kukkonen and Landrum (1995a) was to choose a conservative tracer polydimethylsiloxane (PDMS) that had a similar distribution within the sediment to the study compound (benzo[a]pyrene). This was an attempt to ensure that the study compound and conservative tracer were ingested in similar proportion. However, whilst both approaches were able to assess *Lumbriculus variegatus* assimilation efficiency, Kukkonen and Landrum (1995a) found that neither method was appropriate for *Diporeia* spp. This was attributed to the extremely selective nature of feeding in *Diporeia* leading to differential ingestion of tracer and study compound.

In another example, Forbes and Forbes (1997) tackled the problem of extreme selective feeding by sampling the gut contents of a group of organisms after an exposure duration of less than one complete gut turnover. The ratio of conservative tracer and study compound was therefore determined in sediment matrix selected by the organisms. This was then combined with tracer ratio analysis of faeces egested from organisms exposed for durations greater than one gut turnover. A final group of organisms were subjected to analysis solely of study compound loading within gut-purged tissues. In this manner, a good estimate of tracer ratios in both ingested and egested sediment as well as study compound within tissues was obtained. Forbes and Forbes (1997) concluded that fluoranthene was assimilated via sediment ingestion with an efficiency that varied from 22 – 46% in two clones of the gastropod study species *Potamopyrgus antipodarum*.

Studies that demonstrate contaminant depletion during deposit-feeder digestion strongly implicate dietary uptake of compound derived from sediment particle surfaces. However, existing studies of contaminant assmilation efficiency cannot rule out pore water solute as the sole source of contaminant uptake. Therefore, such studies prompt investigation into the availability of particle-sorbed contaminant.

In addition to the investigation of desorption kinetics in purely aqueous media (Section 1.1.4), there is evidence that sediment-sorbed compounds can be effectively removed from particles by invertebrate gut fluids (e.g. Mayer *et al.* 1996, Weston and Mayer 1998a, Weston and Mayer 1998b, Mayer *et al.* 2001). This may be a better description of desorption occurring during gut processing of sediment. For example, three studies that have examined potential digestive uptake by using isolated gut fluid to extract sediment-sorbed compounds are summarised in Table 1.2.

Table 1.2 Examples of marine invertebrate species and test compounds used to compare contaminant solubilisation in gut fluid to uptake achieved in whole organisms.

Study	Mayer *et al.* (1996)	Weston and Mayer (1998a + b)	Mayer *et al.* (2001)
Test species	*Arenicola marina* and *Parastichopus californicus*	*Arenicola brasiliensis* and *Urechis caupo*	18 species of marine benthic invertebrates
Test Compound	10 PAH compounds	Phenanthrene and Benzo[a]pyrene	Zinc and Benzo[a]pyrene

A strong indication of a potential risk of contaminant uptake via ingestion of sediments is evident from the studies listed in Table 1.2. Digestive fluid from each of the study organisms from these examples is observed to strip hydrophobic organic contaminants from sediment. Weston and Mayer (1998b) measured contaminant solubilisation achieved in vitro by shaking sediment with extracted gut fluid. Concurrently, gut fluids were extracted from organisms that had been allowed to ingest contaminated sediment. Contaminant solubilisation was qualitatively similar between sediment extractions using in vitro and in vivo gut fluids (Weston and Mayer 1998b). Further, solubilisation of PAH compounds by gut fluids hugely exceeded solubilisation by seawater alone, although a lack of detection in sea water for some contaminants precluded statistical comparisons (Mayer *et al.* 1996). It was also noted that, using gut fluids from different organisms, the amount of each contaminant solubilised varied between species (Mayer *et al.* 1996, Mayer *et al.* 2001). Separate studies have suggested that the increased surfactancy of invertebrate gut fluid is related to increased desorption of hydrophobic organic compounds (e.g. Ahrens *et al.* 2001b). In one study listed in Table 1.2, digestive fluids extracted typically 1 – 10% of total sediment contaminant loadings (Mayer *et al.* 1996). It is possible for digestive extraction efficiency to influence bioavailability, since contaminant extraction is not 100% efficient and is variable between species.

In summary, therefore, there are strong implications that uptake via digestive stripping of contaminated sediment particles could occur. Evidence from methods providing estimates of contaminant removal efficiency following gut processing as well

as the demonstrated enhanced solubility in marine deposit feeder gut fluids offers support for this. Such methods provide a firmer basis for this notion than the implied importance of digestion provided by spiked dietary matter. Contaminant solubilisation in gut fluid that far exceeds that of aqueous media suggests that aqueous desorption kinetics cannot always adequately model digestive desorption. The notion of particle-sorbed compound uptake is supported by these examples. Further, it is possible that interspecific variation in dietary desorption may influence bioavailability.

1.1.6 Potential for multi-route uptake

On the basis of the various approaches into understanding sediment contaminant assimilation outlined above (i.e. Sections 1.1.2–1.1.5), a number of conclusions can be drawn. Aqueous desorption behaviour can potentially indicate bioavailability. This is due to the observed variation of aqueous desorption (in parallel with variation in bioavailability) of individual compounds between different sediments. Toxicity induced solely by aqueous solutes in separate experiments is a good indication that pore water is an effective exposure medium. However, many of these aqueous approaches note that it is not possible to ascribe aqueous solute as the only bioavailable fraction. Where the assumption that compounds insufficiently soluble to exhibit aqueous toxicity lack the potential to be toxic in sediment is made, there is a lack of experimental evidence directly supporting this view. Such an assumption is called further into question by the suggestion that digestive processes have the potential to induce contaminant stripping from sediment particles that exceeds simple aqueous partitioning or solubility. There is also potential for interspecific variation in the amount of chemical assimilated via digestive stripping.

The above information suggests that both the aqeuous solute and particle sorbed fractions of a compound are viable sources of uptake from sediment. It has previously been suggested that both of these uptake routes can operate (van Leeuwen and Hermens 1995). Fugacity models of exposure invoked by EqP theory by Di Toro *et al.* (1991) acknowledge the potential for uptake to occur simultaneously via ingestion and pore water. However, in EqP models, it is assumed that exposure to one route would result in the same tissue concentration as exposure to both ingested sediment and pore water. For this to operate the system must be assumed to be at equilibrium. As previously

stated, this can be an unrealistic assumption in ecological systems. Other modelling approaches assume that tissue uptake from each route is additive e.g. toxicokinetic models. This is in agreement with the notion that both uptake routes operate; the most important uptake route depending upon the physicochemical properties of the compound and habitat as well as physiological characteristics of the organism (van Leeuwen and Hermens 1995). An example of toxicokinetic modelling of uptake route variation determined by compound physicochemistry is given in Section 1.1.7.

1.1.7 Example of modelled variation in uptake route contribution with compound distribution

The uptake of five organic contaminants from both pore water and sediment particles was modelled in *Pontoporeia hoyi* by Landrum and Robbins (1990). Change in organism contaminant concentration through time was modelled as the sum of the uptake from sediment and pore water minus the elimination of contaminant. This is the basis of the toxicokinetic approach. The kinetic models cited in Landrum and Robbins (1990) were run (and reported in the same study) using both maximum and minimum possible parameter values to give a range of estimates of uptake from each route. For example the sediment desorption rates of contaminants were determined under 'gas stripping' scenarios which artificially maintain the maximum desorption potential by removing any desorbed material from the system. Desorption rates derived in this manner greatly exceed rates supported by natural systems. Consequently the pore water concentration would be maintained at an artificially high value. In contrast the model was also run assuming infinitely slow desorption. This lead to a very large predicted potential range of minimum pore water volumes that an organism would need to ventilate if the measured tissue concentration of test substance were to be maintained (e.g. 8.2 µl to 560 ml for hexachlorobiphenyl). Using the assumed most realistic values for all parameters (derived or taken from literature) the toxicokinetic model was used to estimate the importance of uptake route for five different organic compounds (Table 1.3).

Table 1.3 Estimated contaminant contribution from interstitial water and sediment ingestion for *Pontoporeia hoyi*: based on a study by Landrum and Robbins (1990)

	Source	
Compound (Kow)	**Interstitial water**	**Particle Ingestion**
Benzo[a]pyrene (NS)	0%	100%
Hexachlorobiphenyl (6.7)	52%	48%
Tetrachlorobiphenyl (5.9)	80%	20%
Pyrene (5.2)	74%	26%
Phenanthrene (4.4)	88%	12%

NS = Not stated in Landrum and Robbins (1990).

Through various iterations of this model it is apparent that it is very sensitive to two key factors. Variation in both the aqueous desorption term and ingestive uptake clearance had large consequences upon predicted accumulation. It is further suggested from this study that, in *P. hoyi*, ingestion is the most important uptake route for compounds that sorb most strongly to the sediments (Landrum and Robbins 1990). This was due to observed accumulation that could not be supported by the rate of toxicant desorption obtained under gas stripping scenarios. This maximum rate of aqueous desorption is unlikely to be exceeded in natural systems (Landrum and Robbins 1990).

In the above example, the potential for variation in proportional contribution from aqueous and particle-sorbed uptake routes is noted. The model suggests that the degree to which a compound sorbs onto sediment particles influences the importance of each uptake route. The use of estimated parameter values prompts experimental quantification of proportional uptake route contribution.

1.1.8 Ecological determination of uptake route

Uptake of compounds from the sediment is suggested to occur from fractions within that sediment. It is intuitively true that the greatest uptake will occur in organisms that have the greatest degree of contact with those fractions. Water column

species are not exposed to the same contaminant fractions experienced by benthos when compounds are associated with sediment (Ankley *et al.* 1991, Egeler *et al.* 1997). This means that the ecology and behaviour of test organisms has the potential to determine the degree of exposure to sediment bound contaminants. The potential of selective particle size feeding to modify contaminant exposure has already been described with respect to amphipods (Section 1.1.4). This behaviour is also reported in the clam *Macoma nasuta* that preferentially ingests fine organic enriched particles but does not ventilate pore water. Instead overlying water is siphoned over the respiratory surfaces in isolation from pore water (Boese *et al.* 1990). In addition behaviours such as sediment avoidance at high contaminant concentrations (e.g. in *Lumbriculus variegatus*: Kukkonen and Landrum 1994; *Diporeia spp.*: Landrum *et al.* 1991; *Hyallela azteca*: Kane-Driscoll *et al.* 1997b) reduce the effective exposure of benthic fauna to sediment-bound contaminants. Tube building in species such as *Chironomus* spp. and *Hexagenia* spp. reduce the amount of pore water exposure by ventilating burrows with overlying water (Harkey *et al.* 1994a, Landrum and Robbins 1990). Thus the ecology of these species delimits the contaminant carrying phases to which they are exposed. In addition to overall uptake depending upon organism biology, it may be possible that proportional contributions to overall uptake may differ in response to organism behaviour. For example, increased ventilation of the clam *Macoma balthica* may result in greater proportional uptake from the aqueous phase (Kaag *et al.* 1997). However, since this coincides with a cessation of sediment ingestion, it is unclear whether the lack of sediment ingestive uptake or the increase in aqueous ventilation is responsible. This is further complicated by the potential for proportional uptake route to be strongly influenced by compound partitioning (Section 1.1.7).

The above examples imply that organism biology can influence overall uptake of a particular contaminant. Additionally, the amount of processing of aqueous fractions for respiratory exchange has the potential to vary between species. However, it is currently unclear whether contaminant fractions present in the aqueous phase can be accumulated in greater proportion via increased aqueous ventilation.

1.1.9 Assessment of assimilation within tissues

Studies of bioaccumulation often use measurements of the amount of compound associated with homogenised complete organism tissues (e.g. Rubinstein *et al.* 1984,

Kane-Driscoll *et al.* 1997b). For the purposes of determining the amount of compound assimilated within tissues, it is necessary to ignore compound that remains external to the organism. To this end, the requirement to allow sediment-ingesting organisms to purge their guts of contaminated sediment prior to tissue loading measurement is recognised (e.g. Mount *et al.* 1999). Kukkonen and Landrum (1995b) found that, even though PDMS could not cross biological membranes to be assimilated, small amounts were measured in tissue homogenates. This was attributed to PDMS presence in non-purged gut contents and also surface sorption of the compound onto the external integuement (Kukkonen and Landrum 1995b). Therefore, if a compound that is expected to be non-bioavailable is measured in homogenised tissue, it is desirable to confirm whether that compound is genuinely assimilated. This consideration is appropriate to investigations of assimilation in strongly particle-sorbed contaminants.

1.2 Thesis aim and objectives

The overall aim of this thesis is to address the hypothesis that uptake of particle-sorbed contaminants is a viable assimilation route: the relative importance of which may vary in response to both chemical partitioning and organism biology. In order to address this aim, a series of experimental objectives were proposed. Each objective is described below accompanied by the chapter number in which it is reported.

It is evident from the review presented in this chapter that compound fractions that become incorporated within internal sediment-particle structures are generally non-bioavailable. However, the assumption that compounds that are strongly resistant to aqueous desorption due to hydrophobicity and attraction for the sorbant-particle surface may be questionable. Solubilisation in gut fluids, and dietary uptake of organic compounds that far exceeds desorption achieved in water, suggests that aqueous desorption is not always a perfect model of the bioavailability of ingested sediment contaminants. The potential for interspecific variation in dietary uptake of sediment-sorbed contaminants is also apparent. In addition, it seems likely that both contaminant partitioning and organism processing of the aqueous phase could influence the relative proportion of uptake achieved from particle-sorbed and aqueous phases.

An investigation of whether contaminant uptake can occur from sediment in the absence of desorption into pore water was undertaken. This was carried out via the exposure of four macroinvertebrate species to artificial and field collected sediment dosed with a desorption resistant compound (Chapter 2). Additionally, the experiments of Chapter 2 served to address whether interspecific variation in uptake of particle-sorbed compound was evident.

In order to robustly confirm that strongly particle-sorbed contaminant is assimilated, whole body residues were measured in feeding and non-feeding as well as moulted and non-moulted individuals (Chapter 3). Visual confirmation of compound assimilation was also sought via autoradiography of organism sections in combination with radiolabelled study compound (Chapter 3).

As an adjunct to the observed variation in uptake of particle-sorbed contaminant, the possible factors that drive such variation were investigated (Chapter 4). Such investigations included both experimentally measured biological attributes and literature derived characteristics.

Finally, the potential for variation in the proportional contribution of uptake route was investigated in three PAH compounds (Chapter 5). The influence of compound partitioning and organism ventilatory appendages upon the proportional contribution of aqueous uptake was investigated. Novel apparatus enabling a separate estimation of the contribution from pore water was utilised (Chapter 5).

Chapter 2: Uptake of a desorption-resistant compound (DODMAC) from sediment in four aquatic macro-invertebrates

2.1 Introduction

2.1.1 Issues

As stated in Chapter 1, sediments form an important potential source of hydrophobic organic contaminants to aquatic foodwebs (Section 1.1.1). However, the process of contaminant transfer from sediments into biota is unpredictable and poorly understood (Section 1.1.1). Equilibrium partitioning (EqP) approaches can only provide broad estimates of bioavailability, and cannot be applied to polar contaminants (Section 1.1.2). Further, EqP cannot distinguish between uptake achieved from aqueous and particulate sources (1.1.2). The general bioavailability of aqueous contaminant fractions are well established (Sections 1.1.1, 1.1.2, 1.1.3 and 1.1.4). In contrast, the bioavailability of strongly particle-sorbed contaminants is not well characterised (Section 1.1.1). The uptake of particle-sorbed contaminant has been implicated to occur via dietary accumulation. Such dietary accumulation is suggested to potentially vary between species in response to differential gut physiology (Section 1.1.5). Conversely, other studies assume that compounds that sorb strongly to sediment particles and resist desorption into aqueous media are not bioavailable to sediment ingesting species (Section 1.1.4).

Therefore, whilst it is clear that it will always be possible to identify a relationship between compound desorption and bioavailability (due to compounds in solution being bioavailable); it seems more pertinent to question whether particle-sorbed contaminants can be assimilated via ingestion. Further, whether any observed uptake from sediment particles appears to vary between species. Thus it is possible that bioavailability is solely determined by the proportion of compound that desorbs into aqueous solution. Alternatively, processes that determine the degree to which compounds are stripped from sediment particles in the gut may influence the amount of contaminant assimilated from sediment. The work presented here is interpreted in the light of these two alternative standpoints.

2.1.2 Aim and approach

The aim of this study was to elucidate whether uptake: (i) could occur solely from sediment particles, in the absence of desorbed compound in aqueous solution; (ii) varies between benthic species with different feeding habits. Two specific hypotheses were addressed:

2.1 That uptake of organic compounds from sediment, in the absence of uptake from pore water, may occur via the ingestion of sediment particles and the subsequent removal of sorbed compound from those particles by digestive processes. This was addressed by testing the prediction that organisms ingesting spiked sediment would achieve measurable tissue loadings of a model compound sorbed solely to sediment particles.

2.2 That the degree to which a species feeds on sediment particles will control the exposure, and hence uptake, experienced by that species. This was addressed by testing the prediction that species that extensively ingest sediment particles achieve higher tissue loadings than species that do not routinely feed on fine sediment particles.

These predictions were tested by exposing benthic macroinvertebrates to a model compound bound to sediment particles. This compound would not undergo desorption into pore water once sorbed onto sediment solids. Thus, any observed uptake could be attributed to assimilation from sediment particles. Uptake under such conditions is not modelled by EqP predictions due to the lack of contaminant flux between sediment particles and pore water. Similarly a lack of aqueous desorption should be equated to a lack of bioavailability in approaches that propose aqueous concentrations determine bioavailability (hereafter "desorption/bioavailability" approaches).

2.1.2.1 Species selection

Four freshwater benthic macroinvertebrates were investigated: *Lumbriculus variegatus* (Muller), *Asellus aquaticus* (L.), *Gammarus pulex* (L.) and larval *Chironomus riparius* (Meigen). Sediment contact and ingestion in each of the species was assumed to follow the ranking *Lumbriculus variegatus* > *Chironomus riparius* > *Asellus aquaticus* = *Gammarus pulex*. Although both *Lumbriculus variegatus* and

Chironomus riparius ingest fine sedimentary particles, it is the oligochaete worm that is classified as an extensive sediment re-working species (Cooke 1969, Brinkhurst and Jamieson 1971). This contrasts with the tube dwelling, deposit feeding behaviour of *Chironomus riparius* larvae (Oliver 1971, McCall and Tevesz 1982, Rasmussen 1984, Armitage *et al.* 1995). Therefore, it was predicted that this difference in habit would lead to a greater degree of exposure in the worm species than in the larval insect species. Further, the epibenthic habit of both *Asellus aquaticus* and *Gammarus pulex* (Graça *et al.* 1994a) was seen to place these species at a lower degree of sediment contact compared to the two infaunal species. These two crustacean species obtain nutrients via the maceration and ingestion of coarse particulate material, e.g. leaf litter and associated fungal masses (Anderson and Sedell 1979, Sutcliffe *et al.* 1981, Graça *et al.* 1994b), and were not predicted to extensively ingest the spiked sediment.

The feeding biology of both crustacean species does not suggest the ingestion of fine sediment particles as a common feeding mode (Anderson and Sedell 1979, Sutcliffe *et al.* 1981, Graça *et al.* 1994b). Therefore both crustaceans were predicted to achieve lesser, or immeasurably low, tissue loadings of a desorption-resistant compound. Thus, uptake of sediment-bound contaminants was expected to follow the same ranking as the predicted degree of sediment ingestion. Such that tissue loadings of organisms exposed to dosed sediments would be *Lumbriculus variegatus* > *Chironomus riparius* > *Asellus aquaticus* = *Gammarus pulex*.

2.1.2.2 Model compound

The model compound used in this study was DODMAC (Dioctadecyl-dimethyl ammonium chloride), a quaternary dialkyl dimethyl ammonium compound in which the alkyl chains are saturated C_{18} only. The empirical formula is given as follows $C_{38}H_{80}NCl$ (ECETOC 1993). It is the major component of dihydrogenated tallow dimethyl ammonium chloride (DHTDMAC), which has been used as a rinse added fabric softener (ECETOC 1993). DHTDMAC (and its major component DODMAC) adsorbs strongly to mineral and organic surfaces and is stated to be present in natural systems almost entirely adsorbed to particulate matter (ECETOC 1993). Additionally the same report states that the aqueous solubility of DODMAC is essentially immeasurable but is quoted as being less than 1 pgl^{-1}. Elsewhere (e.g. Laughlin *et al.* 1992) the aqueous solubility of DODMAC has been described as "vanishingly small".

Laughlin *et al.* (1992) describe, in a paper characterising the physical chemistry of the DODMAC/water system, that under particular temperature or physical shear conditions (i.e. heating to just below the Krafft discontinuity and continuous sonication, respectively), water containing micelles of DODMAC may be formed. However, the formation of such micelles is essentially instantaneous and the lack of DODMAC in aqueous solution is again stressed (Laughlin *et al.* 1992). As well as a high potential for adsorption to solid phases and extreme hydrophobicity, DODMAC appears to be available for assimilation by fish (ECETOC 1993). Finally, in desorption studies using sediment particles suspended in aqueous medium, this material is classified as "desorption resistant" (ECETOC 1993).

Confusion and wide variability in reported physical characteristics of DODMAC are also evident (Table 2.1). For example, the third draft of the comprehensive risk assessment report (BAA 1998) cites values for solubility in water that vary between 2.7 mgl^{-1} and <1 pgl^{-1}. However, the higher solubility measured by Kuneida and Shinoda (1978) was found to be based upon a method for measuring critical micelle concentrations and not solubility (BAA 1998).

Table 2.1 Summary of measured DODMAC physical properties

Physical Property	Value
Water solubility	<1 pgl^{-1} † – 2.7mgl^{-1} ††
Octanol/water partition coefficient (K_{ow})	3.80 †††
Sediment/Water partition coefficient ($K_{sed\text{-}wat}$) for 3 different sediments	3833, 10775 and 12489 lkg^{-1} ††††

† **Laughlin *et al.* (1990)**

†† **Kuneida and Shinoda (1978) – N.B. method inappropriate for solubility determination**

††† **Sanchez-Leal *et al.* (1994), cited in BAA (1998) N.B. K_{ow} noted to be inappropriate characterisation of environmental partitioning of surfactant compounds such as DODMAC.**

†††† **ECETOC (1993)**

The draft risk assessment also suggests that the solubility of <1 pgl^{-1} determined by Laughlin *et al.* (1990) as far more reliable and states "...it is highly probable that the estimate of solubility by Kuneida and Shinoda is far too high, perhaps as much as by ten orders of magnitude (10^{10})." (BAA 1998). The report concluded "In sewage or surface waters, DODMAC is not really dissolved but always adsorbed onto particles or included in vesicles together with other organics." (BAA 1998).

It is possible, therefore, for DODMAC to be dispersed within the water column, either as vesicles or sorbed to suspended particulate matter. Concomitantly, there is wide variation in reported aqueous toxicity values using different aqueous media/exposure apparatus (BUA 1998). Again, the presence of sorptive particles within the test environment is invoked as a mechanism by which bioavailability (to pelagic test species) is reduced in environmentally realistic exposures (BUA 1998). However, the ability to disperse non-dissolved DODMAC within the water column and derive aqueous toxicity values (from both nominal and measured aqueous loadings) is well illustrated by the reported toxicity tests (e.g. ECETOC 1993, BAA 1998, BUA 1998). Aqueous toxicity values are noted to be well above the accepted aqueous solubility of DODMAC due to stable dispersions within water – and not aqueous solute (BAA 1998).

Since DODMAC readily sorbs to both mineral and organic particles (and does not sorb preferentially to sediment organic carbon), calculation of an organic carbon partitioning coefficient (K_{oc}) is not appropriate (BAA 1998). Similarly, and in consideration of the lack of distinction between aqueous solute and aqueous dispersion of DODMAC, the calculation of an octanol/water partition coefficient is also inappropriate. A more meaningful characterisation is suggested to be case-specific sediment/water partitioning behaviour ($K_{sed\text{-}water}$) (BAA 1998). Individual characterisation is suggested following observed variation between different sediments from 3833 – 12489 lkg^{-1} (ECETOC 1993). For the technical grade commercial material (that is 80-90% DODMAC), a value of 85000 lkg^{-1} has been cited (ECETOC 1993). Despite the extent of adsorption to mineral and organic components of sediment, the process has been observed to be rapid and to reach equilibrium values within a few hours (Larson and Vashon 1983). Adsorption to mineral and organic particles has been found to be non-reversible when particles are eluted with water (ECETOC 1993, BAA 1998).

In summary, DODMAC is a cationic surfactant with extremely low aqueous solubility. It binds readily to organic and inorganic particulate matter. It is expected to bind exclusively to sedimentary/particulate matter in the aqueous environment. Further, its classification as "desorption resistant" means that it will not desorb into pore water once sorbed onto sediment particles. However, it is apparently able to pass across biological membranes in fish. These qualities make it an ideal model compound to test for biotic uptake from sediments in the absence of desorption into pore water.

The suitability of DODMAC in addressing uptake that is not accounted for by EqP is also enhanced due to its cationic nature, since EqP models specifically apply to non-polar organic compounds. In this study ^{14}C DODMAC was used exclusively. Two samples were synthesised at Unilever Research Port Sunlight (SEAC Environment, Unilever Research, Quarry Rd. East, Bebbington, Wirral, CH63 3JW). The first sample (used to expose *Chironomus riparius*, *Asellus aquaticus* and *Gammarus pulex* in artificial sediment) had a specific activity of 5.3 $\mu Ci\ mg^{-1}$. The second sample (used to expose all species in natural sediment plus *Lumbriculus variegatus* in artificial sediment) had a higher specific activity of 13.8 $\mu Ci\ mg^{-1}$.

2.2 Materials and Methods

2.2.1 Test organisms

2.2.1.1 Lumbriculus variegatus

Lumbriculus variegatus was cultured in the Department of Animal and Plant Sciences within The University of Sheffield. The culture system was adapted from that used at US EPA testing laboratories, Duluth (USEPA 1993). Culture vessels were rectangular perspex tubs (30 cm long, 15 cm wide and 10 cm deep) containing 1.5 litres of artificial pond water (APW), (HSE 1982, Naylor *et al.* 1989) over 2-cm depth of non-bleached shredded paper towel substrate. The shredded paper towels were soaked for 24 hours in de-ionised water. This softened the substrate prior to use and also, with the decanting of the water used for soaking, helped to remove potential contaminants that may have been present on the substrate surface.

Cultures typically contained approximately 300 - 500 individual worms and were aerated with compressed air passed through 2-cm long by 1-cm diameter aquarium airstones. Cultures were maintained at 20°C and a photoperiod of 16 h light to 8 h dark. The organisms were fed with powdered catfish food ("Catfish pellets", JMC Aquatics, 59 Stubley Lane, Dronfield, Sheffield, S18 6PG) every Tuesday and Thursday. Sufficient food was added to support healthy individuals without clouding the overlying water between each daily water change and was typically between 150 and 180 mg on each feeding occasion.

2.2.1.2 Chironomus riparius

Chironomus riparius larvae were also maintained in culture. The culture system was a modification of the system described by Credland (1973) and resembles that used for *Lumbriculus variegatus*. The substrate used was sand ("SUPAMIX" play pit sand, Pioneer supamix Ltd, Griff Lane, Griff Clara, Nuneaton, Warwickshire, CV10 7PP) rather than paper towel but the temperature, photoperiod, culture vessels and overlying water were the same as specified for *Lumbriculus variegatus* cultures.

Cultures were initiated, usually in pairs, by allowing 4 or 5 egg masses to hatch into approximately 10 ml of APW. This provided larvae from several females with which to initiate each culture, thus maintaining genetic variation within each culture. Subsequently, approximately 200 first instar larvae were transferred to each new culture via a disposable pasteur pipette. Chironomids were fed with powdered tropical fish flake ("Premier tropical flake" JMC Aquatics, 59 Stubley Lane, Dronfield, Sheffield, S18 6PG) at the same quantities and frequency as previously described (Section 2.1.1). Aeration, with compressed air, was provided by hypodermic needles and this was initiated 2 days after larval addition.

In contrast to *Lumbriculus variegatus* culture procedures, overlying water was not changed daily. Instead evaporative losses were replaced with de-ionised water to maintain the concentration of constituent minerals used in the artificial aqueous medium. Cultures were discarded at 4 – 6 weeks old.

2.2.1.3 Asellus aquaticus and *Gammarus pulex*

Asellus aquaticus of uncharacterised sex (approx 10 – 15 mm in length) and *Gammarus pulex* adult males were collected from field populations (Ordnance Survey NGR, SK 315 881 and Ordnance Survey NGR, SK 497 744 respectively) and then maintained in 20-l aquaria of aerated APW. Organisms were held at 20°C (± 2°C) and were fed dried leaf material collected from alder (*Alnus glutinosa*) after abscission but prior to leaf fall. Aquaria holding 300 – 400 individuals received 10-15 leaves per week. Two days prior to use in studies, the feeding of both crustacean species was suspended by removing any leaf material remaining in the aquaria. Crustaceans were

retained in laboratory aquaria for a maximum of two weeks prior to use in experimental procedures.

2.2.2 Test system

2.2.2.1 Sediment collection and preparation

Natural sediment was collected from a pond free of pesticide application for at least five years prior to sediment extraction (Ordnance Survey NGR, SE 280 692). Properties of this sediment were determined by Covance Laboratories Harrogate (Otley Road, Harrogate, HG2 1PY).

These properties are given as follows:

- pH in water - 7.4
- Organic carbon content - 2.0%

Particle size distribution:

- Sand - (63 - 2000 μm) - 80%
- Silt (2 - 63 μm) - 14%
- Clay (<2 μm) - 6%

Artificial sediment was prepared from sand (Proprietary acid washed sable sand (0.1-0.3 mm) from BDH Laboratory supplies, Poole, BH15 1TD, U.K.), kaolin (Proprietary acid washed Kaolin (light), Fisher Scientific U.K. Ltd. Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG, U.K.) and powdered α-cellulose (α-cellulose, non-nutritive bulk; CAS: 9004-34-6, SIGMA Chemical CO. P.O. Box 14508, St Louis MO. 63178 USA). Dry constituents were mixed together in the dry weight proportions of 70% sand : 20% kaolin : 10% cellulose. Organic carbon content and pH of one batch of such sediment was measured following preparation. The following properties were, therefore, determined for the artificial sediment:

- pH in water – 6.8
- Organic carbon content – 11%

Particle size distribution:

- Sand – (63 - 2000 μm) - 70%
- Clay/Cellulose (<2 μm) – 30%

The test system consisted of 200-ml glass jars each containing 30 g dry weight of an artificial sediment or the wet weight equivalent of 20 g dry weight of natural sediment. Ten samples (15 – 27 g) of natural sediment were dried to a constant weight at 60°C for 24 h. The mean proportional dry weight was calculated to be 44% of the initial wet weight (min = 43.66%; max = 44.53%). The calculated wet mass of sediment equivalent to 20 g dry sediment was added to all vessels (i.e. 45.45 g).

2.2.2.2 Sediment dosing

Sediment was dosed with a nominal concentration of 4.3 μg DODMAC per g dry weight of sediment. A stock solution for dosing (dosing stock) was prepared by dissolving DODMAC at the loading appropriate to the sediment dry weight in each vessel. For the 30 g of artificial sediment in each vessel a loading of 64.50 μg DODMAC per ml of HPLC grade ethanol was prepared. The loading was adjusted to 43 μg per ml for the 20 g samples of field collected sediment (i.e. 2.15 μg DODMAC per ml ethanol for every gram of sediment dry weight). Dosing stocks were prepared in conical flasks of the appropriate size. Ethanol solvent was added via a 100-ml graduated measuring cylinder. Half the volume of solvent was added to the conical flask prior to the addition of DODMAC. Subsequently the remaining required volume of solvent was added; thus promoting mixing of the stock solution. The DODMAC used to prepare each dosing stock solution was itself stored dissolved in ethanol. Therefore the appropriate volume of stored DODMAC solution was transferred to the dosing stock via a 100-μl glass Hamilton syringe. The volume of such transfers was typically approximately 500 μl of stored DODMAC solution added to approximately 200 ml of ethanol. Dosing was achieved by adding 2.0 ml of the dosing stock solution to the sediment in each test vessel and mixing until homogenous in appearance. The ethanol was then allowed to evaporate over three days' storage of test vessels prior to use. During this time the sediments were stirred thoroughly on one occasion per day to facilitate such dissipation.

2.2.2.3 Exposure system

Artificial sediment in each test vessel (whether dosed or non-dosed) was moistened by mixing with 2 ml of APW to allow it to be gently tamped down. This produced a cohesive pellet of sediment that was less likely to become suspended upon addition of overlying water. Field collected sediment already formed moist cohesive pellets and therefore did not receive this initial manipulation. Particle suspension in the water column was further limited in all vessels by placing a disc of buoyant plastic on top of the sediment pellet and pouring the overlying water onto this. Such "settling discs" were cut from commercial "bubble wrap" packing material. Each floating disc was removed and discarded following the addition of overlying water to test vessels. APW was used as overlying water and 100 ml was added to each test vessel. The addition of overlying water delimited the start of the exposure, occurring just prior to animal addition.

Animals were added to dosed test vessels and non-dosed vessels (Section 2.2.2.4). The number of vessels and the treatment groups prepared in exposures using artificial and field collected sediment are given in Table 2.1. Of the vessels without animals, groups of dosed and non-dosed vessels were analysed at the start of the exposure period (T0). Similarly a group of dosed vessels lacking animals was analysed at the end of the 48-h exposure period (T48; Section 2.2.2.5). The exposures in artificial sediment were carried out separately for each species; each exposure including unique "non animal" reference vessels. In contrast the field collected sediment exposures were conducted simultaneously for all species with a single set of reference vessels lacking animals.

2.2.2.4 Animal exposure and gut purge

Animals were added to 20 dosed test vessels and either 20 or 15 non-dosed vessels (Table 2.2). Each vessel contained either five *Gammarus pulex*, *Asellus aquaticus* or *Chironomus riparius* larvae (>5 mm length, $3^{rd}/4^{th}$ instar) or 20 *Lumbriculus variegatus*. All animals had been starved for 24 h at 20°C before addition to test vessels. Addition of each group of organisms to individual test vessels proceeded non-sequentially (i.e. in an arbitrary order). The organisms were exposed to sediment for a 48-h period before being removed to gut purge vessels for 24 hours. Gut purge vessels consisted of 200-ml beakers each with a 1.5 cm depth of sand

("SUPAMIX" play pit sand, Pioneer supamix Ltd, Griff Lane, Griff Clara, Nuneaton, Warwickshire, CV10 7PP) and 2.5 cm depth of APW. After the 24-h gut purge, each

Table 2.2 Allocation of vessels within treatment groups and test format for artificial and field collected sediment exposures.

	Artificial sediment		Field collected sediment	
		Vessel totals for four species		Vessel totals for four species
No. non-dosed T48[b] vessels (+animals)[a]	20 per species	80	15 per species	60
No. dosed T48 vessels (+animals)	20 per species	80	20 per species	80
No. non-dosed T0[b] vessels (-animals)[a]	20 per species	80	Single group of 15	15
No. dosed T0 vessels (-animals)	20 per species	80	Single group of 20	20
No. dosed T48 vessels (- animals)	20 per species	80	Single group of 20	20
	Total for exposure of 4 species	400	**Total for exposure of 4 species**	195

a = T0 and T48 denote the time (h since start of test) at which vessels were sampled for sediment, pore water and overlying water.

b = "+animals" or "–animals" respectively indicates the presence or absence of animals within that group of vessels.

organism was observed and the absence of artificial sediment within the gut confirmed visually.

Exposure and gut purge were performed in a temperature-controlled facility maintained at a nominal 20 ± 2°C. In order to minimise disturbance and since toxicity

was not the focus of these studies, water quality measurements such as pH and dissolved oxygen were not taken. It was assumed that water quality would not substantially influence the presence or absence of compound uptake. The animal tissue collected from each gut purge vessel was rinsed, blotted and then weighed prior to addition to scintillation vials for analysis.

2.2.2.5 Sampling

One-millilitre samples of overlying water, pore water and sediment were taken at the start of the exposure period (T0) and at the end of the 48-h exposure period (T48). Samples were taken from both non-dosed and dosed vessels at T0 to determine the initial status of the system, along with comparative "post test" samples of dosed and non-dosed vessels at T48. These T48 samples were further split between vessel groups in which animals were present or absent. In all cases the mean detected radioactivity in non-dosed vessels were assigned to represent background radioactivity. Dose vessel sample radioactivity values minus mean calculated background values were attributed to ^{14}C DODMAC. Background values were specific to each sample type since "dose" pore water, overlying water, sediment and tissue samples were compared to the background activities of "non-dose" pore water, overlying water, sediment and tissue samples respectively. Finally, the single mean background value for each sample type was subtracted from the relevant individual sample values; giving residual activity values that approximate to zero in non-dosed samples, and an appropriate measure of ^{14}C-labelled test compound in dosed samples.

2.2.3 Analysis of samples

2.2.3.1 Sediment sample preparation

Weighed samples of solid sediment, for both artificial and field collected sediment, were taken after 7 day air-drying of sediment samples removed from test vessels. In all cases 18 ml of Packard Emulsifier Scintillator Plus™ (Packard Bioscience B.V, Rigaweg 22, 9723 TH, Groningen, The Netherlands.) was added to each sample and shaken until thoroughly mixed. Sediment samples were retained after the addition of scintillant fluid and counted several times until, after two weeks, increases in dosed sediment measured activity reached an asymptote (Appendix 2.1).

The solid sediment disintigrations per minute (dpm) values were used to generate measured dose values for each sample replicate by dividing measured dpm values by the specific activity of the DODMAC sample. Subsequently this figure was divided by the mass of the sample to give μg DODMAC per g sediment sample.

This methodology was validated in advance via the amendment of non-dosed sediment with known weights of dosed sediment and the subsequent fitting of the regression curve to weight of dosed sediment in each sample versus measured dpm values (Appendix 2.2).

2.2.3.2 Aqueous sample preparation

At each sampling time a 2.0-ml sample of overlying water was taken from each vessel, the remaining overlying water being discarded in each case. Pore water (2 ml) was taken from each test vessel following one-minute centrifugation at 1500 rpm of the whole test vessel. Each 2-ml overlying water and pore water sample was then centrifuged at 3000 rpm for one hour before removing 1-ml samples for scintillation counting. Ten millilitres of Packard Emulsifier Scintillator Plus™ was added to each 1-ml sample and counted immediately.

2.2.3.3 Tissue sample preparation

The organisms recovered from each individual gut purge vessel comprised a single tissue sample. Tissues were solubilised, according to common methodology (e.g. Bransome 1970, Neame and Homewood 1974) for three days using 2 ml Soluene™ prior to addition of 10 ml Hionic Fluor™ scintillator fluid (Packard Bioscience B.V, Rigaweg 22, 9723 TH, Groningen, The Netherlands.). The samples were left in darkness for a further 24 h to allow for attenuation of chemiluminescence before counting. Preliminary sample processing indicated that 24 h was a sufficient duration to allow effective attenuation of chemiluminescence.

2.2.3.4 Counting

Samples were counted using a Tricarb 3000 Liquid Scintillation Counter with a single ^{14}C counts window (0 - 156 Kev emission spectrum). The external standards

method (Bransome 1970, Neame and Homewood 1974) was used to correct for quench and each vial was counted for 2 minutes. Depending upon the sample used each μg of DODMAC is calculated to produce either 11766 or 29748 disintegrations per minute (dpm). Therefore, the dpm values from each sample were divided by the appropriate figure after subtraction of the background value to give μg DODMAC. In the case of aqueous samples this figure is equivalent to μg per ml, however division by sample weights of tissue and sediment samples is required to convert solid sample figures to μg per g.

2.3 Results

2.3.1 Analysis of sediment particles and aqueous phases

2.3.1.1 Sediment versus aqueous samples

The measured DODMAC loadings in the dosed sediment agreed well with the nominal value of 4.3 μgg^{-1} in both artificial and field collected sediments (Fig. 2.1). Additionally it is clear that the great majority of the DODMAC associated with thesediment particles rather than the aqueous phase. Mean values of 4.452 μgg^{-1} for artificial sediment and 4.342 μgg^{-1} for field-collected sediment were determined. In comparison, the mean values determined for aqueous samples in artificial and field-collected sediment respectively were 0.00294 μgml^{-1} and 0.00019 μgml^{-1}. Therefore, calculated sediment/water partition coefficients ($K_{sed\text{-}water}$) are in the region of 1514 lkg^{-1} and 22852 lkg^{-1} for artificial and field-collected sediment respectively (Fig. 2.1).

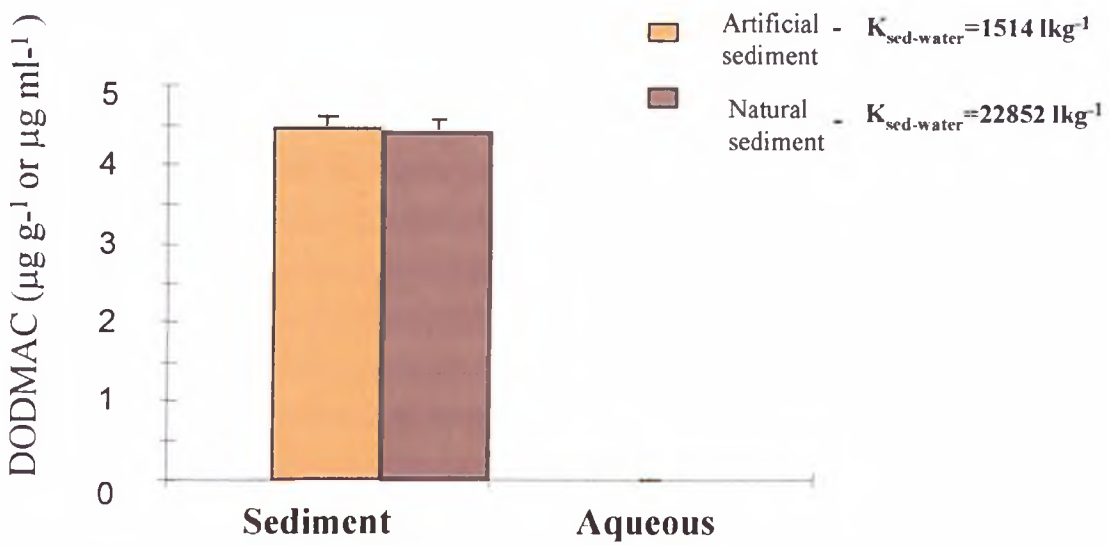


Fig. 2.1 Mean (+SE) measured DODMAC loadings in whole sediment samples and aqueous samples taken from all vessels containing sediment spiked with DODMAC for exposure of four freshwater macro-invertebrate species. Sediment/pore water partition coefficients ($K_{sed\text{-}water}$)are given for both artificial and natural sediments (lkg^{-1})

2.3.1.2 *Aqueous samples from dosed sediment versus aqueous samples from non-dosed sediment*

When the dpm values for non-dosed aqueous samples were compared with the samples from dosed vessels, it is apparent that DODMAC was present in a number of samples from both sediment types (Figs. 2.2 and 2.3). These data did not conform well to the assumptions of parametric testing and could not be simply transformed in order to meet such assumptions. Therefore, non-parametric Kruskall-Wallis analyses were applied. In artificial sediment aqueous samples non-parametric pairwise comparisons of treatment medians to non-dosed samples (statistic "Q'", Zar 1984) indicated that a number of treatment groups did not differ significantly from non-dosed samples. Specifically samples of overlying water from T0 dose minus animals, *Lumbriculus variegatus*, *Asellus aquaticus* and *Gammarus pulex* vessels did not differ significantly from non-dosed samples (maximum Q'= 2.59, where critical value at k=13; $p<0.05$ is Q'= 2.866). Conversely, all remaining sample groups were found to contain significantly more radioactivity than non-dosed samples (minimum Q'= 4.06, where critical value at k=13; $p<0.001$ is Q'= 3.925). Similarly, non-parametric pairwise comparisons between samples taken from field collected sediment vessels detected significantly more radioactivity in a number of dose treatment groups than in non-dosed samples. Specifically T0 dose minus animal pore water and dosed *Lumbriculus variegatus* overlying water samples were found to have significantly higher median dpm values than non dose samples at $p<0.05$ (Q'= 3.30 and 3.31 respectively where critical value at k=13 for $p<0.02$ is Q'= 3.114). In addition, median dpm values in dosed *Chironomus riparius* pore and overlying water as well as dosed *Lumbriculus variegatus* pore water were found to significantly exceed those of non dose samples at $p<0.001$ (minimum Q'= 4.179, where critical Q'= 3.935). The remaining treatment groups were not found to contain significantly higher radioactivity levels than non-dosed samples at $p<0.05$ (maximum Q'= 2.39, where critical Q'= 2.866).

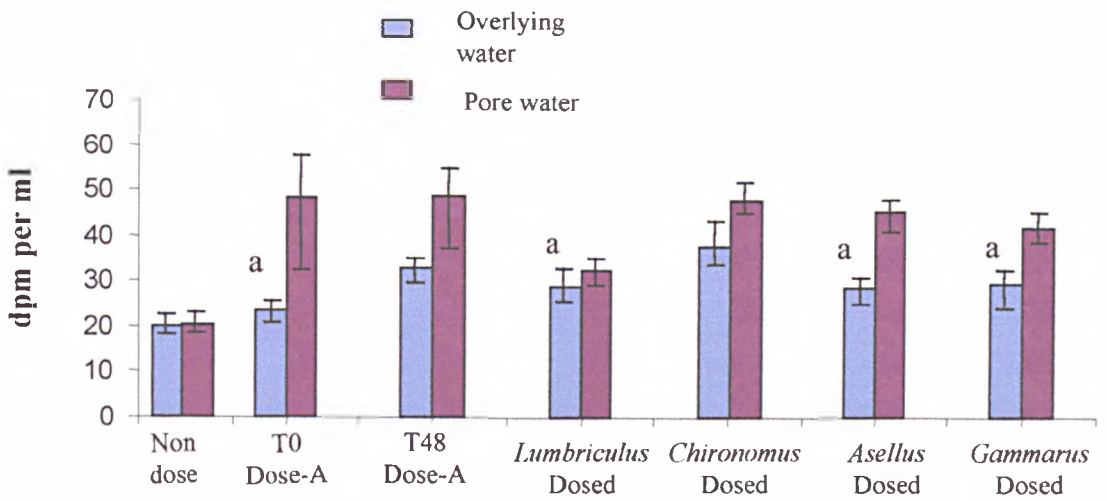


Fig 2.2 Median (± Interquartile range) disintigrations per minute (dpm) per ml measured in aqueous samples taken from artificial sediment vessels. Groups annotated "a" were not detected to differ significantly from activity measured in non-dose vessels at $p<0.05$. All groups, except "non dose", lacking this annotation were detected to have significantly greater activity than non-dose samples at $p<0.001$.

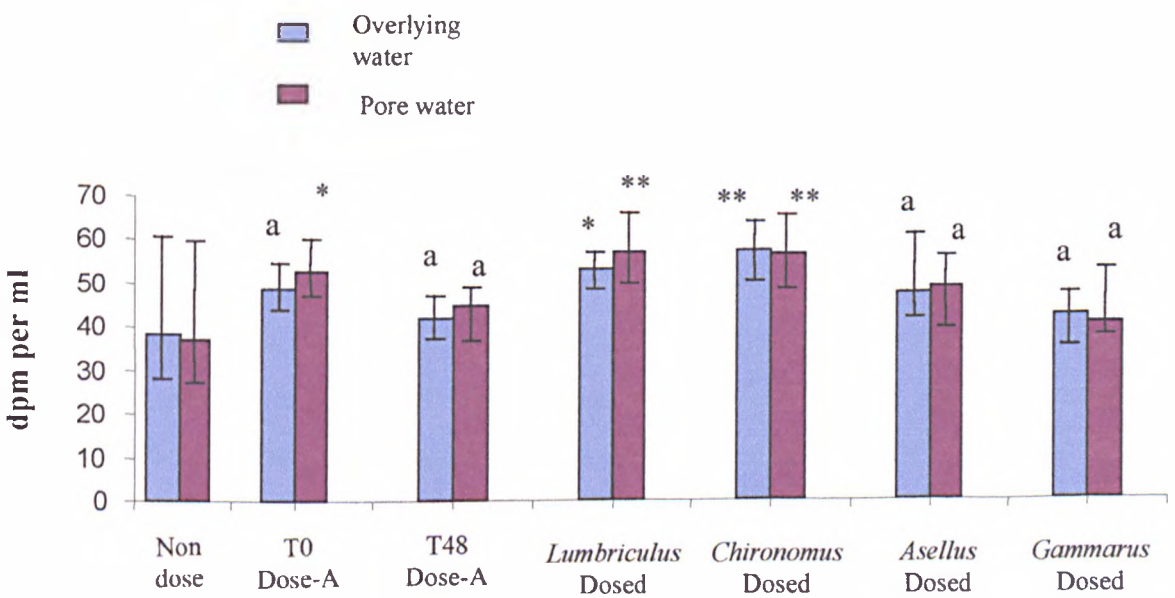


Fig 2.3 Median (± Interquartile range) disintigrations per minute (dpm) per ml measured in aqueous samples taken from field collected sediment vessels. Groups annotated "a" were not detected to differ significantly from activity measured in non-dose vessels at $p=0.05$. The annotations "*" and "**" denote differences detected at $p<0.05$ and $p<0.001$ respectively.

2.3.2 Tissue loadings

As in the case of the aqueous data described in Section 2.3.1.2, the data for tissue loadings of DODMAC were most suitably analysed via non-parametric methods. Again, Kruskall-Wallis analyses were applied to tissue samples exposed using both artificial and field collected sediment.

2.3.2.1 Tissue loadings achieved in artificial sediment

Measured loadings of DODMAC within tissues exposed to dosed artificial sediment varied between non-dosed and dosed artificial sediment groups ($H_7 = 132.32$; $p < 0.001$, Fig. 2.4). All dosed sediment groups achieved significantly higher loadings than non-dosed groups (Q' for *Lumbriculus variegatus* vs background =3.27; $p<0.005$). In addition, tissue loadings varied between dosed groups ($H_3 = 57.37$; $p < 0.001$). Median tissue loadings of groups exposed to dosed sediment were compared via non-parametric pairwise comparisons of all treatments (statistic "Q", Zar 1984). Tissue loadings in *Asellus aquaticus* did not differ significantly from those of *Gammarus pulex* ($Q = 0.67$; $p>0.05$). However *Gammarus pulex* tissues had significantly higher loadings than *Lumbriculus variegatus* tissues ($Q = 3.11$; $p<0.02$) and *Chironomus riparius* tissue loadings were significantly greater than *Asellus aquaticus* tissue loadings ($Q = 3.96$; $p<0.001$). Therefore, all species achieved measurable tissue loadings of DODMAC from dosed artificial sediment. These loadings varied between species, but not as predicted from the expected sediment ingestion hierachy; *Lumbriculus variegatus* >*Chironomus riparius* >*Asellus aquaticus* = *Gammarus pulex*. Rather, the results obtained suggest that the uptake hierachy from the artificial sediment used is *Chironomus riparius* >*Asellus aquaticus* = *Gammarus pulex* >*Lumbriculus variegatus*.

2.3.2.2 Tissue loadings achieved in field collected sediment

Tissue loadings of DODMAC varied between groups exposed to non-dosed and dosed sediment ($H = 99.31$; $DF = 4$; $p < 0.001$, Fig. 2.5). All dosed sediment groups achieved significantly higher loadings than non-dosed groups (Q' for *Gammarus pulex* versus background tissues =3.76; $p<0.001$). Tissue loadings varied between dosed groups ($H = 37.9$; $DF = 3$; $p < 0.001$). *Chironomus riparius* loadings did not differ

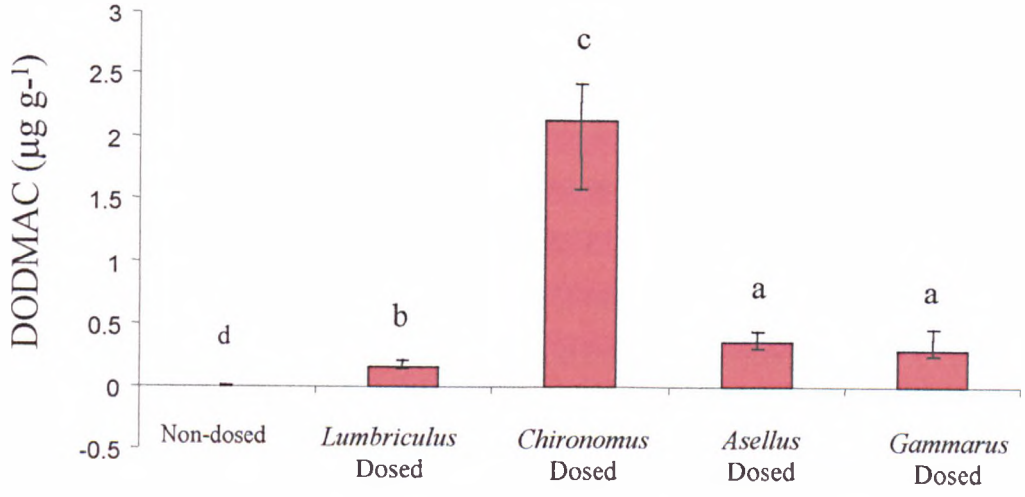


Fig. 2.4 Median (± Interquartile range) tissue loadings of DODMAC in organisms exposed using artificial sediment. Groups annotated with the same letter do not differ significantly from each other at $p<0.05$. Non-dosed treatment is pooled results for tissues of all species exposed to non-dosed sediment.

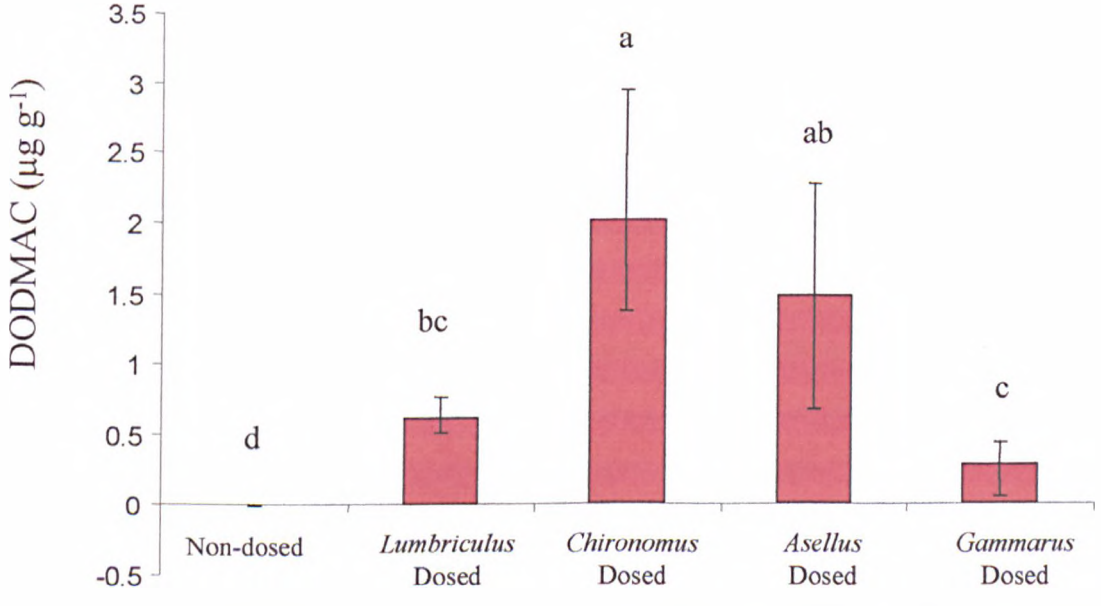


Fig. 2.5 Median (± Interquartile range) tissue loadings of DODMAC in organisms exposed using field collected sediment. Groups annotated with the same letter do not differ significantly from each other at $p<0.05$. Non-dosed group is pooled results for tissues of all species exposed to non-dosed sediment.

significantly from *Asellus aquaticus* loadings (Q = 1.14; p>0.05). *Asellus aquaticus* loadings did not significantly exceed *Lumbriculus variegatus* loadings (Q = 2.36; where critical Q value for p<0.05 = 2.81). *Lumbriculus variegatus* loadings did not significantly exceed *Gammarus pulex* loadings (Q = 2.07; p>0.05). However *Chironomus riparius* tissue loadings were significantly greater than *Lumbriculus variegatus* loadings (Q = 3.68; p<0.01) and *Asellus aquaticus* loadings significantly exceeded *Gammarus pulex* loadings (Q = 4.31; p<0.001). Again all species achieved measurable tissue loadings of DODMAC and such tissue loadings differed between species. In field collected sediment, the tissue loading hierachy differed from both the *a priori* prediction and from that achieved in artificial sediment exposures. The hierachy measured in these exposures to field collected sediment can be expressed as follows:

C. riparius A. aquaticus L. variegatus G. pulex

Species sharing an underscored line were not detected to differ significantly from each other at p<0.05.

2.4 Discussion

Two hypotheses were addressed in this chapter (Section 2.1.2). The first hypothesis was that:

"uptake of organic compounds from sediment, in the absence of uptake from pore water, may occur via the ingestion of sediment particles and the subsequent removal of sorbed compound from those particles by digestive processes."

In order to address this hypothesis, it was necessary to expose one or more species of sediment-ingesting invertebrate to a compound that was resistant to aqueous desorption when sorbed to sediment particles. The measured tissue loadings of DODMAC appear to support the prediction that ingestion of sediment particles could lead to the assimilation of a desorption resistant compound from such particles (Sections 2.3.2.1 and 2.3.2.2). However, this finding is subject to a number of caveats; pertaining both to the behaviour of the model compound within the test system and also to the interpretation of bulk tissue loadings as being representative of true assimilation.

Measurements of DODMAC in sediment and water samples showed that the compound predominantly associated with sediment particles. Although, contrary to assumptions that DODMAC would be absent from aqueous samples, levels of radioactivity in aqueous samples from dose vessels appeared to be elevated above levels found in non-dosed vessels. It is clear that DODMAC was present within a number of aqueous samples derived from dosed sediment. It is not clear, however, from measurements of gross radioactivity whether the substance is sorbed to co-sampled particulate matter, or whether such loadings are a measure of true aqueous dissolution. The suggestion (BAA 1998, section 2.1.2.2) that case-specific sediment/water partition coefficients are used to characterise the behaviour of DODMAC within exposure scenarios resulted in figures that corresponded broadly to previously measured values (Fig. 2.1/section 2.3.1.1 and Table 2.1/section 2.1.2.2 respectively). Caution was urged in section 2.1.2.2 in regarding aqueous loadings above 1 pgl^{-1} as aqueous solute rather than sorbed to suspended particles or dispersed in stable vesicles. In the application of EqP models where measured pore water concentrations greatly exceed predicted values, a correction for sorbed material is invoked (e.g. Ankley *et al.*1994). The fraction of sorbed compound along with the freely dissolved fraction are quantified and, together, deemed to make up the total measured pore water loading (DiToro *et al.* 1991). Dissolved organic carbon (DOC) was identified as a likely co-sampled sorbant material in DiToro's paper. The sorbed fraction within pore water is accorded the same status as material bound to bulk sediment particles, whilst compound bioavailability is inferred from the estimated freely dissolved concentration by Equation 2.1 (after DiToro *et al.* 1991):

$$\text{dissolved pore water concentration} = \frac{\text{total measured pore water concentration}}{1 + [K_{DOC} \cdot m_{DOC}]} \qquad \textbf{Eq. 2.1}$$

In Equation 2.1, K_{DOC} is the DOC partition coefficient for the test compound and m_{DOC} is measured DOC concentration within pore water (kgl^{-1}). DOC was not measured in either of the two sediment types used in the current study, however, DiToro *et al.* (1991) and Ankley *et al.* (1994) exemplify the concept that measured total pore water loadings do not necessarily represent aqueous concentrations of hydrophobic organic chemicals. The phenomenon of sorbed chemicals boosting apparent aqueous concentrations may not, though, universally depend on DOC as the sorbant. For

example, Senseman *et al.* (1995) describe how both Ca-montmorillinite clay as well as humic acids were responsible for sorbing hydrophobic organic compounds within pore water. In both field collected and artificial sediments used here to expose four species to DODMAC, there are potential sorbant materials present that may act to elevate measured pore water loadings above those attainable via true aqueous solution of the test compound. Such difficulties in characterising true aqueous solute of DODMAC (and the commercial grade dihydrogenatedtallowdimethylammonium choride DHTDMAC) are well illustrated by the following statement: "Separating the suspended solids by centrifugation and analysing the aqueous phase for DHTDMAC will give the "dissolved" DHTDMAC. The accuracy of this method depends on the efficiency of isolating the suspended solids and colloidal particles from the solution. Difficulties in achieving good separation can result in values of "dissolved" DHTDMAC being above the limit of solubility" (ECETOC 1993).

Therefore, based upon the rigorously determined water solubility of DODMAC and its strong affinity for particulate matter (Laughlin *et al.* 1990, ECETOC 1993, Larson and Varshon 1994, BAA 1998, BUA 1998), the application of desorption/bioavailability theories would predict immeasurably low bioavailability of sediment-sorbed DODMAC. Additionally EqP and empirical measurements introduce the possibility that total measured aqueous loadings may not consist solely of compounds in free solution. It seems very unlikely that the measurement of DODMAC within aqueous samples obtained here represents freely dissolved compound (measured loadings of, exceptionally, up to $4\mu gl^{-1}$ versus solubility of $<1pgl^{-1}$). Further, the variation in measured pore water concentration does not appear to be tracked by the variation in tissue loadings between the species. This contradicts the suppositions that: i.) measured pore water loadings truly reflect freely dissolved DODMAC and ii.) such freely dissolved DODMAC concentrations determine uptake. Finally, in the most extreme case, pore water samples from artificial sediment exposure of *Asellus aquaticus* reached activities equivalent to approximately 4ng DODMAC ml^{-1}. Even if this represented a true aqueous solution, a 300-mg *Asellus aquaticus* would need to process more than 37 ml of pore water with an assimilation efficiency of 100% in order to achieve the tissue loading measured in this species. Since each 30 g of artificial sediment yields only approximately 5 ml of pore water it is unlikely that uptake can be solely attributable to the aqueous route. This is the case even though it appears that an unrealistically high concentration of DODMAC is used in this estimation.

The second issue related to the potential uptake of a desorption resistant compound from sediment is the validity of inferring uptake from total tissue loadings. It is crucial that total tissue loadings of DODMAC reflect genuine assimilation of the compound and not just adsorption of contaminant to the integuement.

It seems unlikely that the tissue loadings obtained in exposure of DODMAC to the four species used in this study were due to compound that had desorbed into aqueous solution. The selection of DODMAC as a suitable compound to test whether uptake can occur in a manner not considered by EqP approaches, and contrary to predictions of desorption/bioavailability approaches, appears sound. The tissue loadings measured in all species could not be predicted using an EqP approach, since the cationic nature of DODMAC precludes application of EqP. Neither would uptake of DODMAC be predicted by approaches linking aqueous desorption to bioavailability. Measured pore water loadings are unlikely to reflect true aqueous concentrations and aqueous loadings do not adequately address the observed uptake.

Toxicokinetic modelling predictions of Landrum and Robbins (1990) and potential digestive uptake mechanisms highlighted in a number of papers authored by Mayer and co-workers (Mayer *et al.* 1996, Weston and Mayer 1998 and Mayer *et al.* 2001) offer alternative mechanisms of uptake from sediments. In the case of toxicokinetic modelling, it is suggested that compound partitioning between aqueous phases and varying sediment particle size fractions influences the potential exposure route, rather than fundamental availability, of a compound (Landrum and Robbins 1990). In general these authors suggest that uptake from sediment particles becomes increasingly prevalent as compound affinity for sediment solids increases. Thus under this approach, DODMAC may be viewed as potentially bioavailable via a predominantly ingestive route. Of course, this operates with the proviso that the study species must process the contaminant-holding sediment fraction and must also be able to successfully remove the contaminant from sediment particles. Therefore, studies that use gut fluids derived from sediment biota as an effective extraction agent for a range of organic contaminants (i.e. Mayer *et al.* 1996, Weston and Mayer 1998, Mayer *et al.* 2001) show that not only is ingestive uptake theoretically predicted; it is also mechanistically viable in a range of marine benthic invertebrates.

The risk of ingestive uptake of sediment-sorbed compounds inferred from toxicokinetic models and gut fluid extractions requires confirmation. A source of such

confirmation would be the observation of genuine assimilation of a desorption resistant compound from dosed sediment. Therefore the finding, in both artificial and field collected sediment, that all species achieved tissue loadings of a desorption resistant compound offers support for Hypothesis 2.1 (Section 2.1.2). If the tissue loadings detailed in Sections 2.3.2.1 and 2.3.2.2 represent truly assimilated DODMAC; then this would represent key evidence in the confirmation of uptake of particle-sorbed contaminant via the ingestive route. In turn DODMAC assimilation from sediment would indicate that aqueous fractions are not the sole determinant of bioavailability from sediments. However, it is possible that the measured tissue loadings of DODMAC could simply be the result of externally adhering material; and not represent true assimilation at all. This issue is addressed in Chapter 3.

The second hypothesis that was addressed in this chapter was that:

" The degree to which a species feeds on sediment particles will control the exposure, and hence uptake, experienced by that species."

This was addressed by exposing four different species to sediment-bound DODMAC. The species were predicted to vary in the amount of sediment that they would ingest. *Lumbriculus variegatus* and *Chironomus riparius* were expected to extensively ingest sediment particles. Conversely, *Gammarus pulex* and *Asellus aquaticus* were not expected to ingest sediment at all (Section 2.1.2.1). The tissue loadings achieved in each species were measured and compared to their predicted sediment-ingestion behaviour.

Interspecific variation in tissue loadings was measured. Such variation did not agree with species' predicted hierarchical positions (Section 2.1.2.1). However, contrary to predicted behaviour, all four species were observed to extensively ingest both artificial and field collected sediment. Thus our *a priori* expectations of sediment ingestion rates were flawed. Therefore, the hypothesis that differential sediment ingestion between species may influence uptake of sediment-sorbed compounds is not adequately addressed in these exposures. Accordingly there is good evidence to suggest that differences in sediment ingestion behaviour within and between benthic species can determine the relative uptake from sediment particles. Kaag *et al.* (1997), for example, suggested that the body burdens in two sediment ingesting species could not be accounted for by uptake from the aqueous phase. The facultative deposit

feeding *Macoma balthica* showed intermediate accumulation between the filter feeding *Mytilus edulis* and the exclusively deposit feeding *Arenicola marina* (Kaag *et al.* 1997). Further, when exposed within a test vessel that also contained *M. edulis*, the facultative deposit feeding clam *M. balthica* increased its ingestion of sediment; resulting in a concurrent increase of contaminant accumulation (Kaag *et al.* 1997). Therefore, in the absence of contributions from aqueous phase uptake (i.e. in a desorption resistant compound such as DODMAC) variations in total uptake can potentially be governed by ingestion behaviours.

The specific particles that are ingested from a given sediment can also vary between species. Harkey *et al.* (1994) observed that the uptake of both benzo[a]pyrene and hexochlorobiphenyl by *Diporeia* spp. was greatest in a sediment consisting solely of particles between 20 and 63 μm in diameter in comparison to all other particle size fractions. The difference was not assessed statistically, but is in agreement with observations made by other authors that suggest this species exhibits extreme feeding selectivity (Kukkonen and Landrum 1995). The significance of feeding selectivity is highlighted by observations that chemicals can associate with different particle sizes within the sediment (Harkey *et al.* 1994, Kukkonen and Landrum 1995, Kukkonen and Landrum 1996). Organisms that preferentially ingest particles of a compound-specific size fraction will preferentially ingest that compound. Thus the potential of selective particle size feeding to modify contaminant exposure in amphipods is apparent. This behaviour is also reported in the clam *Macoma nasuta* that preferentially ingests fine organic enriched particles and does not ventilate pore water (Boese *et al.* 1990). Clearly, therefore, there is a need to properly quantify the actual sediment ingestion behaviours of each of the species exposed to DODMAC. These factors, along with other potentially influential characteristics, are investigated in Chapter 4.

2.4.1 Conclusions

The exposures described in this chapter have indicated apparent uptake of a desorption resistant compound from both artificial and field collected sediment. Further experimentation is required to discern whether this apparent uptake is representative of true assimilation (Chapter 3). Additionally, variation in apparent uptake between species was observed. The ingestion behaviour, as well as the variation in apparent DODMAC assimilation of the organisms, was not well predicted

within the test system. Interactions between organisms and sediment (both artificial and field collected) are implicated as potentially important influences upon uptake. Similarly the sorption of DODMAC in differing particle size fractions may result in differential exposure between species. Further investigation of factors influencing exposure and uptake are therefore explored in Chapter 4.

Chapter 3: Total Tissue loadings as a measure of true uptake

3.1 Introduction

The results presented in Chapter 2 suggest that aquatic invertebrate species achieve measurable tissue loadings of a desorption resistant compound via exposure to spiked sediment, a situation not accounted for by equilbrium partitioning (EqP) approaches (i.e. Di Toro *et al.* 1991) and contrary to desorption/bioavailability approaches (e.g. Lawrence *et al.* 2000, Kraaij *et al.* 2001). Exposures of *Gammarus pulex*, *Asellus aquaticus*, larval *Chironomus riparius* and *Lumbriculus variegatus* to sediment dosed with ^{14}C labelled Dioctadecyldimethyl ammonium chloride ([^{14}C]-DODMAC) all resulted in measurable tissue loadings.

Tissue loadings were derived using the amount of contaminant associated with the entire body of the study organisms. Whilst gut purging ensured the removal of the confounding influence of dosed sediment within the gut (after USEPA 1994, ASTM 1997 and reviewed by Mount *et al.* 1999) it may still be possible that dosed sediment could adhere to the outermost integuement, especially in *Gammarus pulex* and *Asellus aquaticus*. It is possible, therefore, that adhesion of the contaminant to outer body surfaces of the study organisms, rather than uptake into tissues, could account for all of the contaminant associated with organisms. If this were to be the case, then the results presented in Chapter 2 would not constitute a challenge to the precepts of aqueous desorbtion/bioavailability approaches.

Clearly, it is important to examine the proportion of the total tissue loading that is accounted for by material associated with external body surfaces. This is especially true when considering a desorption resistant compound such as DODMAC; since application of aqueous desorption theory to such a compound would suggest little risk of transfer from sediments into aquatic foodwebs. The objective of the work described in this chapter is to distinguish between true assimilation and surficial adsorption of material in apparent uptake of sediment-sorbed contaminants.

There are a number of aspects of the biology of the four study species that may be utilised to elucidate whether DODMAC is likely to be truly assimilated via ingestion. For example, it is possible to compare uptake in feeding and non-feeding

individuals in *Lumbriculus variegatus*. Leppanen and Kukkonen (1998a) noted that the onset of architomic reproduction (fragmentation) in *Lumbriculus variegatus* resulted in a cessation of sediment ingestion. This was found to be due to the regeneration of a new prostonium (at the anterior terminus of the posterior fragment) or a new tail section to the anterior fragment (Leppanen 1999). The ability to discern feeding and non-feeding individuals based upon their growth and reproduction status was utilised as a tool to separate uptake due to sediment ingestion from that due to exposure of the integuement to pore water (Leppanen and Kukkonen 1998b). Uptake due to ingestion was calculated as the tissue loading in feeding animals minus the tissue loading achieved in non-feeding animals (Leppanen and Kukkonen 1998b). This idea has been extended further by the deliberate production of non-feeding worms via the ablation of the apical segments encompassing the prostonium (Conrad *et al.* 2000).

Non-feeding *Lumbriculus variegatus* are simple to produce and may be used to compare uptake achieved via ingestion of sediment to the contribution of contaminant associated with outer body surfaces. Similarly, trials carried out at The University of Sheffield revealed that it is possible to prevent *Asellus aquaticus* individuals from ingesting sediment via a novel method (detailed in Section 3.2.2.2). However, no appropriate methodology to produce non-feeding *Chironomus riparius* or *Gammarus pulex* individuals could be established.

The three arthropod species *Chironomus riparius*, *Asellus aquaticus* and *Gammarus pulex* possess a shared biological characteristic that may be utilised to discern whether a compound is assimilated internally. All arthropods have a cuticle formed from a secreted integument containing the nitrogenous polysaccharide, chitin (Parker and Haswell 1972). Post-embryonic growth and development proceeds via a succession of moults (ecdyses) of this chitinous cuticle (Parker and Haswell 1972). In the crustaceans *Asellus aquaticus* (Isopoda) and *Gammarus pulex* (Amphipoda) morphological development is essentially complete upon hatching; apart from the development of the appendages of the eighth thoracic segment (thoracomere) in *Asellus aquaticus* (Schram 1986). This gives rise to very distinctively shaped exuviae upon moulting at all stages of development. Additionally, the two crustacean species have relatively bulky cuticles (when compared to the larval chironomid exoskeleton) due to the calcification of crustacean cuticle (Neville 1975). Chironomid larvae cuticles lack calcification (Neville 1975) and possess a much simpler external morphology (Oliver 1971, Armitage *et al.* 1995). These factors were found to produce highly visible and

easily identifiable exuviae in the crustacean species but highly cryptic exuviae from larval chironomids. Identifying and isolating larval chironomid exuviae was extremely difficult and consequently, the onset of pupation was used to indicate that moulting had occurred in this species.

The three arthropod species exposed in the experiments described in Chapter 2 periodically shed their entire outer body surfaces at clearly identifiable instances. Thus, following exposure to dosed sediments, tissue loadings of arthropod individuals can be determined in non-moulted individuals and individuals that have moulted subsequent to sediment exposure. This allows the determination of the amount of compound remaining within somatic tissues following the loss of the external body surfaces. However, due to the lack of moulting behaviour in Oligochaeta, this mechanism cannot be examined in *Lumbriculus variegatus*.

The final feature of the test system that may allow determination of true assimilation versus external adherence of DODMAC is the use of radiolabelled compound. If histological sections of the organisms can be successfully exposed to photographic emulsion, then it may be possible to visualise the distribution of [^{14}C]-DODMAC within the tissues of the organisms via established methods of autoradiography (e.g. Gahan 1972, Baker 1989).

Therefore the null hypothesis that external surficial adhesion accounts for most or all of the measured DODMAC tissue loadings was tested in a series of experiments utilising the characteristics noted above. The following predicted results would lead to rejection of the null hypothesis:

3i Organisms feeding on dosed sediment achieve tissue loadings significantly higher than individuals that are in contact with, but not feeding on, dosed sediment.

3ii In moulting species, the loss of exuviae from individuals previously exposed to dosed sediment does not reduce tissue loadings to a level equivalent to non-dosed tissues.

3iii Microautoradiographs of histological tissue sections demonstrate compound associated with internal tissues of organisms exposed to dosed sediment.

3.2 Materials and Methods

3.2.1 General methods

All sediment dosing was performed as described in Section 2.2.2.2. The DODMAC sample of 13.8 μCi mg^{-1} (Section 2.1.2.2) along with the artificial sediment (Section 2.2.2.1) were used throughout for all exposures addressing the three predictions detailed in this chapter. Sediment loading of DODMAC was 4.3 μg DODMAC per g dry weight of sediment in all cases. The identity, source and husbandry of the four test organisms are given in Section 2.2.1. Pre-and post-dosing test vessel preparation and overlying water medium is detailed in Section 2.2.2.3. However, the number and size of test vessels vary between the exposures performed in this chapter and are detailed individually. Similarly, whilst all exposures terminate with the addition of organisms to gut purge vessels (Section 2.2.2.4), the numbers of organisms allocated to each gut purge vessel and the subsequent duration spent within these vessels varied between exposures. Details of organism number and duration within gut purge vessels are given individually for each exposure. All exposures and gut purges were carried out at 20°C ± 2°C apart from the gut purging occurring during the transport of organisms for microautoradiography (Section 3.2.2.8). During transport the temperature approximated to 20°C but was not formally regulated.

3.2.1.1 Tissue analysis via liquid scintillation counting (LSC)

Tissues analysed via liquid scintillation counting (LSC) were processed as described in Section 2.2.3.3 and counted as described in Section 2.2.3.4. The collection of post gut-purge tissue varied between exposure regimes and is detailed individually.

3.2.1.2 Tissue analysis via Microautoradiography

All microautoradiography was carried out at Unilever Research Colworth (Unilver R&D Colworth, Sharnbrook, Bedford, MK44 1LQ) by Helen Minter. Tissues were frozen in liquid nitrogen, sectioned sagitally (*G. pulex* and *A. aquaticus*) or dorso-ventrally (*L. variegatus* and *C. riparius*) at 8 μm thickness and stained prior to

exposure to photographic emulsion according to standard methodologies (e.g. Gahan 1972 and Baker 1989). The specific procedure adopted is given in Appendix 3.1.

3.2.2 Specific methods for each exposure regime

3.2.2.1 Influence of feeding upon tissue loading

a.) Production of non-feeding organisms

Non-feeding worms were produced via the removal of the apical (mouthpart) segments. This was performed using a scalpel with worms held in a small amount of artificial pond water (APW, Section 2.2.1.1) within a petri dish (after Conrad *et al.* 2000). Non-feeding *Asellus aquaticus* (Fig. 3.1) were produced by masking the mouthparts with 3-5-mm wide x 25-mm strips of stretched parafilm "M" (American National Can™, Chicago IL. 60631), secured behind the cephalic region with cyanoacrylate based glue ("Super Glue", UHU (UK.) Ltd. 551 London Road, Isleworth, Middlesex, TW7 4DS).

b.) Pre-exposure

Groups of ten worms (either feeding or non-feeding) were held within 20-ml scintillation counting vials containing approximately 15 ml of APW overnight at 20°C. Each vial was labelled according to the feeding or non-feeding nature of the organisms within. In total there were twenty vials containing ten non-feeding worms and forty vials each containing ten feeding worms.

After securing the parafilm mask, ten non-feeding *Asellus aquaticus* individuals were added to a 600-ml beaker of APW. Two groups of ten feeding *Asellus aquaticus* individuals were held simultaneously in two further identical beakers. The organisms were retained in the beakers at 20°C for approximately 2 h prior to the initiation of the experimental exposure.

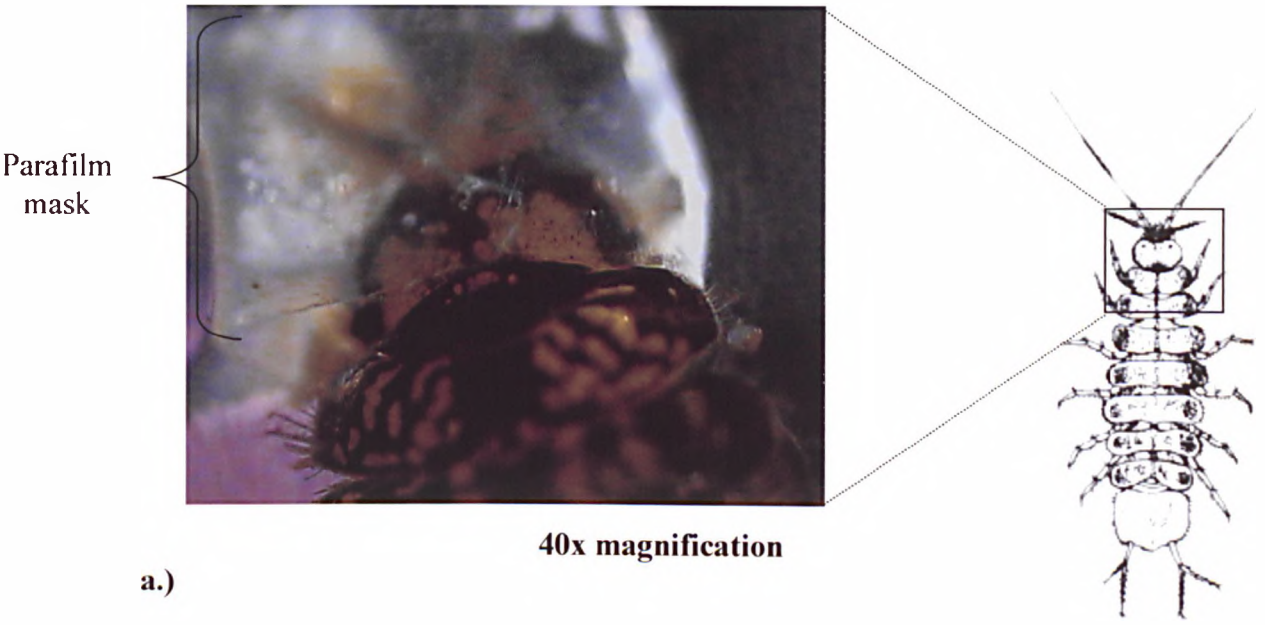


Fig. 3.1 *Asellus aquaticus* "non-feeding manipulation" (a) close-up and (b) whole organism

c.) Exposure

Both *Lumbriculus variegatus* and *Asellus aquaticus* tissue loadings were compared between feeding and non-feeding individuals resulting from exposure to sediment bound DODMAC. Feeding and non-feeding organisms were concurrently exposed to 4.3 μg per g [^{14}C]-DODMAC dosed sediment. A third group of organisms (feeding) were also exposed to non-dosed sediment to determine background tissue radioactivity.

Lumbriculus variegatus were exposed in 200-ml glass jars containing 30 g artificial sediment and 100 ml APW (Section 3.2.1). Twenty vials each containing ten feeding worms and 20 vials each containing ten non-feeding worms were each added to one of 40 dosed sediment vessels. Such additions were performed in an arbitrary sequence. Twenty vials each containing ten feeding worms were also emptied into each of 20 non-dosed sediment vessels. After 48 h, organisms were recovered from sediment using plastic pasteur pipette and added to gut purge vessels for 24 h. All of the organisms recovered from each individual exposure vessel were transferred communally to one of sixty gut purge vessels. Each individual gut purge vessel was labelled with the organism feeding status during the exposure as well as the dosed or non-dosed nature of the sediment. The total tissue contents of each gut purge vessel comprised a single tissue sample. Each tissue sample was analysed via liquid scintillation counting (LSC, Section 3.2.1.1).

Asellus aquaticus were exposed using two plastic 20-cm diameter vessels each containing 60 g of artificial sediment and 200 ml of APW (Section 3.2.1). Exposure of feeding and non-feeding *Asellus aquaticus* was initiated via the addition of 10 feeding and 10 non-feeding individuals to a single vessel containing dosed sediment. The remaining ten feeding organisms were added to the non-dosed sediment vessel. Therefore the exposure to sediment lacked replication. The individual organisms were treated statistically as if they were independent, however the fact that tissue loadings of "feeding" and "non-feeding" individuals were derived from one exposure vessel must be borne in mind. At the end of the 48-h exposure period, the parafilm masks were loosened and removed using a mounted needle before organisms were placed in gut-purge vessels for 24 h. Individual animals were placed in each gut purge vessel and their exposure feeding status as well as dose or non-dose sediment exposure was

recorded via labelling of the gut purge vessels. The contents of each gut purge vessel (i.e. individual organisms) were analysed by LSC (Section 3.2.1.1).

3.2.2.2 Influence of moulting upon tissue loading

Groups of 30 individuals of the three arthropod test species (*Gammarus pulex*, *Asellus aquaticus* and *Chironomus riparius*) were exposed to artificial sediment dosed with DODMAC (Section 3.2.1). Organisms were exposed communally in plastic pots measuring 20 cm in diameter and 6 cm depth. Concurrently groups of 15 individuals of each species were exposed to non-dosed sediment in order to determine background tissue radioactivity. Dry artificial sediment was allocated in 90-g batches to each of the three dosed exposure vessels and three non-dosed exposure vessels. Overlying APW (250 ml) was added along with the appropriate test organisms to initiate the test (Section 3.2.1). Exposure periods of 48 h were terminated by transferring organisms to individual gut purge vessels (Section 2.2.2.4). Each gut purge vessel held only one organism. Gut purge vessels were labelled with the dosed or non-dosed status of the sediment to which the organisms within were exposed. Individual organisms were retained within the individual gut purge vessels to await moulting (identified via pupation in *Chironomus riparius*).

Observation of moulting resulted in the removal of moulted individuals along with a non-moulted counterpart for tissue analysis (i.e. moulted and non-moulted pairs were removed at the same timepoint following the termination of exposure to dosed sediment). For each day that at least one pair of moulted and non-moulted organisms were collected from dosed sediment exposure, a non-dosed organism was also sampled in order to derive background tissue radioactivity. Individual organisms collected from each gut purge vessel were analysed by LSC (Section 3.2.1.1). In addition the exuviae from moulted *Asellus aquaticus* and *Gammarus pulex* were also collected and analysed in the same way. No organisms moulted within 24 hours of the exposure to sediment, therefore at least 24-h gut purging occurred in all cases. Collection of moulted/pupated individuals was completed within 14 days of the termination of exposure to artificial sediment. Organisms that could not clearly be identified as moulted, or that did not survive were disregarded after 14 days. Again, the comparison between post-exposure "moulted" and "non-moulted" individuals resulted from exposure within the same (non-replicated) exposure vessel. The results of statistical

analyses that assume independence between samples should therefore be interpreted in this light.

3.2.2.3 Visualisation of DODMAC distribution

Both *Asellus aquaticus* and *Gammarus pulex* were exposed in groups of 10 to sediment-bound DODMAC for 48 h using plastic pots measuring 20 cm in diameter and 6 cm in depth. Each pot contained 90 g dry weight of artificial sediment and 250 ml overlying water (Section 3.2.1). Groups of 20 *Chironomus riparius* and *Lumbriculus variegatus* were exposed using 200-ml glass jars containing 30 g dry weight of dosed sediment and 100 ml overlying APW. One exposure vessel per species was used.

At the end of the 48-h exposure, all animals were recovered from the sediment and placed into gut purge vessels modified from those described in Section 2.2.2.4. One gut purge vessel per species was used and was labelled to identify the species contained within. The substrate and overlying water used was the same as previously described, but the glass jars were of one litre capacity rather than 200 ml. Overlying APW was added to a volume of approximately 300 ml and screw caps were used to seal the vessels. The 700-ml headspace capacity above the overlying water provided sufficient oxygen to maintain the organisms during transport to Unilever Research Colworth (Unilver R&D Colworth, Sharnbrook, Bedford, MK44 1LQ) for autoradiography. Sealed gut purge vessels were placed in a polystyrene "cool box" and held at approximately 20°C during the 3-4-h road transportation.

After transportation, screw caps were removed from the gut purge vessels to facilitate organism ventilation. Organisms were inspected to confirm that the gut purge duration was sufficient to secure the absence of sediment within the digestive tract. Having satisfied this condition, organisms were removed from the gut purge vessels. Once removed from gut purge vessels, the organisms were frozen using either immersion in liquid nitrogen or by placing upon a frozen glass plate before storage at -80°C. For *Chironomus riparius*, *Asellus aquaticus* and *Gammarus pulex*, immersion in liquid nitrogen was utilised. This was achieved by submerging each organism within a foil "boat" inside a flask of liquid nitrogen. Conversely, *Lumbriculus variegatus* individuals were placed upon a glass microscope slide that rested on top of a bed of "dry ice" pellets to achieve freezing in a flat plane. Microautoradiography was used to

visualise the distribution of DODMAC within the tissue samples (Section 3.2.1.2 and Appendix 3.1).

3.2.3 Statistical analyses

A number of the datasets generated during this study contained data that were not suitable for parametric analyses. Since the data could not be suitably transformed in these instances, non-parametric tests were applied. However, where appropriate, parametric analyses were used. Pairwise analyses were applied to experiments comparing moulted to non-moulted individuals sampled at the same timepoint. One-tailed analyses were applied where tissue loadings in moulted (dosed sediment exposed) individuals were predicted to be greater than non-dosed tissue loadings. Similarly, one-tailed analyses were applied where feeding individuals were predicted to achieve higher tissue loadings than non-feeding individuals exposed to dosed sediment. Two tailed analyses were applied where the direction of difference was not specifically predicted in advance (i.e. comparison of moulted and non-moulted organisms exposed to dosed sediment).

3.3 Results

3.3.1 Influence of feeding upon tissue loading

The null hypothesis that tissue loadings consist solely of externally adhering DODMAC would be rejected if organisms feeding on dosed sediment achieved tissue loadings significantly higher than individuals that are in contact with, but not feeding on, dosed sediment (Prediction 3i).

Feeding was manipulated using *Lumbriculus variegatus* and *Asellus aquaticus*. The activity levels and physical contact with the sediment did not appear to differ between feeding and non-feeding individuals in either species. Feeding individuals of both species achieved much greater tissue loadings than non-feeding individuals (*Lumbriculus variegatus*; $t_{19} = 9.45$, $p<0.0001$, *Asellus aquaticus*; $U_{10, 10} = 3$; $p<0.001$). Further, DODMAC loadings measured in non-feeding individuals indicated that only a mean loading of $0.00314 \mu g g^{-1}$ in *Lumbriculus variegatus* (Fig. 3.2) and a median of $0.0694 \mu g g^{-1}$ in *Asellus aquaticus* (Fig. 3.3) was associated with external body surfaces.

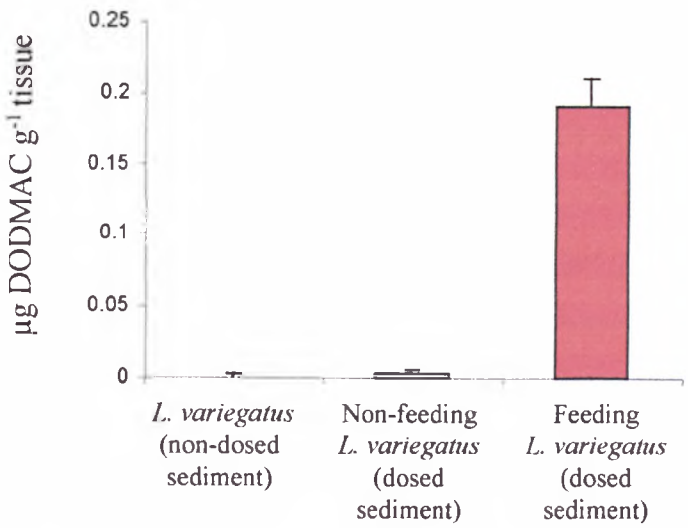


Fig. 3.2 Mean (+SE) DODMAC tissue loading in feeding and non-feeding *Lumbriculus variegatus* exposed to dosed sediment with reference to feeding organisms exposed to non-dosed sediment

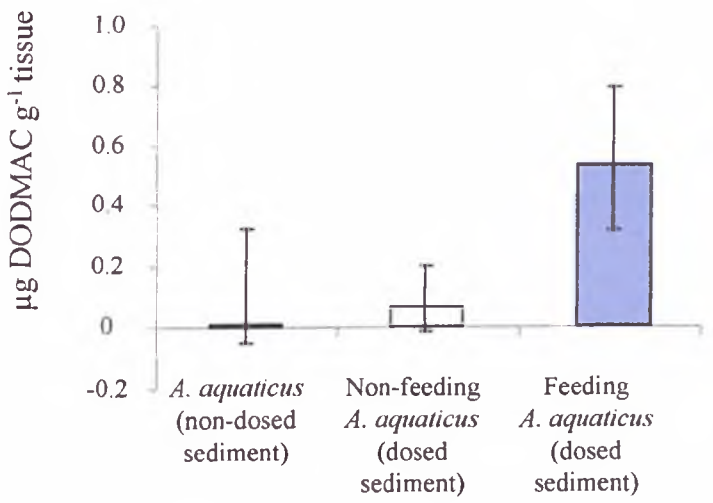


Fig. 3.3 Median (± interquartile range) DODMAC tissue loading in feeding and non-feeding *Asellus aquaticus* exposed to dosed sediment with reference to feeding organisms exposed to non-dosed sediment

3.3.2 Influence of moulting upon tissue loading

The null hypothesis that tissue loadings consist solely of externally adhering DODMAC would be rejected, in moulting species, if the loss of exuviae from individuals previously exposed to dosed sediment does not reduce tissue loadings to a level equivalent to non-dosed tissues (Prediction 3ii).

The median tissue loading remaining in moulted individuals of *Gammarus pulex*, *Chironomus riparius* and *Asellus aquaticus* exposed to dosed sediment are all significantly greater than that in non-dosed tissues ($U_{22,10} = 18$; $p<0.001$; $U_{18,10} = 26$; $p<0.005$; and $t_{11} = 4.07$; $p<0.01$ respectively). This indicates genuine internal assimilation of DODMAC in these species (Figs. 3.4 and 3.5).

Post exposure *Gammarus pulex* and *Chironomus riparius* individuals showed a reduction in tissue loading in moulted individuals when compared to non-moulted individuals sampled at the same timepoint (Wilcoxon signed-rank test: $T-<T_{0.005\,(1),\,22}$; $p<0.005$ and Wilcoxon signed-rank test: $T-<T_{0.0005\,(1),\,18}$; $p<0.0005$ respectively). However, no such difference was detected between moulted and non-moulted Asellus aquaticus individuals ($t_{12}= 2.09$, $p=0.06$).

3.3.3 Visualisation of DODMAC distribution

The null hypothesis that tissue loadings consist solely of externally adhering DODMAC would be rejected if microautoradiographs of histological tissue sections demonstrated compound associated with internal tissues (Prediction 3iii). Figures 3.6 – 3.9 show longitudinal sections through each of the study species and in all cases radiolabelled compound can be seen associated with internal tissues. In *Chironomus riparius*, DODMAC can clearly be seen associated both with the gut lining and also the underlying musculature (Fig. 3.6). In *Lumbriculus variegatus*, the most obvious concentrations of the compound appear to be associated with the musculature of the gut as well as the muscles attached to the inner surfaces of the external cuticle (Fig. 3.7). In addition to the longitudinal section there is also a fortuitous transverse section through a

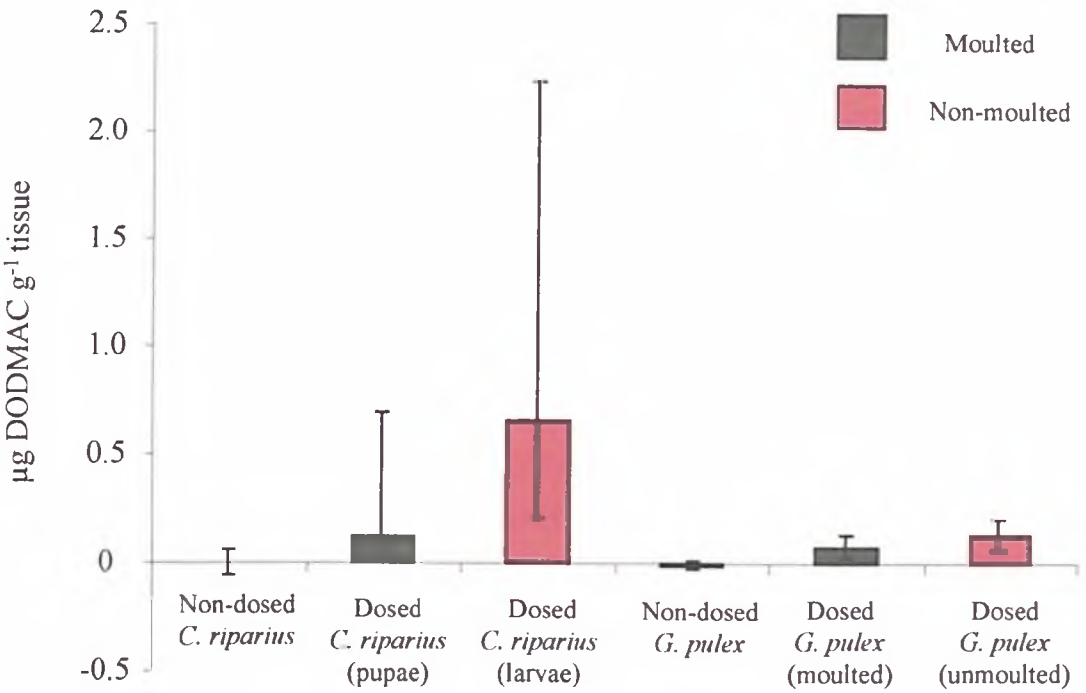


Fig. 3.4 Median (± interquartile range) DODMAC tissue loading in moulted and non-moulted *Chironomus riparius* and *Gammarus pulex.*

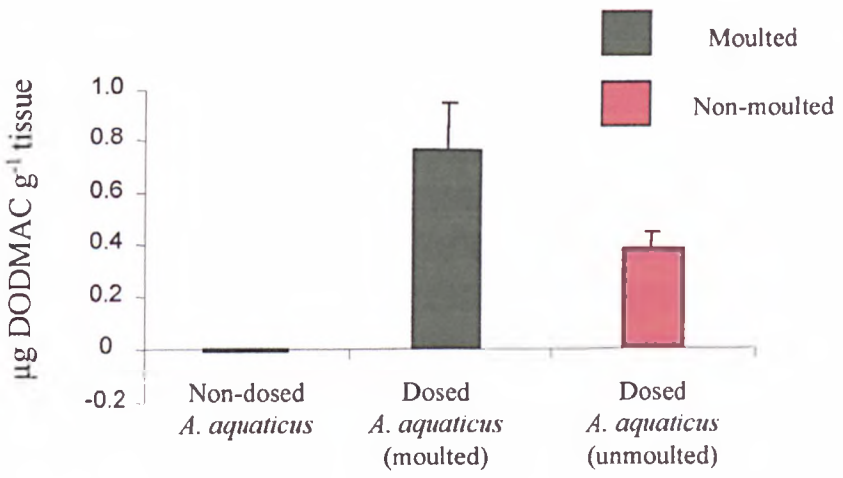


Fig. 3.5 Mean (+SE) DODMAC tissue loading in moulted and non-moulted *Asellus aquaticus.*

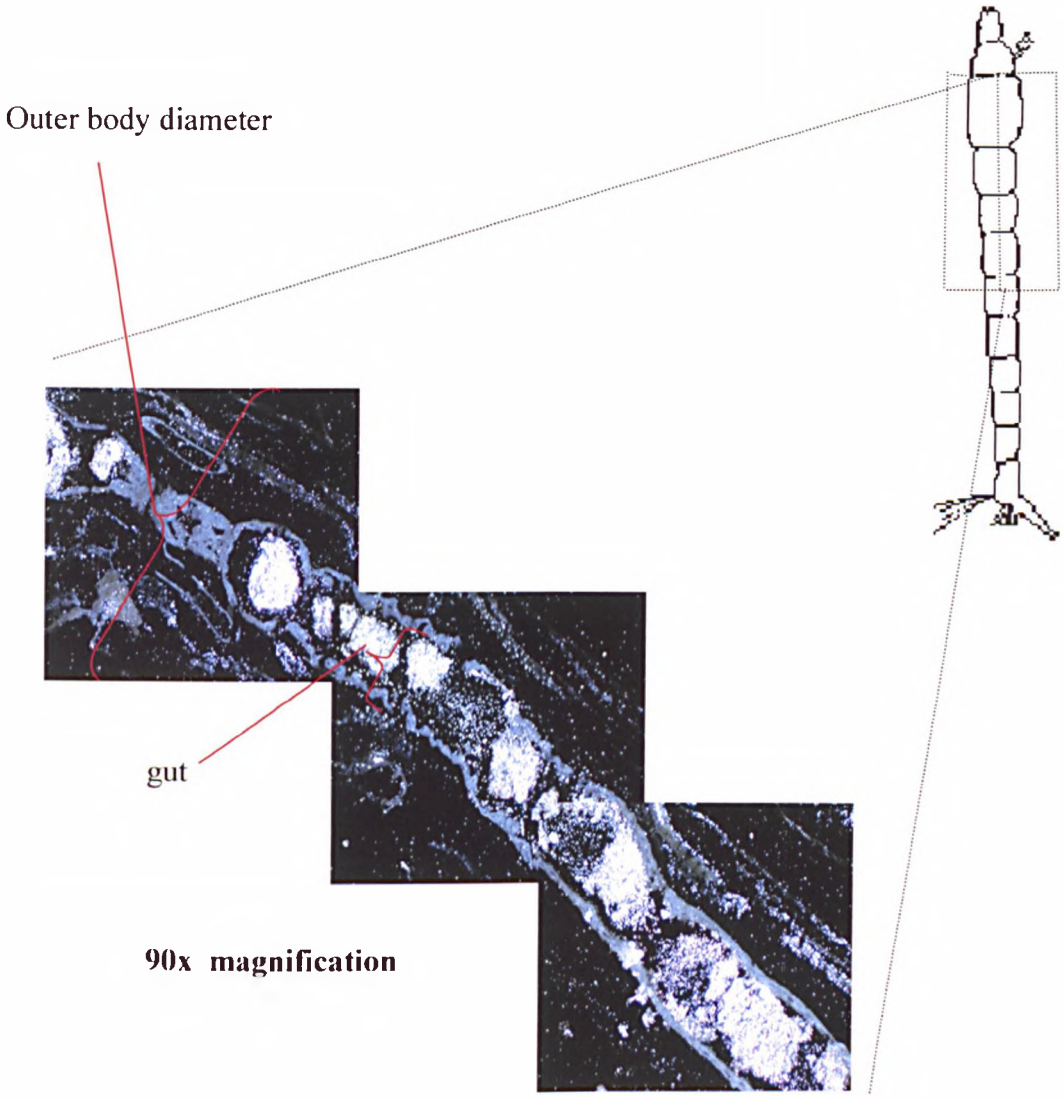


Fig. 3.6 Autoradiograph of a dorso-ventral longitudinal section taken along the centre line of a larval *Chironomus riparius* exposed to artificial sediment dosed with ^{14}C-DODMAC. Silver grained areas correspond to radioactive disintigrations.

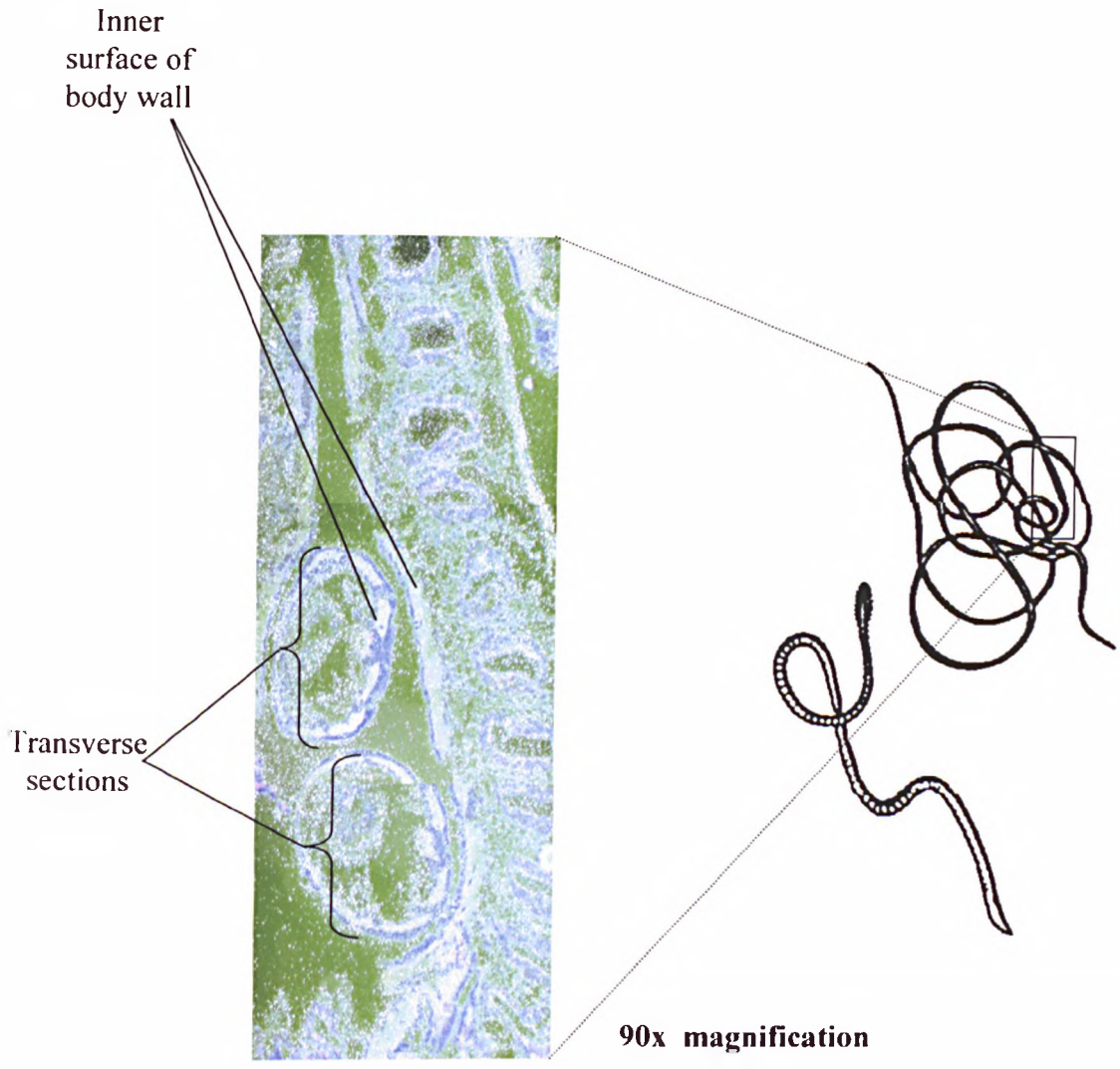


Fig. 3.7 Autoradiograph of a dorso-ventral longitudinal section taken along the centre line of *Lumbriculus variegatus* exposed to artificial sediment dosed with ^{14}C-DODMAC. Silver grained areas correspond to radioactive disintigrations.

"u" shaped loop that appears in the lower left corners of Fig. 3.7. Again this shows that DODMAC appears to associate with the solid internal masses more readily than the gut lumen or haemocoel in *Lumbriculus variegatus.*

In *Gammarus pulex* there is some distortion of the section as the telson is compressed somewhat so as to be pushed toward the pleopod on the ventral side of the organism (Fig. 3.8). The greatest visible concentration of the compound appears to be associated with the external cuticle, although there is some compound associated with internal tissues. Unfortunately, problems with handling and exposure of *Gammarus pulex* sections mean that only one section was successfully exposed and developed. It is possible that sections more comprehensively encompassing internal anatomy would detect localised concentrations of compound with greater clarity.

Interestingly there is a very clearly identifiable concentration of radioactivity associated with the surface of lobes of the hepatopancreas (or midgut gland) of the *Asellus aquaticus* specimen section (Fig. 3.9).

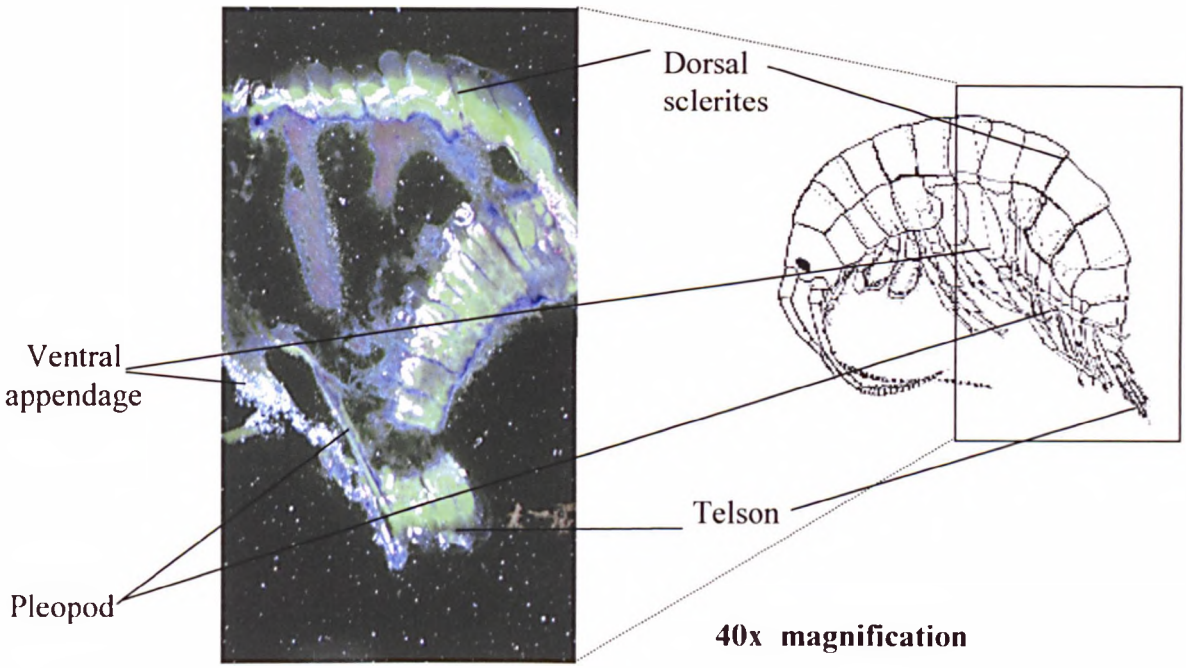


Fig. 3.8 Sagittal autoradiograph section of *Gammarus pulex*. Section does not include gut cavity. Majority of detected radioisotope appears to be associated with external structures

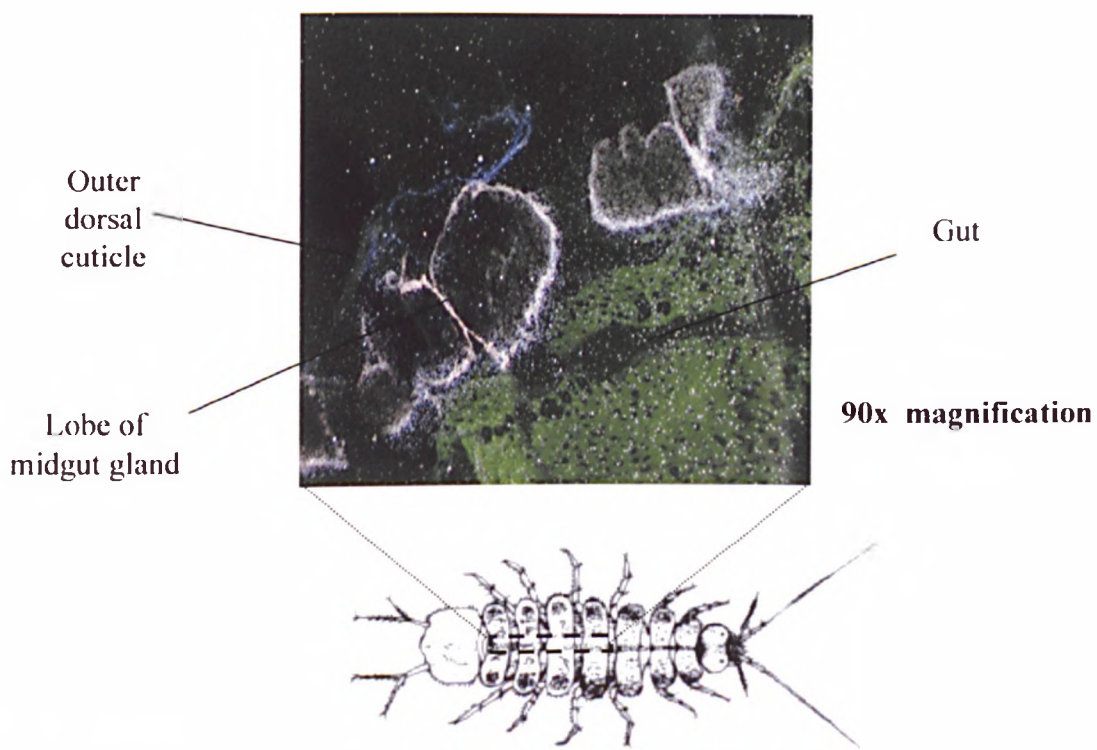


Fig. 3.9 Sagittal autoradiograph section of *Asellus aquaticus*. Concentrations of radioactivity clearly associated with lobes of midgut gland

3.4 Discussion

The results of each experiment described in this chapter contradicted the null hypothesis that surficial adhesion of the test compound accounts for the measured tissue loadings of benthic organisms exposed to sediment-bound DODMAC. In the exposures of feeding and non-feeding *Lumbriculus variegatus* there is very clear evidence that it is impossible for surficial adhesion to account for the tissue loadings observed in Chapter 2. Additionally, the results show the importance of ingestion in assimilation of sediment-bound compounds. Indeed, the subtraction of non-feeding tissue loadings from feeding *Lumbriculus variegatus* tissue loadings (after Leppanen and Kukkonen 1998b) indicates that assimilation is almost entirely due to ingestive sources. In *Asellus aquaticus* the available data also support the notion that, in the exposure reported here, feeding on dosed sediment was responsible for the majority of the measured DODMAC tissue loadings. Thus in two of the four species exposed in Chapter 2 there is evidence that DODMAC is truly assimilated and that ingestion of contaminated sediment appears to mediate this assimilation.

The evidence for genuine assimilation of DODMAC in all species is further supported by the results of Section 3.3.2. The act of moulting did not reduce the tissue loading to a level equivalent to that of non-dosed tissues. In *Asellus aquaticus*, there may even be a tendancy for the tissue loading to become more concentrated following the loss of the cuticle, although this is not fully elucidated by the available data. The fact that moulting (post-exposure) did not reduce tissue loadings is particularly compelling in the light of the derivation of gut cuticle in *Asellus aquaticus*. Unlike many other crustacea, the ectodermally derived foregut and hindgut meet, and the endodermal "midgut" contribution to the digestive system is confined to the caeca (Schram 1986). Therefore, almost the entire alimentary passage is lined with ectodermally derived cuticle that is moulted at ecdysis. Consequently, with the loss of moulted cuticle, all remaining DODMAC must be associated with internal organs and tissues. Such DODMAC must have been stripped from sediment particles, transported across the gut lining and transferred to internal tissues within the 72 hours between initiation of exposure and termination of gut purge. Of the three arthropod species, *Gammarus pulex* has the smallest proportion of the gut lining made up of ectodermal cuticle; having only limited ectodermal invaginations at the extremeties of the fore and hindgut (Schram 1986). In *Chironomus riparius* larvae, the proportion of ectodermal

cuticle lining the alimentary canal is greater than in *Gammarus pulex*, but less than that of *Asellus aquaticus* (Wigglesworth 1965, Schram 1986).

The use of autoradiography could potentially offer greater insight regarding the internal distribution of the compound. In general, the visualisation of DODMAC further supported the geniune assimilation of compound, especially in *Asellus aquaticus*, *Chironomus riparius* and *Lumbriculus variegatus*. In these three species, compound could be easily identified as associating with either internal musculature or organs. However, the available images of *Gammarus pulex* sections were not able to identify DODMAC distribution with the same clarity. Therefore, whilst it is clear that all species strip compound from sediment particles and retain tissue loadings that are too great to be accounted for by adhesion to external integuement; a comprehensive account of the distribution within *Gammarus pulex* tissues is not discernable from these results.

These observations contradict the predictions of a lack of bioavailability in desorption resistant compounds yielded by desorption/bioavailability studies (e.g. Lawrence *et al.* 2000, Kraaij *et al.* 2001). Further, they are in much closer agreement to kinetic modelling approaches (e.g. Landrum and Robbins 1990) as well as experimental measurements of contribution of ingestive uptake to tissue loadings (Kaag *et al.* 1997, Wang and Chow 2002). The results support the notion that reduced representation of a compound within aqueous fractions e.g. via high K_{ow} values (Landrum and Robbins 1990) or biotic removal from overlying water (Kaag *et al.* 1997), increases the contribution of ingestion to assimilation. In turn, this highlights the difference between a compound's propensity to desorb from sediment particles into pore water compared to the propensity to desorb within the gut environment. Again, this is in agreement with studies that highlight the efficacy of gut fluids and the gut environment in extracting a range of sediment-bound compounds (Mayer *et al.* 1996, Weston and Mayer 1998, Ahrens *et al.* 2001, Mayer *et al.* 2001, Griscom *et al.* 2002).

3.4.1 Conclusions

The prediction of total bioavailability from the propensity to desorb into aqueous media appears to be not universally correct. Comparisons between feeding and non-feeding, as well as moulted and non-moulted organisms indicate the genuine assimilation of DODMAC. Similarly, autoradiographic visualisation of DODMAC

within tissue sections of all four species also indicates assimilation of radiolabelled compound. Therefore, the experiments reported in this chapter support the conclusion that tissue loadings measured in Chapter 2 were indicative of genuine assimilation of sediment-sorbed DODMAC.

Chapter 4: Examination of factors influencing interspecific variation in DODMAC uptake

4.1 Introduction

This chapter aims to examine a number of differences between organisms that may affect the assimilation of sediment-sorbed compounds. The tissue loadings measured in the experiments described in Chapter 2, and confirmed in Chapter 3, suggest variation in the degree of assimilation of DODMAC between the four invertebrate species exposed to this compound. Several intrinsic factors could potentially result in interspecific variation in the bioaccumulation of organic compounds including differences in behaviour (e.g. selective feeding), physiology (e.g. metabolic enzymes, gut retention and chemistry) and morphology (e.g. tissue lipid content). Such factors are considered subsequently. However, the first factor to receive consideration below examines whether differential uptake rates (howsoever achieved) could be responsible for this pattern of tissue loadings.

4.1.1 Differential uptake rate

With reference to Equation 4.1 (taken from Lee 1992), it is apparent that there is the potential for interspecific variation in the trajectory of uptake curves modelled by kinetic approaches.

$$\frac{dCt}{dt} = \sum \left(Fs \times CPs \times EPs\right) - E - GD \qquad \textbf{Eq. 4.1}$$

where:

$\frac{dCt}{dt}$ = weight specific change in tissue residue with time: µg/(g tissue x time)

Fs = weight specific flux of sediment through organism: g/(g tissue x time)

CPs = concentration of compound in sediment: µg/g

EPs = fraction of pollutant extracted (assimilated) from sediment (decimal fraction)

E = elimination rate of compound from depuration of the parent compound and metabolic degradation: µg/(g tissue x time) and

GD = growth dilution: µg/(g tissue x time)

Also, the uptake rate term (*Fs x CPs x EPs*) of a specific compound in a particular organism does not, necessarily, reflect the final tissue loading of that compound (e.g. van Leeuwen and Hermens 1995). Therefore, given that the tissue loadings of DODMAC in each of the four species exposed in Chapters 2 and 3 were achieved in 48-h exposures, it is possible that the rate of uptake could be responsible for the differences in tissue loadings. From 48-h exposures to artificial sediment it was not clear whether, with increased exposure duration, the apparent difference between tissue loadings would disappear. Additionally, dramatic increase in 48-h DODMAC tissue loadings of *Asellus aquaticus* and *Lumbriculus variegatus* in 48-h exposures via dosed field collected sediment were noted (Chapter 2). It is possible that assimilation from field collected sediment in *Asellus aquaticus* and *Lumbriculus variegatus* follows a far steeper trajectory of tissue loading through time. The specific cause of the interspecific variation in trajectory of tissue loading with time may not be simple to determine. Nonetheless, there is utility in determining that difference in assimilation rate, rather than an overall capacity for assimilation, is sufficient to produce the pattern of tissue loadings achieved in 48 h of exposure under standardised conditions. Potential mechanisms mediating the trajectory of uptake through time, or the ultimate tissue loading, are also considered below.

4.1.2 Behaviour

Interspecific differences in behaviour may result in variation in exposure to environmental contaminants. Sediment-dwelling organisms can feed on specific particle size ranges and consequently sediment particles ingested by benthic invertebrates do not, necessarily, have the same particle-size distribution as bulk sediment (Klump *et al.* 1987, Harkey *et al.* 1994, Kukkonen and Landrum 1996). In addition to selectivity in particle sizes, organisms may also exhibit selectivity for the organic content of ingested particles (Kukkonen and Landrum 1995, Kukkonen and Landrum 1996, Rodriguez *et al.* 2001). Feeding preferences will affect the fractions of sediment ingested by different species. This is potentially important as sediment

contaminants can associate with specific fractions depending on both the size range and the organic content of particles (Harkey *et al.* 1994, Kukkonen and Landrum 1995, Kukkonen and Landrum 1996). Therefore, the interactions between contaminant distribution and the selective ingestion of specific sediment fractions can substantially influence the exposure to sediment contaminants experienced by benthic organisms. A further potential form of particle selectivity is the detection and avoidance of contaminant-enriched particle fractions. Millward *et al.* (2001) found that tubificid worms altered their particle-size preference when exposed to sediments with a contaminant loading that exceeded a threshold level. Faeces collected from worms exposed to sediment-sorbed pyrene above this threshold level contained significantly fewer fine particles (<3.5μm) than worms exposed to control sediment or sediment containing lower pyrene loadings (Millward *et al.* 2001). Faeces from organisms exposed above the identified threshold level also contained more medium sized (3.5 - 43μm) particles than all other groups (Millward *et al.* 2001). This was linked to a reduction in biota-sediment accumulation factor (BSAF, the ratio of assimilated compound to sediment loading) in organisms exposed above the threshold loading level (Millward *et al.* 2001). Therefore, if species vary in their ability to detect contaminant loading or their ability to effect particle selectivity, there may be interspecific variation in amelioration of contaminant tissue loading by what could be termed "avoidance selectivity".

Therefore, even when considering a standardised sediment dosed with a single compound, there is potential for interspecific variation in exposure to that chemical mediated by feeding selectivity based on particle-size, particle organic quality or particle contaminant loading; or any combination of all three. However, for feeding selectivity to operate, the contaminant must show a differential association between specific particle fractions.

4.1.3 Tissue Morphology

Lipophilic sediment contaminants, such as DODMAC, are often assumed to reside primarily within tissue lipids following assimilation (e.g. Mackay 1982, Di Toro *et al.* 1991, Lee 1992). It has been suggested that tissue lipid content may limit the maximum tissue loading of such contaminants, with increased lipid content corresponding to higher tissue loadings of lipophilic compounds (e.g. Mackay 1982,

Landrum and Robbins 1990, Di Toro *et al.* 1991, Jager 1998). Thus, variation in lipid content between species may drive the differences in DODMAC tissue loadings reported in Chapter 2.

In contrast to quantifying the tissue-fraction considered likely to accommodate lipophilic compounds (i.e. lipid content), it may be possible that a "non-accommodating" fraction within tissues could indirectly influence DODMAC tissue loading. The use of dry-weight measurements in the determination of contaminant tissue loadings has been suggested to reduce measurement variability (Lee 1992). Additionally, since the solubility of DODMAC in water is likely to be "vanishingly small" (Laughlin *et al.*1992), tissue water content potentially represents the proportion of tissue that is unavailable for DODMAC sequestration. Hence, variation in DODMAC tissue loadings may be inversely related to tissue water-content.

For each species exposed to DODMAC, measurements of tissue lipid and water could be compared to the tissue loading of the study compound. Such comparisons could be used to examine a potential relationship between DODMAC tissue loading and two factors that appear likely to influence the inherent storage capacity of organism tissue. A positive relationship would be expected between lipid content and DODMAC loading. Conversely, water content would be expected to be negatively related to the tissue loading of DODMAC in each species.

4.1.4 Physiology

(a) Biotransformation

Organisms, including invertebrates, that assimilate synthetic organic compounds from the environment may be able to metabolise parent compounds to produce smaller, more water-soluble and hence more easily excretable metabolites (van Leeuwen and Hermens 1995). Examples of the biotransformation of organic compounds in benthic invertebrates include the metabolism fluorene by the sediment-dwelling copepods Schizopera knabeni and Coullana sp. (Lotufo 1998) and anthracene by the amphipods *Diporeia sp.* (Landrum 1982) and *Hyalella azteca* (Landrum and Scavia 1983). However, not all species metabolise organic compounds to the same extent or at the same rate. For example, exposures of larval *Chironomus riparius*, *Hyalella azteca* and *Lumbriculus variegatus* to the organophosphate pesticides diazinon, chlorpyrifos and azinphos-methyl elicited differing degrees of

biotransformation (Ankley and Collyard 1995). Whereas both *H. azteca* and *Chironomus riparius* were able to metabolise each of these pesticides via a cytochrome P450 pathway, *Lumbriculus variegatus* could not (Ankley and Collyard 1995). Similarly, whereas exposure of larval *Chironomus riparius* to sediment-bound benzo[a]pyrene resulted in body burdens comprised of 83% metabolites and 17% parent compound, exposure of the mollusc, *Sphaerium corneum,* under the same conditions resulted in body burdens comprised of 26% metabolites and 74% parent compound (Borchert *et al.* 1997). Variation in ability to biotransform compounds is also evident between species belonging to the same taxonomic group. For example, the two crustaceans *Hyalella azteca* and *Diporeia sp* are both able to biotransform fluorene but *H. azteca* has a greater metabolic capability than *Diporeia* (Kane-Driscoll and Landrum 1997). The findings of the studies cited above serve to highlight both the occurrence of, and the variation in, biotransformation of organic contaminants by benthic invertebrates. This implies that the potential exists for species to vary in their capability to reduce tissue loadings of an assimilated compound via biotransformation pathways.

(b) Gut residence time

The results reported in Chapter 3 suggest that DODMAC is absorbed across the gut wall; comparisons of feeding and non-feeding individuals of both *Lumbriculus variegatus* and *Asellus aquaticus*, as well as autoradiography of histological sections, indicate ingestive assimilation. Understanding factors that influence the efficiency of such assimilation could potentially explain differences in tissue loadings achieved in different species. One such factor is the duration that ingested material spends within the gut of aquatic organisms. Bioenergetic-based toxicokinetic models of contaminant uptake due to sediment ingestion (e.g. cited in Lee 1992) suggest that increasing the throughput of sediment per unit time increases the amount of compound assimilated as illustrated by Equation 4.1 (taken from Lee 1992). Assuming other terms remain constant, increases in the term "*Fs*" (sediment flux or throughput rate) are expected to yield increases in tissue loading by this model. An increase in gut throughput rate is synonymous to a decrease in the residence time within the gut. Hence, a decrease in "gut residence time" would seem to predict an increase in overall assimilation occurring via the gut. Instances of a positive relationship between nutrient assimilation

rate, as well as contaminant assimilation rate, and gut residence time are documented. For example, Boyce *et al.* (2000) report an increase in both nutrient assimilation efficiency and assimilation rate with a concurrent decrease in measured gut residence time. Similarly, for contaminant assimilation by the oligochaete *Limnodrilus hoffmeisteri*, Millward *et al.* (2001) observed an 80% reduction in egestion rate that corresponded to a reduction in total assimilation of pyrene from sediment. If a slowing of egestion rate equates to greater durations of gut residence time, it would appear that increased gut residence time results in a decrease in total assimilation. However, Millward *et al.* (2001) did not quantify the relationship between gut residence duration and egestion rate. In this example a reduction in egestion rate could simply reflect an intermittant cessation of feeding (and hence faecal pellet production), rather than a slowing of the speed of passage through the gut. However, both of these examples highlight the importance of the assimilation efficiency term ("*EPs*" in the example of Equation 4.1) to overall uptake.

In contrast to studies that find variation in assimilation efficiency apparently varying inversely with gut residence time, there are also examples of the opposite relationship. An increase in the proportion of food digested was measured within larval fish with reduced gut passage velocity (i.e. with increased gut residence time) when feeding upon crustacean zooplankton (Johnston and Mathias 1996). The potential for gut residence time to influence the extraction efficiency ("*EPs*" from Eqn. 4.1) as well as the bulk amount of substrate processed is highlighted by this observation. Indeed, the absorption of material by the gut (both food and contaminants) is stated to be a time-dependant process (Kofoed *et al.* 1989, Selck *et al.* 1999). Further, Selck *et al.* (1999) highlight that the gut passage rate in a deposit feeding polychaete (*Capitella* sp. I.), will be higher for easily digestible food when compared to more refractory substrate. This pattern appears not to be restricted to a single species, as there are examples of increased gut retention time for more highly refractive foods in fish. For example shelled versus unshelled food in Boyce *et al.* (2000) and lipid rich foods in Basimi and Grove (1985), Grove *et al.* (1985) and Medved (1985) both elicit extended gut retention time in a range of fish species. Finally, there is evidence to suggest that lengthening gut retention time can increase contaminant assimilation as well as absorption of food. Wang and Chow (2002) found that, in response to a decrease in food availability, the gut retention time in the mussel *Perna viridis* increased - resulting in a concurrent increase in the assimilation

efficiency of benzo[a]pyrene sorbed to food particles. Both phytoplankton and sediment diets were assessed by Wang and Chow (2002). Similarly, Ahrens *et al.* (2001a) found that increased gut retention time, acting in concert with enhanced digestive chemistry, yielded higher absorption efficiencies of sediment-bound hydrophobic organic contaminants. Therefore, the generality of increased gut retention time aiding assimilation efficiency in refractive foods exemplified previously (Basimi and Grove 1985, Grove *et al.* 1985, Medved 1985, Boyce *et al.* 2000) seems, also, to apply to contaminant assimilation in sediment ingesting invertebrates as described by Selck *et al.* (1999). Implicit in the notion forwarded by Selck *et al.* (1999) is that increasing gut residence time affords greater opportunity for contaminant absorption, as long as suitable physiology exists to liberate the compound from the substrate. Therefore, in the sediment exposures without food additions, and in consideration of a fatty alkyl quaternary ammonium compound such as DODMAC, one might expect that longer gut retention times would provide greater opportunity for dietary assimilation.

(c) Gut structure and function

As alluded to above, differences in gut physiology provide a further mechanism by which organisms may achieve differential DODMAC assimilation. The adaptations in gut physiology comprise structural adaptations that provide the exchange surfaces as well as the metabolic "machinery" for secretory and absorptive processes. Here, distinction is made between physical structural adaptations and the secretory products of such structures, such that these aspects are considered separately. Firstly, the physical adaptations of the digestive tract are considered, with subsequent separate examination of the secretory products of particular organs and cells.

In a review of nutrient absorptive organs and processes in invertebrates, Ahearn *et al.* (1992) state that "physiological mechanisms of gastrointestinal absorption and secretion of organic and inorganic solutes amongst invertebrates remain severely underinvestigated". Whilst this situation has not, to date, significantly improved; relevant literature covering aspects of invertebrate gut physiology does exist. Particular emphasis is placed upon the existence of a specialised mid-gut diverticulum (often termed the hepatopancreas) and the function thereof. In echinoderms, molluscs and arthropods such diverticula (correctly termed the mid-gut gland) have been specifically identified as performing the dual function of nutrient absorption and digestive secretion

(Gibson and Barker 1979, Dall and Moriarty 1983, Ahearn 1987, Ahearn *et al.*1992). The suggestion that the mid-gut gland is responsible for digestive secretions was first made by Hoppe-Seyler (1876; cited in Gibson and Barker 1979) and finally conclusively demonstrated in primary cell culture of midgut gland epithelial cells by Ahearn *et al.* (1992). This, in common with the majority of studies pertaining to the midgut gland, was obtained from studies of decapod Crustacea. However, the basic mechanism of action of the mid-gut gland has also been observed in gammarids (*Gammarus minus*; Shultz 1976) and terrestrial isopods (e.g. Clifford and Witkus 1971) as well as a broad range of decapod crustaceans (extensively reviewed in Gibson and Barker 1979 and Dall and Moriarty 1983). Embryonic (undifferentiated) epithelial cells arise at the apex of the midgut gland and then migrate down the tubule towards the opening into the gut lumen. During this migration the embryonic cells differentiate into those bearing microvillus borders and those containing secretory vacuoles (Dall and Moriarty 1983). The contents of the secretory vacuoles are liberated into the gut lumen to promote extracellular digestion with the resultant breakdown products being assimilated via the absorptive cells of the epithelia (Dall and Moriarty 1983). In addition to the endogenous secretion of digestive enzymes, crustacea also commonly utilise exogenous enzymes derived from gut dwelling bacteria (Dall and Moriarty 1983, Calow and Petts 1992). Therefore, it is likely that both *Asellus aquaticus* and *Gammarus pulex* display considerable investment into development of specialised digestive structures and an attendant complement of extracellular digestive enzymes. Similarly, via a comparison between the midgut epithelia of a polychaete annelid (*Melinna palmata*) and two insect species (*Locusta migratoria* and larval *Chironomus plumosus*), Newell and Baxter (1936) showed that chironomid larvae possess a similar microvillus-lined epithelia to those observed in Crustacea. Additionally chironomid larvae were also found to incorporate a rich flora of motile spirillid bacteria as well as embedded rod-like bacteria that appeared to be involved in nutrient digestion and translocation (Newell and Baxter 1936). In contrast, the annelid showed a ciliated epithelial border and an apparent lack of the rich flora observed in *Chironomus* (Newell and Baxter 1936). However, it is apparent that larval chironomids lack the further invagination of this "brush border" epithelium to form the midgut gland that is evident in crustacea and adult insects (Wigglesworth 1965, Ahearn *et al.* 1992).

In contrast to the arthropod species considered above, the microdrili (aquatic oligochaetes, including *Lumbriculus variegatus*) have much simpler digestive systems.

Indeed, the absence of any branching and lack of differentiation of the epithelium beyond pharangeal and intestinal sections is attributed specifically to the relative ease of meeting nutritional needs in aquatic muds (Stevenson 1930). Comparisons between primitive and more highly evolved Lumbricidae (including terrestrial earthworms) revealed that as species spread to habitats containing more recaltricent nutrient sources, the complexity of the gut physiology concurrently increased (Stevenson 1930). The confirmation of this idea was provided by the reversion to a rudimentary unbranched intestinal gizzard in *Allolobophora dubiosa*, a descendant of more complex terrestrial forms, upon its "second reversion to a limicolous habit" (Stevenson 1930). In *Lumbriculus variegatus* specifically, the unbranched intestinal epithelium consists of ciliated cells, secretory glandular cells, lymph cells and excretory phagocytic amoebocytes (Stevenson 1930). In *Lumbriculus variegatus* the chromophil cells, secretory cells of digestive enzymes, do not discharge into the gut lumen. Rather, they appear to effect the breakdown of corpuscles within the coelom - in preparation for the reassimilation of suitable material across the coelomic wall (Stephenson 1930). There is also an apparent lack of the rich gut flora evident in arthropod digestive physiology (Stephenson 1930).

Therefore, it appears that, through feeding on a range of more refractive compounds (Cummins 1973, Rasmussen 1984, Graça *et al.* 1994b), the arthropod test species are likely to have more highly developed digestive structural physiologies than the microdrile oligochaete (Stephenson 1930).

(d) Gut secretion physiology

In addition to differential investment in structural gut adaptations, there is potential for variation in endogenous or exogenous digestive substances consistent with the variation in uptake of the fatty alkyl quaternary ammonium compound DODMAC. For example, the potential for interspecific differences in the efficacy that gut fluids strip sediment-sorbed contaminants is documented in a range of marine species (Mayer *et al.* 1996, Weston and Mayer 1998a, Weston and Mayer 1998b, Ahrens *et al.* 2001b, Mayer *et al.* 2001, Griscom *et al.* 2002). The important role of digestive surfactants in determining the bioavailability of sediment-sorbed hydrophobic organic contaminants (HOCs) tetrachlorobiphenyl and hexachlorobenzene to marine deposit feeders is particularly highlighted (Ahrens *et al.* 2001b). Greater sediment desorption and gut

absorption efficiencies of HOCs are related to greater gut fluid surfactancy and micelle concentration in *Neries succinea* and *Pectinaria gouldii* (Ahrens *et al.* 2001b). This is likely to be particularly relevant since DODMAC is, not only an HOC, but also a surfactant compound (ECETOC 1993). Further, the presence of lipase or esterase enzymes could be used to infer competence in dietary acquisition of fatty compounds from ingested material (as in e.g. Dadd 1973, Dall and Moriarty 1983). Whilst not, necessarily, specifically involved in the physical stripping of fatty acids from sediment particles, the existence of such enzymes would imply investment in specialised lipid assimilatory physiology.

In contrast to gut secretions that may indicate enhanced stripping of DODMAC from intact sediment particles, van Leeuwen and Hermens (1995) state that the ability to digest the substrate particles themselves greatly enhances uptake from sediments. Clearly, therefore, the ability to solubilise artificial sediment components is also likely to be important. Here, investigations would be constrained through lack of information upon the comprehensive characterisation of the field-collected sediment. Therefore, the obvious candidate component, given the structurally intransigent nature of kaolin and silica sand, would be the powdered cellulose used as a carbon source within the artificial sediment. Digestion of this form of cellulose (as cited in e.g. Monk 1977), irrespective of whether due to exogenous or endogenous enzymes, is likely to confer greater uptake of sediment-sorbed contaminants. This is especially true if, as suggested by Di Toro *et al.* (1991), the organic fraction of the sediment forms a substantial sorptive fraction for the hydrophobic organic test compound.

Therefore, there is potential for variation in gut-fluid components to control variation in dietary extraction efficiency of sediment-sorbed organic compounds. The presence of surfactants, lipases and cellulases within the gut fluids of each of the species exposed to sediment-sorbed DODMAC is likely to be important in this respect. The presence of particular digestive secretions, in combination with both the physical secretory structures and structures that allow efficient absorption, represent the range of investments that organisms make in digestive competence. Thus, there is potential for variation in the digestive environment by both physical and biochemical means.

4.1.5 Hypotheses and predictions

Based upon each of the factors outlined in the preceding sections, a number of hypotheses about potential mechanisms for interspecific variation in DODMAC assimilation from sediment were generated. These are given below.

(a) Uptake kinetics

Irrespective of the specific underlying mechanism, it may be possible for differential kinetic trajectories to cause 48-h DODMAC tissue loadings that, over longer periods, arrive at the same asymptote (Hypothesis 4.1). Observations consistent with this hypothesis would be obtained if extending the exposure period from 48 h to two weeks resulted in equivalent tissue loadings between all four species (Prediction 4.1). It is also possible, for *Lumbriculus variegatus* and *Asellus aquaticus*, that the extended exposure period may yield tissue loadings that approximate to steady-state values and that the variation between substrate type is rate-driven (Hypothesis 4.2). If both these conditions are satisfied, then the much higher (c.f. 48-h artificial sediment) species-specific tissue loadings of *Lumbriculus variegatus* and *Asellus aquaticus* observed in 48-h field sediment exposures should not significantly exceed the proposed steady state achieved in 2-week artificial sediment tissue loadings (Prediction 4.2). However, since the phenomenon of rate-driven variation can operate without specific reference to the mechanisms responsible, consideration to the identification of such mechanisms is given in the following paragraphs.

(b) Behavioural determination of exposure: Selective Feeding

It is possible that DODMAC may associate differentially between the fine particulate (kaolin and cellulose) and coarse particulate (sand) fractions of the artificial sediment. In turn it is also possible that organisms differ in their proportional ingestion of this highly contaminated fraction (Hypothesis 4.3). There are a variety of potential observations that would provide evidence in support of this hypothesis. Firstly, separating fine from coarse particles in dosed artificial sediment would be expected to reveal a higher loading of DODMAC within the fine particle fraction (Prediction 4.3i). If the observed measurements conformed to this prediction, the following further predictions would also be addressed. Exposure of organisms to a single fraction of the

sediment, uniform in both nature and size range, would be expected to remove the potential for either inherent or avoidance selectivity – and result in equal tissue loadings between all species (Prediction 4.3ii). Additionally, the proportion of sand particles found in faecal matter from each species following ingestion of artificial sediment should be inversely related to the DODMAC tissue loadings (Prediction 4.3iii). However, even if the ratio of ingested fine to coarse particles does not vary as predicted, it is possible that the organic content of ingested fine particulate fraction may differ between species. If this is the case, the organic content (measured by loss on ignition) of the faecal pellets, and hence proportion of cellulose ingested, would be positively related to DODMAC tissue loading (Prediction 4.3iv).

(c) Tissue morphological control of inherent capacity for assimilation: Lipid and water content

Lipid content of tissues may delimit the maximum achievable tissue loading of the lipophilic compound DODMAC (Hypothesis 4.4). It follows, therefore, that measured lipid content should be positively related to DODMAC tissue loading (Prediction 4.4). Whether or not lipid content is primarily responsible for tissue loading variation, the proportion of non-accommodating tissue fraction (water) could potentially determine the remaining proportion of combined accommodating tissue fractions (i.e. lipid, protein, carbohydrate etc., Hypothesis 4.5). Tissue water content would, therefore, be expected to relate inversely to DODMAC tissue loading (Prediction 4.5).

(d) Physiology: Biotransformation

The existence of interspecific variation in biotransformation capability would result in differing degrees of DODMAC tissue loading amelioration by catabolic processes in each species (Hypothesis 4.6). If this were occurring in the species exposed to DODMAC, tissue loadings would be negatively related to the proportion of metabolites to parent compound achieved in each species (Prediction 4.6).

(e) Physiology: Gut retention time

Following the synopsis in Section 4.1.4, in consideration of a strongly-sorbed fatty alkyl quaternary ammonium compound in a sediment of low nutrient value, increased gut retention time would provide increased opportunity for assimilation of DODMAC from sediment particles (Hypothesis 4.7). Consequently, gut retention time would be predicted to be positively related to DODMAC tissue loading across the exposed species (Prediction 4.7).

(f) Physiology: Structural and secretory adaptations of the gut

There is an apparent potential for invertebrate species to vary considerably in the structural and secretory adaptations to efficient dietary assimilation of nutrients. Concurrently, there appears to be variation in the efficacy with which species assimilate sediment-sorbed contaminants via digestive processes. Therefore, species displaying digestive physiological adaptations that enhance rapid absorbtion, sediment component solubilisation and/or lipid absorption may be able to enhance their DODMAC assimilation capacity (Hypothesis 4.8). It was predicted that, using suitable existing literature, the inability of *Lumbriculus variegatus* to achieve 48-h tissue loadings comparable to any of the arthropod species could be related to a lack of suitable gut physiological adaptations (Prediction 4.8).

4.2 Materials and Methods

All organisms were derived from the sources described in Section 2.2.1. Artificial sediment was prepared as described in Section 2.2.2. Similarly the DODMAC sample is the higher specific activity sample (13.8 $\mu Ci\ mg^{-1}$) detailed in Section 2.1.2.2.

4.2.1 Assessment of uptake rate kinetic trajectory

Twelve replicate DODMAC-dosed and 12 replicate non-dosed artificial sediment vessels (Section 2.2.2) were used to expose each of the species: *Lumbriculus variegatus*, *Chironomus riparius*, *Gammarus pulex* and *Asellus aquaticus*. The exposure duration was extended to 14 days followed by removal to gut purge vessels

(Section 2.2.2.4) for 24 h. Tissue analysis for ^{14}C proceeded as described in Section 2.2.3. Tissue loadings of *Lumbriculus variegatus* and *Asellus aquaticus* determined in 48-h exposure to field collected sediment dosed with DODMAC were taken from the results of Chapter 2.

4.2.2 Assessment of selective feeding

(a) Measurement of DODMAC distribution between fine and coarse particle fractions

The relative DODMAC loading in the sand particle fraction of dosed artificial sediment was determined by adding 50 ml of distilled water to 30-g samples of dry sediment in a 150-ml glass jar. The vessel was then capped and shaken vigorously for approximately 30 seconds. The overlying water/fine particulate suspension was then immediately decanted into a separate 150-ml glass jar. For each of 20 replicate 30-g dosed sediment samples, a pair of glass jars were left to air dry in a laminar flow cupboard for two weeks; one jar contained the sand particles from the original dosed sediment whilst the second contained the suspension of the fine particle fraction. This yielded 20 replicate samples of sand particles and 20 replicate samples of fine particulate fraction derived from whole artificial sediment dosed with DODMAC. In addition, ten samples of non-dosed sediment were separated and dried in the same way. Subsequently, small (~0.5 g) samples of sand particles and fine particulate fraction from each replicate sediment sample were analysed for ^{14}C by liquid scintillation counting (Section 2.2.3). The mean value of the non-dosed samples was used to determine background radioactivity and subtracted from each value obtained from the dosed samples (as per Section 2.2.3).

(b) Assessment of uptake in the absence of selective feeding

Kaolin clay (Section 2.2.2.1) was used to substitute the artificial sediment used in the exposures detailed in Section 2.2. Twenty dosed and twenty non-dosed vessels were prepared for each of the four species: *Lumbriculus variegatus*, *Chironomus riparius*, *Gammarus pulex* and *Asellus aquaticus*. The details of substrate dosing procedures, exposure conditions, exposure duration, vessel type, overlying water, test-organism identity and addition, gut purge procedure and apparatus as well as sample analysis were all identical to those described in Chapter 2. The only difference was that organisms were exposed, not to whole artificial sediment, but to a substrate made

up of one pure fraction of that sediment. The tissue loadings achieved in the 48-h exposures to particle-sorbed DODMAC were used to determine whether tissue loadings varied in the absence of the opportunity to feed selectively.

(c) Determination of selectivity for sand and organic content of ingested particles

Vessels containing 1-cm depth of undosed artificial sediment and a 2-cm depth of artificial pond water (APW) (Section 2.2) were prepared for each test species. *Chironomus riparius* and *Lumbriculus variegatus* were exposed in 8-cm diameter plastic cups and *Asellus aquaticus* and *Gammarus pulex* were exposed in 1.5-l rectangular plastic culture vessels (Section 2.2). Two vessels, each containing 50 organisms, were used for both *Asellus aquaticus* and *Gammarus pulex* (total of 4 vessels). For *Lumbriculus variegatus* and *Chironomus riparius* a single vessel each containing 100 individuals was used (total of 2 vessels). Organisms were exposed for 48 h before collection of faecal pellets or gut contents from each test species proceeded as follows.

Faecal pellets, produced at the substrate surface, by a group of 100 *Lumbriculus variegatus* were collected using a pastuer pipette. The faecal pellets were found to be suitably consolidated, so as to retain their integrity during collection in this manner. Subsequent to transfer into a petri dish containing de-ionised water, the pellets could be placed individually onto a glass microscope slide using a pair of fine forceps. For *Chironomus riparius*, *Asellus aquaticus* and *Gammarus pulex* it proved more feasible to collect each of 100 individual organisms that had been exposed to non-dosed artificial sediment for 48 h and to dissect out the peritrophic membrane and its contents. The intact entire gut contents pellet could then be extruded onto a glass microscope slide.

The pellets from each of the four species were examined under a light microscope (40x magnification). Sand particles were identified (where present) and counted following the crumbling of the pellet with a mounted needle. Measurements of the dimensions of the pellet were made (Nikon Eclipse E-600 microscope, Nikon House, 380 Richmond Road, Kingston, Surrey, KT2 5PR with video capture and screen measurement software, LUCIA: Laboratory Imaging s.r.o., Nad Úpadem 901/63, CZ - 149 00 Praha 4 – Háje) so that, if required, an estimate of sand particles per pellet volume could be made.

For determination of faecal pellet organic content, organism exposure and faecal pellet collection were performed as described for sand particle content analysis. All weighings were performed on a suitably sensitive microbalance (Mettler ME30, Mettler-Toledo Ltd., 64 Boston Road, Beaumont Leys, Leicester, LE4 1AW, U.K.). After collection, the faecal pellets were allowed to dry at room temperature for a week following addition to pre-weighed foil weighing boats. Pellets were allocated to foil boats such that the complete gut contents from a single *Chironomus riparius, Gammarus pulex or Asellus aquaticus* individual occupied a single weighing boat. Conversely, individually egested faecal pellets were allocated singly to weighing boats for *Lumbriculus variegatus*. Such pellets were invariably shorter than the complete gut length of the organism.

The foil boats and their contents were weighed before being placed in a furnace and brought to 550°C. The samples were held at this temperature for two days to ensure complete combustion, and then the temperature was allowed to return to the ambient value. Subsequently the foil boats were carefully removed and reweighed. Following the subtraction of the weight of the foil boat from both pre- and post-combusted samples, the "loss on ignition" values were calculated for each sample. This was effected by the subtraction of post-combusted sample weight from the pre-combusted sample weight. The difference between the two values was then expressed as a percentage of the pre-combusted sample weight. A calibration curve (Appendix 4.1) to confirm the reliability of detecting cellulose content in this manner was produced. A range of known masses of cellulose were added to clay and sand particles prior to "loss on ignition" determination as above. The linear regression of "cellulose mass added" against the loss on ignition values was used to determine that the methodology could reliably detect changes in cellulose content (Appendix 4.1).

4.2.3 Assessment of lipid content

The lipid content determination of a pooled tissue sample (weighing between ~2-5 g) was carried out for each of the species *Lumbriculus variegatus, Chironomus riparius, Gammarus pulex* and *Asellus aquaticus*. Pooled masses of groups of whole organisms of each species were frozen at -20°C at The University of Sheffield (Western Bank, Sheffield, S10 2TN). Frozen tissue was then shipped in dry ice to Unilever Research Port Sunlight (SEAC Environment, Unilever Research, Quarry Rd East, Bebbington, Wirral, CH63 3JW). A modified version of the Bligh and Dyer lipid

content determination methodology (Bligh and Dyer 1959) was utilised by Mr. Stephen Harding at Unilever Research and is detailed in Appendix 4.2.

4.2.4 Assessment of water content

The percentage water content of each of the four species was determined by weighing 20 blotted individuals of each species (Mettler College 150, Mettler-Toledo Ltd., 64 Boston Road, Beaumont Leys, Leicester, LE4 1AW, U.K.) and then adding them to pre-weighed foil weighing boats. These were then held at 60°C for two weeks to ensure complete dryness. The weighing boats and their contents were then re-weighed and the foil boat mass subtracted from each of the dried sample masses. The difference between the two tissue masses was then expressed as a percentage of the blotted wet weight of each individual organism.

4.2.5 Assessment of biotransformation

A pooled tissue mass consisting of all whole organisms recovered from gut purge vessels for each individual species were taken following 48-h exposure to artificial sediment dosed with DODMAC (exposure methodology and gut purge procedure described in Section 2.2.2). Following 24-h gut purging, the tissue samples were shipped as described in Section 4.2.3.

Biotransformation of DODMAC in each of the four species was assayed via thin layer chromatography (after e.g. Smith and Feinberg 1965) in combination with ^{14}C detection. Stephen Harding carried out the analyses at Unilever Research Port Sunlight (SEAC Environment, Unilever Research, Quarry Rd. East, Bebbington, Wirral, CH63 3JW) and the specific modified methodology is detailed in Appendix 4.3.

4.2.6 Assessment of gut retention time

Since the artificial sediment used in previous exposures (Section 2.2.2.1) was found to be visible in the digestive tract of each of the four test species, it was possible to track its progress following ingestion. Forty individuals of each species were allowed to ingest artificial sediment (2-cm depth of sediment covered by 2-cm depth overlying APW in an 8-cm diameter plastic tub). After approximately two hours organisms were removed to individual gut-purge vessels (Section 2.2.2). The time of removal for each individual was recorded along with the unique identification used to label each gut-purge vessel. The organisms were then left for 15 – 30 minutes and upon re-examination, both the time elapsed (minutes) and the distance (mm) between the trailing (most anterior) edge of the sediment pellet and the pharynx was recorded to the nearest millimetre. The total length of the alimentary canal was also recorded for each organism at this time. All length measurements were performed using a transparent scale marked at 1-mm intervals. The time required for one complete gut passage was estimated by dividing the total length of the alimentary canal by the calculated pellet velocity (i.e. distance moved by trailing edge of pellet/time).

4.2.7 Assessment of gut structural and secretory attributes

It was not practicable, within the scope of this project, to experimentally determine the characteristics of the gut physiology of each of the test species. Therefore, an investigation of existing work detailing the characteristics of digestive physiology within relevant taxonomic groups of organisms was undertaken. Recent literature, 1981 to present, was examined using a searchable database (Web of Science, http://wos.mimas.ac.uk). Literature published prior to this was examined by using a combination of citations in more recent papers and by examination of catalogues of references held at The University of Sheffield. Subsequently, relevant references cited in publications prior to 1981 were then also examined. In addition a database of some 17,000 references covering all aspects of annelid biology was found to be available via the world wide web (http://www.keil.ukans.edu/~worms/bibliog/lreadme.html). The resultant overall body of consulted literature consisted of textbooks and articles from periodical publications that spanned nearly 300 years (from 1705 – 2002). It was determined, to within as specific a taxonomic group as possible, whether there was evidence of particular characteristics that would be likely to enhance digestive

assimilation of DODMAC. Specifically, the characteristics researched were those highlighted in Sections 4.1.4 *(c)* and *(d)*. Information on the presence of a given characteristic, along with the taxonomic level of specificity, were tabulated. Instances where there was evidence for a lack of such characteristics were also tabulated. Finally, where no existing information was found, a record was made of such absences.

4.2.8 Overall approach to statistical analyses of measured factors

Before attempting to relate variation in any measured factor to tissue loading, it was first established (where possible) whether there was evidence of interspecific variation in that factor. In other words, if no interspecific variation could be established then it is unlikely that tissue loading could be proportionally related to that factor. Additionally, for predictions relating to exposure to isolated clay particles (Prediction 4.3ii) and extended exposure (Prediction 4.1), it was necessary to establish whether variation in tissue loading was still exhibited under specific, revised conditions. Generally, for both the above scenarios, this was carried out using parametric analysis of variance (ANOVA). However, the nature of the data collected for predictions relating to lipid content (Prediction 4.4) and biotransformation (Prediction 4.6) preclude such analysis and, as such, interpretation of the measured factor is restricted to qualitative analysis. Where the data consisted of percentage values, the appropriate arcsine transformation was applied before ANOVAs were carried out.

4.3 Results

4.3.1 Assessment of uptake kinetics

Extending the exposure period from 48 h to 14 days did not result in equivalent tissue loadings in four species exposed to DODMAC in artificial sediment ($F_{3, 43}$ = 74.61; $p<0.001$). Tukey's pairwise comparisons of 14-day tissue loadings (Fig. 4.1) revealed that *Chironomus riparius* still achieved significantly higher tissue loadings than any other exposed species ($p<0.05$). However, the remaining three species did converge to

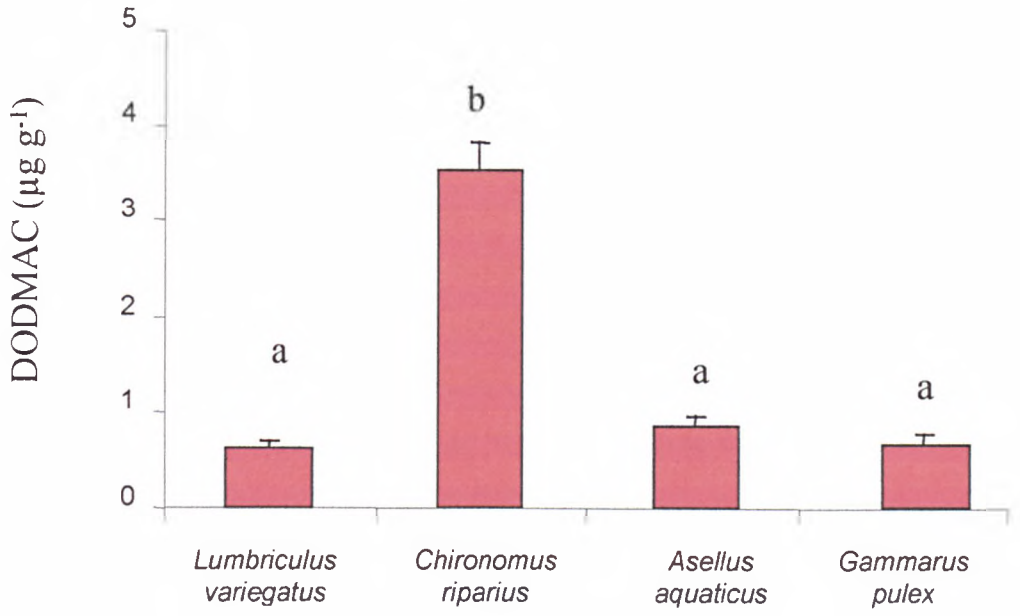


Fig. 4.1 Mean (+ SE) DODMAC tissue loadings in four benthic invertebrate species exposed to dosed artificial sediment for two weeks. Groups sharing the same alphabetic label do not differ at $p<0.05$ (Tukey's pairwise comparisons)

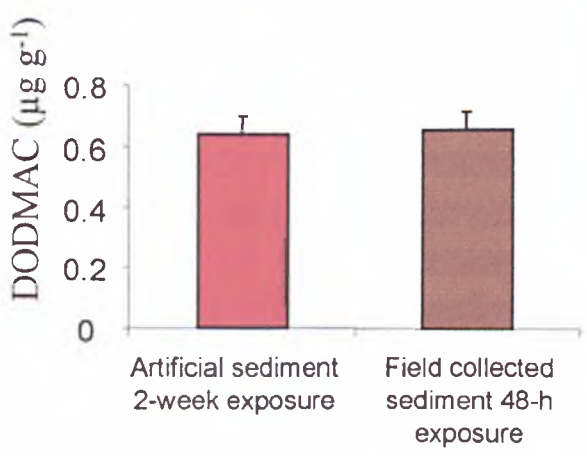


Fig. 4.2 *Lumbriculus variegatus* tissue loadings achieved in extended exposure to artificial sediment and short term exposure to field collected sediment. No significant difference detected ($p>0.05$)

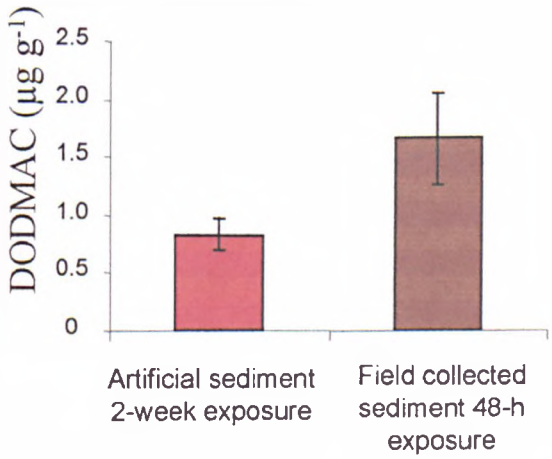


Fig. 4.3 *Asellus aquaticus* tissue loadings achieved in extended exposure to artificial sediment and short term exposure to field collected sediment. No significant difference detected ($p>0.05$)

achieve equivalent tissue loadings in the extended exposure duration (Tukey's pairwise comparisons $p>0.05$).

Species-specific comparisons of DODMAC tissue loadings achieved in 48-h exposures to dosed field sediment (previously noted to greatly exceed 48-h artificial sediment exposure) and two week artificial sediment exposures (assumed to represent maximum steady state tissue loading) revealed no significant differences for *Lumbriculus variegatus* or *Asellus aquaticus* ($t_{\geq 17} \leq 2$; $p>0.05$, Figs. 4.2 and 4.3). It should be noted, however, that *Asellus aquaticus* tissue loadings achieved in 48-h exposure to field collected sediment showed a tendancy to exceed tissue loadings achieved in two week exposure to artificial sediment (Fig. 4.3).

4.3.2 Assessment of selective feeding

Significantly higher DODMAC loadings were measured in isolated fine particles (i.e. cellulose and kaolin) than in sand particles taken from dosed artificial sediment ($t_{20} = 16.84$; $p<0.001$). The mean (SE) loading measured in the fine particulate fraction was 8.79 (0.48) $\mu g g^{-1}$ compared to the 0.29 (0.076) $\mu g g^{-1}$ measured in sand particles (Fig. 4.4). This provides the differential particle-fraction distribution of contaminant necessary to enable selective feeding to operate. However, when the potential for selective feeding was removed by exposure to a single sediment fraction (i.e. kaolin), interspecific variation in DODMAC tissue loadings was still evident ($F_{3, 79} = 40.28$; $p<0.001$). Subsequent analysis via Tukey's pairwise comparisons revealed that *Chironomus riparius* achieved greater tissue loading than any other species tested and that *Gammarus pulex* tissue loadings exceeded those of both *Asellus aquaticus* and *Lumbriculus variegatus*. The latter two species were not detected to differ in tissue loading (Fig. 4.5).

Similarly when exposed to whole artificial sediment there is evidence that the species do not differ in the particle size fraction that they ingest. Table 4.1 illustrates the fact that all species extensively ingest the fine particulate fraction of artificial sediment and that the occurrence of sand particles in ingested matter appears to be anomalous. Consequently, no further quantification of relative sand content per faecal volume was undertaken. No significant variation in faecal "loss on ignition" values was detected between species ($F_{3,68} = 2.72$; $p = 0.053$), although the variation apparently tends toward

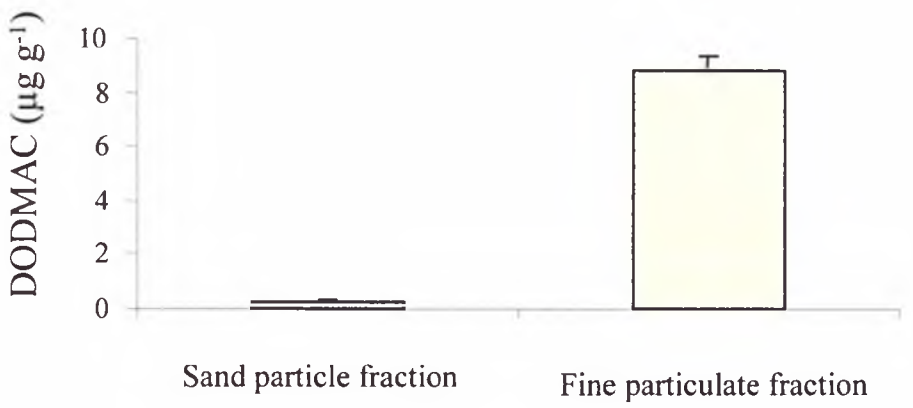


Fig. 4.4 Mean (+ SE) DODMAC loadings in separated sand and fine particle fractions. Fractions were isolated from post-dosed whole sediment.

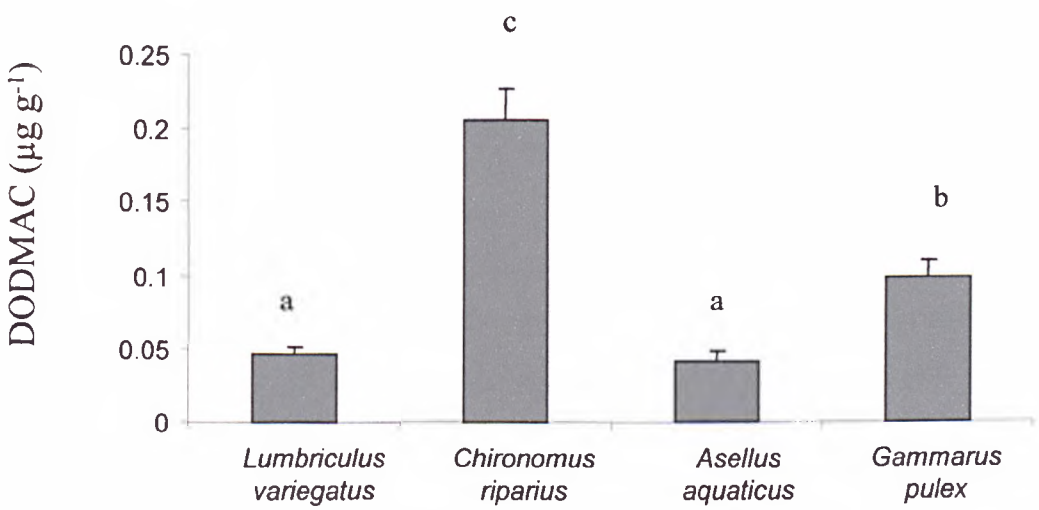


Fig. 4.5 Mean (+ SE) DODMAC tissue loadings in four benthic invertebrate species exposed to dosed isolated clay-particle sediment fraction. Groups sharing the same alphabetic label do not differ at $p<0.05$ (Tukey's pairwise comparisons)

significance. *Asellus aquaticus* faecal pellets had the smallest weight loss on ignition, indicating a possible trend for smaller amounts of cellulose in the material ingested by *Asellus aquaticus* individuals (Fig. 4.6)

Table 4.1 Sand grain content of faecal pellets

Species	Number of individual sand grains found in examination of 100 faecal pellets
Lumbriculus variegatus	4
Chironomus riparius	2
Asellus aquaticus	0
Gammarus pulex	6

4.3.3 Analysis of lipid and water content

Values of measured lipid content differed between species with *Asellus aquaticus* and *Gammarus pulex* having the highest lipid content and *Chironomus riparius* the lowest lipid content (Fig. 4.7). However, this apparent difference between species cannot be statistically validated due to the use of pooled tissue samples used to provide sufficient sample matrix. Additionally, there is no evidence that lipid content is positively correlated with DODMAC tissue loading ($r_{s\ 4} = -0.2$, $p>0.05$). Water losses upon drying tissue at 60°C for each species (Fig. 4.8) did not vary significantly ($F_{3,39} = 2.42$; $p>0.05$).

4.3.4 Gut retention time

The time taken to make one complete passage of the alimentary canal was found to vary between the four species ($F_{3,116} = 7.91$; $p<0.001$) with gut retention time being greatest for *Chironomus riparius* and least for *Gammarus pulex* (Fig. 4.9). There was no significant difference in the gut retention times of *Lumbriculus variegatus, Gammarus pulex* or *Asellus aquaticus* nor was there a significant difference between the

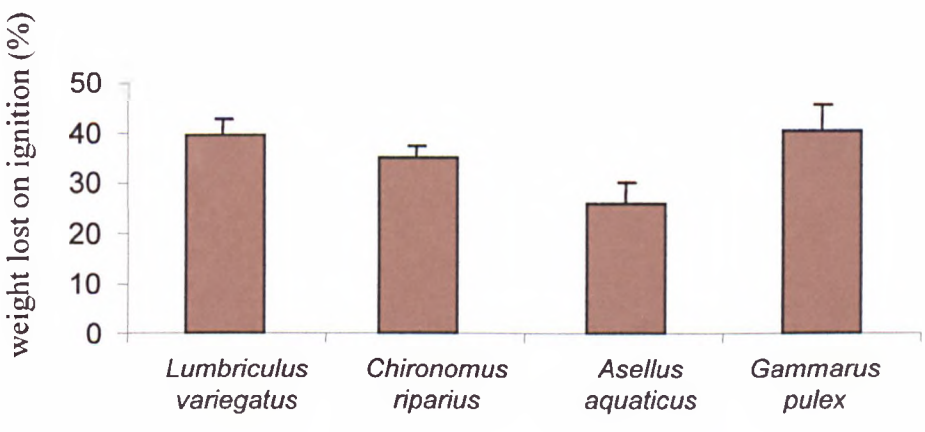


Fig 4.6 Mean (+SE) faecal pellet weight loss after 48 h combustion at 550°C for each of four species of benthic invertebrate.

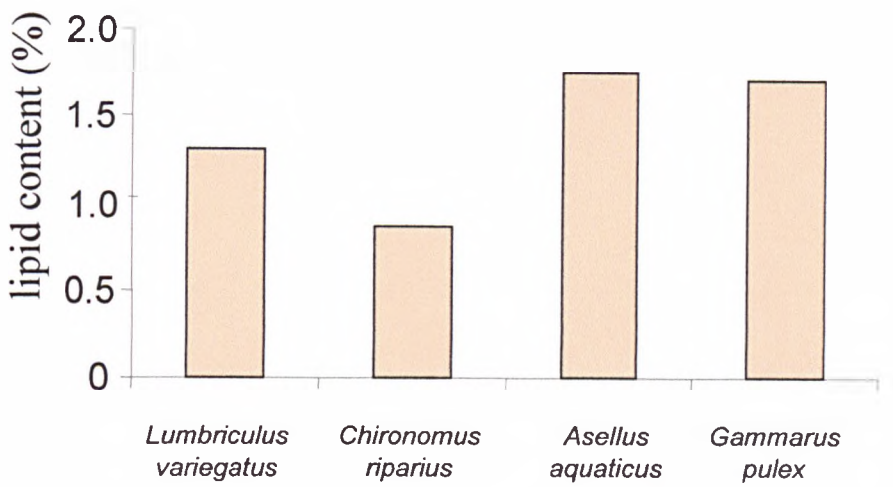


Fig 4.7 Percentage tissue lipid content in each of four benthic invertebrate species exposed to sediment-bound DODMAC. Values derived from mean of three measurements made from single pooled tissue sample for each species

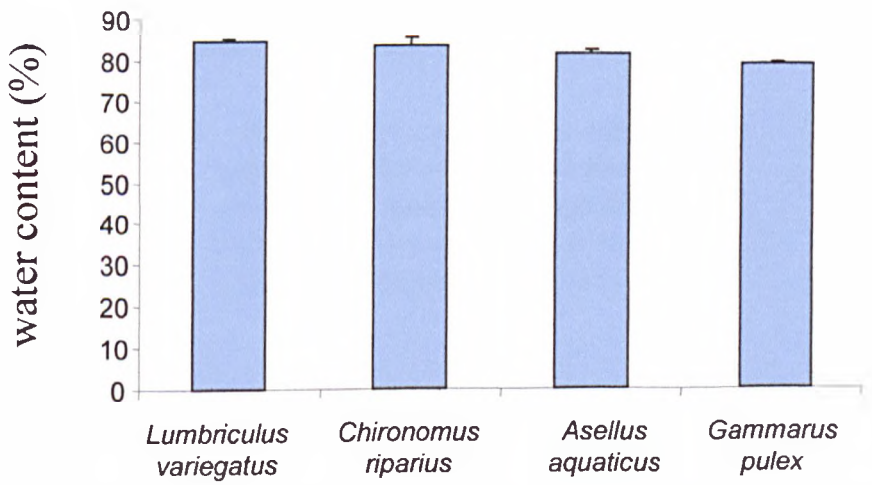


Fig 4.8 Mean percentage (+SE) tissue water content in each of four benthic invertebrate species exposed to sediment-bound DODMAC.

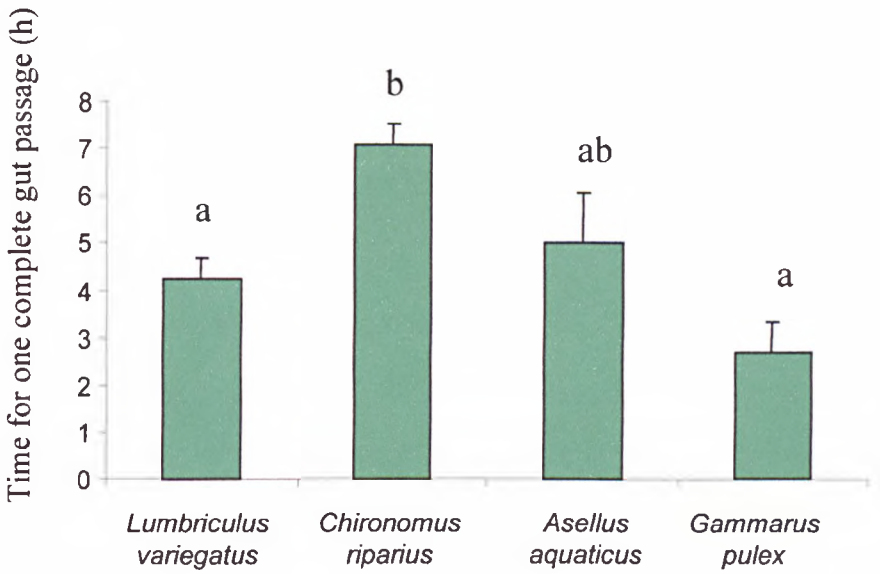


Fig. 4.9 Mean (+SE) time taken for one complete gut passage for each of four benthic invertebrate species. Groups sharing the same letter do not differ at $p<0.05$ (Tukey's pairwise comparisons)

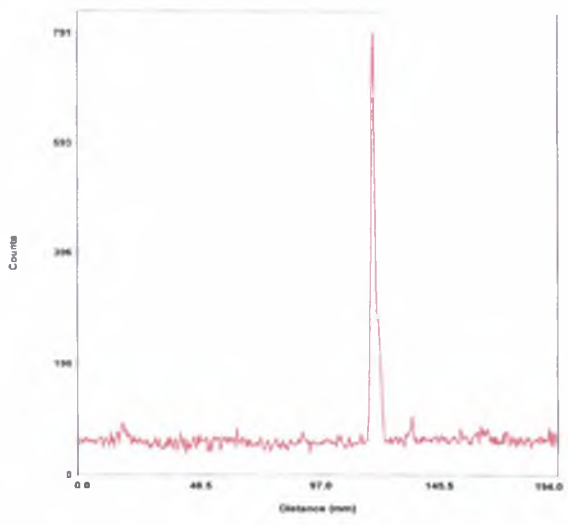
gut retention times of *Chironomus riparius* and *Asellus aquaticus*. Gut retention time appeared to be more variable in *Asellus aquaticus* and *Gammarus pulex* than in the remaining two species (Fig. 4.9). The coefficient of variance for *Gammarus pulex* and *Asellus aquaticus* gut passage time was 110% and 103% respectively. In comparison, the *Lumbriculus variegatus* and *Chironomus riparius* values for the same factor were 62% and 37% respectively.

4.3.5 Biotransformation capability

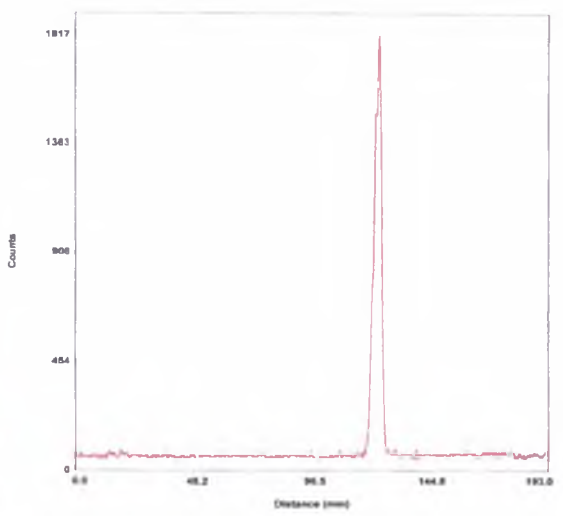
There is good evidence for a lack of variation in biotransformation capacity. No species showed any biotransformation of DODMAC (Figs. 4.10 a to d). Each tissue extract chromatogram consisted of a single peak with the shape and retention time characterised by that of the pure parent material (Fig. 4.11).

4.3.6 Assessment of gut physiological attributes

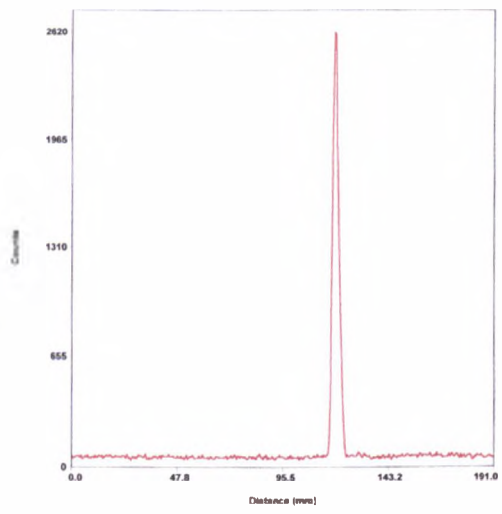
The presence or absence of the specific factors identified in Section 4.2.7, based on available literature, is detailed in Table 4.2. Possibly due to the small size of the study species exposed in Chapter 2, the existing literature that includes comprehensive studies of relevant digestive physiology appears to favour larger invertebrate species. For example in polychaetes such as *Arenicola marina* and *Neanthes virens* there are in-depth studies of the full suite of digestive enzymes, along with details of their distribution within the alimentary tract (Longbottom 1970 and Kay 1974 respectively). There are also fully comprehensive descriptions of the absorptive physiology of all dietary components, with explicit reference between histological/ultrastructural anatomy and the function thereof (e.g. *Arenicola marina*; Kermack 1955). Studies such as the digestion and storage of fat in Polychaeta (e.g. Marsden 1968) or the digestive constraints with reference to feeding ecology (e.g. Penry and Jumars 1990), are generally lacking for the species considered in Chapter 2. Such fundamental descriptions of processes and limitations of digestive physiology are likely to have relevance to contaminant uptake, as well as to the uptake of nutrients. In consideration of DODMAC, a fatty alkyl quaternary ammonium compound, the absorption of fatty material is likely to be of particular relevance. In addition to nutritional fundamentals, there are also examples of studies relating gut conditions to contaminant absorption.



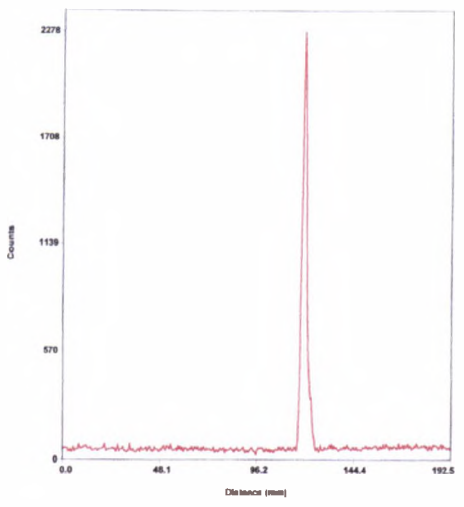
a.)



b.)



c.)



d.)

Fig 4.10 TLC chromatograms of tissue extract taken from: (a) *Lumbriculus variegatus,* (b) *Chironomus riparius*, (c) *Asellus aquaticus* and (d) *Gammarus pulex* individuals exposed to sediment-bound DODMAC

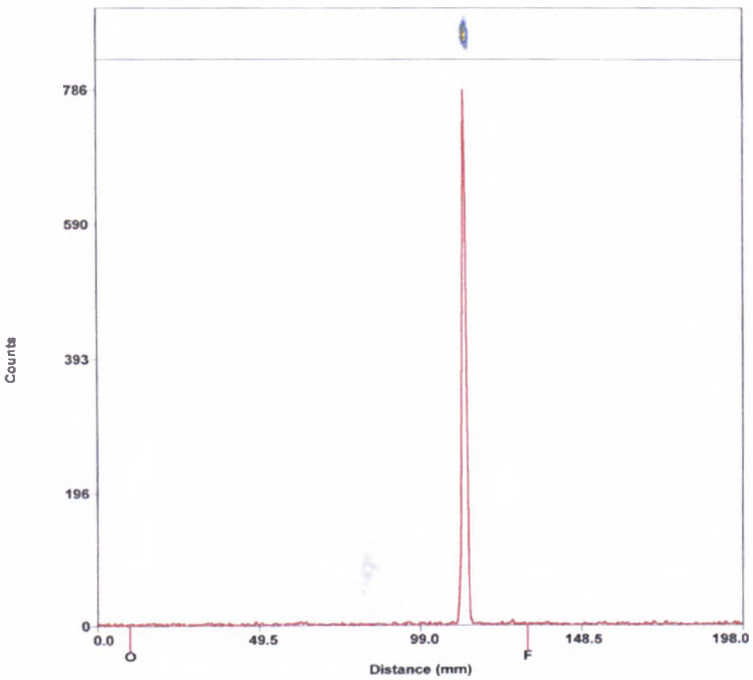


Fig 4.11 TLC chromatogram of DODMAC reference standard (pure material)

Gut fluids isolated from marine invertebrates were found to vary in contaminant extraction efficacy when incubated with sediment-sorbed organic compounds (Mayer *et al.* 1996, Weston and Mayer 1998a, Weston and Mayer 1998b, Ahrens *et al.* 2001a, Ahrens *et al.* 2001b, Mayer *et al.* 2001, Griscom *et al.* 2002). In addition, studies such as Weber and Lanno (2001) and Chen and Mayer (1999), relate in-vivo dietary conditions to bioaccumulation from sediments (in fish and marine invertebrates respectively). The main conclusions of these studies suggest that gut fluids isolated from a range (circa 20 *spp.*) of marine benthic invertebrates can effectively extract both metal and organic pollutants from marine sediments. Additionally, the extraction competence measured in-vitro is related to the tissue loadings achieved in whole organisms. Therefore, gut fluid constitution appears to be an important determinant of sediment-sorbed pollutant assimilation efficiency. Finally, there are also studies of mechanisms of elimination, for example in polychaetes (Dales 1961, Dales 1964, Marsden 1966) the elimination (as well as nutrient uptake) of waste products by coelomic amoebocytes (or coelomocytes) has been described. Documentation of this sort of fundamental mechanism, in relation to contaminants in the species exposed in Chapter 2, is not apparent.

A number of factors were identified in published literature that are relevant to the exposure system and species used in the experiments of Chapter 2. In *Gammarus pulex*, *Asellus aquaticus* and *Chironomus riparius* there is evidence to suggest that cellulase activity is present (Kooiman 1964, Monk 1977, Rasmussen 1984). Conversely, although suggesting that cellulose digestion may occur in terrestrial oligochaetes, Stephenson (1930) suggests that this ability is lacking amongst the microdrili, including *Lumbriculus variegatus*. Where native cellulose (derived from plant cell walls) is the substrate of interest, the presence of a single cellulase enzyme is insufficient to achieve breakdown (Dall and Moriarty 1983). Native cellulose hydrolysis requires the synergistic action of three types of enzymes: endo-β-1, 4-glucanase, exo-β-1, 4-glucanase and β-glucosidase (Dall and Moriarty 1983). Additionally, in some cases of reported native cellulose hydrolysis, the results have been questioned (e.g. *Gammarus sp.*: Chamier and Willoughby 1986 cited in Calow and Petts 1992) due to the use of incubations at excessive temperatures for durations far in excess of normal gut passage times (Calow and Petts 1992). However, powdered cellulose used in preparation of artificial sediment (Section 2.2.2.1) is more readily solubilised (Monk 1977, Dall and Moriarty 1983, Calow and Petts 1992). As such, the

ability to solubilise this form of cellulose is not in question with reference to the form utilised in the artificial substrate of Chapter 2 and the evidence for cellulase activity within the species (Table 4.2). Therefore, there appears to be good evidence that *Lumbriculus variegatus* lacks the ability to digest the organic fraction of the artificial substrate, in contrast to the digestive capability found in the remaining three arthropod study species. This ability is likely to elevate the amount or rate of contaminant absorption from sediments via ingestion compared to species that lack the ability to digest sediment particles (van Leeuwen and Hermens 1995). Therefore, based upon digestion of sediment components, *Lumbriculus variegatus* would be expected to display the lowest rate of accumulation of compounds associated with the organic fraction of the artificial sediment (Section 2.2.2.1).

Another manner in which digestive ability, either through exogenous enzyme acquisition or by some enhancement of translocation, appears to be greater in the three arthropod species is in the presence of rich gut flora. Particular mention is made of the presence, in *Chironomus* larvae, of both motile and epithelially "rooted" bacterial cells that function as conduits for nutrient transport from food bolus to epithelial cell membrane (Newell and Baxter 1936). Gut floras involved in crustacean digestion are also documented, including those of *Gammarus pulex* and *Asellus aquaticus* (Newell and Baxter 1936, Gibson and Barker 1979, Dall and Moriarty 1983, Graça 1993). In contrast, even though many annelid species also exhibit rich gut floras (e.g. Stephenson 1930, Marsden 1968, Longbottom 1970, Kay 1974), there is an apparent lack of microbial gut symbionts in microdrili, including *Lumbriculus variegatus* (Stephenson 1930). In contrast to annelid species with extensive gut floras and feeding largely on plant detrital matter, *Lumbriculus variegatus* obtains nutrition by ingesting micro-organisms ingested along with sediment particles (Brinkhurst and Jamieson 1971). Therefore, again, it appears that the three arthropod test species possess the greatest investment in rapid and effective digestive assimilation of a range of ingested materials.

The distinction between each of the four test species for lipase or esterase activity is less clear. In addition it is not known if, or how, such enzymes would play any part in liberating DODMAC from sediment particles. However, there appears to be a lack of such enzymes in *Chaetogaster spp.* (Stephenson 1930). There is no evidence to suggest that this characteristic is shared by *Lumbriculus variegatus* or other

Table 4.2 Comparison of digestive features between categories of organisms encompassing species exposed to sediment-bound DODMAC.

	Exogenous enzymes/specialised gut flora	Lipases/ esterases	Cellulose digestion	Micelle formation/intestinal detergents	Periodic peaks in Lipid assimilatory activity	Endocytosis/ Intracellular digestion
Annelida	√[1,2,3,4]	√[1,2,3]	√[4]	?	?	√[5,6]
Microdrilli (aquatic oligochaetes)	x[4]	x[4]	x[4]	?	?	√[5,6]
Lumbriculus variegatus	x[4]	?	?	?	?	√[4]
Crustacea	√[7,8,9]	√[9]	√[10,11]	√[7, 9, 12]	√[7](pre-moult peak in crustacean midgut gland)	?
Amphipoda	√[7,8,9]	√[9]	√[10,11]	√[9, 12]	?	?
Isopoda	√[7,8,9]	√[9]	√[10,11]	?	?	?
Gammarus pulex	√[7,8,9]	?	√[10,11]	?	?	?
Asellus aquaticus	√[7,8,9]	?	√[10,11]	?	?	?
Insects	√[8]	√[14]	√[15]	?	√[14]	?
Diptera (*Chironomus plumosus*)	√[8]	√[14]	√[15]	?	√[14](pre-pupation peak in dipteran larvae)	?
Larval *Chironomus riparius*	?/√[8, 13] †	?	√[15]	?	?/ √[14, 13] †	?

1.) Marsden (1968), **2.)** Kay (1974), **3.)** Longbottom (1970), **4.)** Stephenson (1930), **5.)** Kermack (1955), **6.)** Marsden (1966), **7.)** Gibson and Barker (1979), **8.)** Newell and Baxter (1936), **9.)** Dall and Moriarty (1983), **10.)** Monk (1977), **11.)** Kooiman (1964), **12.)** Lester *et al.* (1975), **13.)** Armitage *et al.* (1995), **14.)** Dadd (1973) and **15.)** Rasmussen (1984)

† The near identical nature of larvae of *C.riparius* and *C. plumosus* is noted [13]

The references listed beneath the table are numbered from 1 – 15 and are cited, by number, within the table. The symbol "√" indicates that there is positive evidence for the existence of a particular characteristic. Conversely, the symbol "x" indicates that there is positive evidence for a lack of a given characteristic. Finally, the symbol "?" indicates that no evidence was found, for or against, the existence of a particular characteristic.

microdrili (Stephenson 1930). Further, although the presence of lipases or esterases is documented in insects and crustaceans (Dadd 1973 and Dall and Moriarty 1983 respectively) their presence within each of the test species could not be explicitly confirmed. Therefore, it is not possible to infer that differences in enzymes involved in lipid digestion exist between the study species. However, there is evidence that lipid assimilation periodically peaks within the arthropod species. For crustacean species it appears ubiquitous that the lipid content within the midgut gland (or hepatopancreas) greatly increases during the premoult period (Gibson and Barker 1979). Additionally, all insects characteristically accumulate fats in high concentrations at physiological stages of development preceding pupation and diapause (Dadd 1973). Whilst it is not clear whether *Lumbriculus variegatus* displays periodic variation in assimilation of nutrients related to reproductive or developmental status, it is apparent that there is no reason for individuals used in the experiments of Chapter 2 to display mass synchronicity in developmental stage. This lack of developmental synchronicity is shared by *Asellus aquaticus* and *Gammarus pulex*, and stems from the non-selective allocation of individuals to test vessels. However, for *Chironomus riparius* individuals, the majority of larvae added to test systems would be at the 4^{th} instar stage of development. This developmental stage immediately precedes pupation for *Chironomus riparius* (Oliver 1971, Armitage *et al.* 1995). It is unlikely, based upon the selective feeding assessment, that lipid assimilation could be enhanced by selection for lipid-rich food particles as seen in some trichopteran species (Cargill *et al.* 1985; cited in Calow and Petts 1992). Therefore, for this putative mechanism to operate, some other enhanced investment in lipid assimilation would have to be evident. Though the requirement for experimental verification is abundantly apparent, this represents a potential mechanism by which *Chironomus riparius* DODMAC tissue loadings are elevated.

Apart from lipid-digesting enzymes, the presence of emulsifying agents within gut fluid is cited to enhance dietary lipid assimilation in crustaceans (Lester *et al.* 1975, Gibson and Barker 1979). The presence of such intestinal detergents does not appear to be documented in chironomid larvae or in aquatic oligochaetes. However, in a study that relates specific gut fluid constituents to contaminant assimilation in catfish intestine, Weber and Lanno (2001) report that the formation of lipid/bile micelles

enhances the assimilation of lipophilic contaminants such as benzo[a]pyrene. This is mirrored in annelids (marine polychaetes) where variation in micelle concentration is related to variation in uptake of lipophilic sediment contaminants (Ahrens *et al.* 2001b). The absence/presence or abundance of such surfactants within gut fluids of individual species appears to represent another manner in which organisms may achieve differential assimilation of lipophilic contaminants. However, how this varies between *Lumbriculus variegatus*, *Chironomus riparius*, *Gammarus pulex* and *Asellus aquaticus* is not currently apparent.

4.4 Discussion

The aim of the investigations undertaken in this chapter was to characterise the likely ways in which the organisms could differ to effect the interspecific variation in DODMAC tissue loading. First of all, consideration was given to differences in rates of uptake, howsoever effected, in relation to the duration of exposure. Extending the exposure duration in artificial sediment (Prediction 4.1) resulted in three of the four species achieving equivalent tissue loadings. This implies that, for *Lumbriculus variegatus*, the potential to achieve the same tissue loading as *Gammarus pulex* and *Asellus aquaticus* existed, but could not be realised within 48-h exposure (i.e. a clearly non steady-state condition). Conversely, *Chironomus riparius* was still able to achieve significantly higher tissue loadings of DODMAC than the remaining species. Therefore, both the rate of uptake (over 48 h of exposure) and the ultimate maximum tissue loading of DODMAC in *Chironomus riparius*, appear to be greater than for the remaining three species. Further, it appears that the dramatic increases in tissue loading noted in *Asellus aquaticus* and *Lumbriculus variegatus* when exposed to field collected sediment may be related to variation in rate of uptake between substrate type. Whilst there is evidence to suggest that *Lumbriculus variegatus* tissue loadings of DODMAC approach steady state at around 0.6 $\mu g g^{-1}$ (A.U. Conrad Pers. Comm.), it is not known what the corresponding value is for *Asellus aquaticus* tissues. Thus, for *Lumbriculus variegatus*, maximal tissue concentration is likely to be exhibited in the 2-week exposure and there is no suggestion that the 48-h tissue loading (achieved in field

collected sediment) exceeds this putative capacity. However, since there is a tendency that higher tissue loadings may be achieved in 48-h exposure to field collected sediment in *Asellus aquaticus*, it seems that the 2-week exposure to artificial sediment yields tissue loadings that remain slightly below their maximum potential. Both the rate of uptake and the maximal tissue capacity for DODMAC are therefore implicated to be important determinants of tissue loadings. Interpretations of the investigations into specific mechanisms driving differences in both rate and capacity are considered in the remainder of this chapter.

Based upon the results of the series of experiments detailed in this chapter, it is apparent that many of the measured factors are unlikely to be responsible for the variation in uptake observed in Chapter 2. For example, the potential for selective feeding to operate exists due to the distribution of DODMAC within the sediment, however the species do not appear to vary significantly in their selective behaviour. It must also be acknowledged that the particle-size fractions offered within the artificial sediment (Section 2.2.2.1) consisted only of "sand" (>63μm) and "clay" (<2 μm) particle sizes. This contrasts with the continuum of particle sizes found within natural sediments used in other exposures of benthic invertebrates where selective feeding is apparent (e.g. Kukkonen and Landrum 1995, Kukkonen and Landrum 1996). Therefore, even within the original exposure regime (Chapter 2), there will have been limited scope for particle-size selectivity to influence tissue loadings. Even though there was a trend for *Asellus aquaticus* to ingest a slightly lower proportion of organic particles, the remaining species showed no propensity to vary in this respect. Neither was the tissue loading in *Asellus aquaticus* significantly lower than in *Gammarus pulex*, or *Lumbriculus variegatus* whose organic particle selectivity tended to be greater. It is therefore difficult to relate variation in feeding selectivity to the variation in tissue loadings observed. Additionally, tissue-loading variation still exists, even in the absence of the potential for selective feeding. Thus, exposures to artificial sediment suggest that mechanisms other than selective feeding can contribute to variation in tissue loadings achieved by different species under standardised exposure conditions.

For a number of other measured factors, no interspecific variation could be established that could drive differences in tissue loading. For example, the lack of

biotransformation capacity for DODMAC in any of the studied species and the lack of variation in tissue water content precluded these as mechanisms for differential tissue loading. Though, in dismissing biotransformation as a possible factor, alternative modes of elimination that it was not possible to investigate in this project should be noted. For example, the elimination of hydrophobic substances in vesicles termed "coelomocytes" in annelids (Dales 1961, Dales 1964, Marsden 1966).

Whilst lipid content appeared to vary, the absence of statistical consideration of variation between individuals restricts the conclusions that can be drawn from this trend. It would appear from the measured values that *Chironomus riparius* tissues have lower lipid contents than the remaining three species. The two (apparently equivalent) highest lipid contents were measured in *Gammarus pulex* and *Asellus aquaticus* tissues. The measured value of *Lumbriculus variegatus* lipid content appears to be intermediate between that of *Chironomus riparius* and the crustacean values. Fresh-weight lipid content values measured here approximate previously published values. *Gammarus pulex* total lipid was determined as 6.1% of dry mass (Meier *et al.* 2000). Using the measured water content of *G. pulex* (~80%, Fig. 4.8), an approximate fresh weight lipid content of 1.22% is estimated. *Asellus aquaticus* total lipids have been measured as 0.69% ± 0.26% of fresh weight (Montañés and van Hattum 1995). West *et al.* (1997), determined *Lumbriculus variegatus* fresh weight lipid content as 1.36% ± 0.32% whilst the same study cites fresh weight total lipid contents for 3rd instar *Chironomus tentans* as 0.82% ± 0.01%. West *et al.* (1997) also give the fresh weight lipid content of 4th instar *Chironomus tentans* as 1.08% ± 0.12%.

It appears unlikely that total tissue lipid is the key determinant of the interspecific variation DODMAC in tissue loading, since *Chironomus riparius* has the lowest measured tissue lipid content but the highest tissue DODMAC loading. The larvae of both *Chironomus riparius* and *Chironomus tentans* are biologically very similar (Armitage *et al.* 1995) and this may suggest that the lipid content measured in this chapter is a representative value. Additionally, *Lumbriculus variegatus* has the next lowest measured tissue lipid content after *Chironomus riparius*, but also achieves the lowest tissue DODMAC loading. Unsurprisingly, there is no indication of a significant statistical correlation between lipid content and DODMAC tissue loading. DeJongh *et al.* (1997), and Legierse (1998) have reported that organism protein content (rather

than lipid content) may be an important determinant of contaminant tissue loading. A lack of measured interspecific variation in tissue water content, coupled with lower measured lipid content in *Chironomus riparius*, means that there is a higher proportion of tissue components that remain unaccounted for in this species. Tissue protein measurements could, therefore, provide relevant information relating to the maximum DODMAC loading capacity of each species.

A distinction between the types of lipid present within a study species could offer a useful indication of storage capacity for hydrophobic organic contaminants. Bremle and Ewald (1995) showed that the amount of non-polar (storage) lipids in oligochaetes and chironomids was more important than total lipid (non-polar plus cell membrane lipids) in the determination of PCB uptake from sediments. Further, Legierse (1998) specifically notes that for "lean" organisms (of only around ~0.5% lipid) the proportional representation of polar (cell membrane) lipids is greatly enhanced relative to the non-polar storage lipids. Such increases in the proportion of polar lipids are brought about simply by the reduction in the amount of non-polar storage lipid present. A relative lack of storage lipid was cited to increase the importance of tissue protein as a sink for chlorobenzenes within the freshwater gastropod *Lymnaea stagnalis* (Legierse 1998). Therefore, where measures of total lipid indicate that the organisms of interest are relatively lean, the characterisation of lipids and quantification of tissue protein may be appropriate. However, irrespective of total tissue capacity, the rate or efficiency of extraction from sediment must also be an important determinant of tissue loadings achieved in sediment exposures. For example, *Asellus aquaticus* achieved comparably high tissue loadings to *Chironomus riparius* in exposure to field collected sediment for 48 h (in contrast to much lower *A. aquaticus* tissue loadings in 48-h artificial sediment exposure).

A factor that could statistically be validated to vary between species was gut retention time. As with measured lipid content, gut retention times were found to approximate to previously determined values for organisms comparable to two of the species investigated in this chapter. Brooke *et al.* (1996) state that *Lumbriculus variegatus* and *Chironomus tentans* both clear their gut contents in a period less than 12 h. Literature-based values for gut retention times in *Gammarus pulex* and *Asellus aquaticus* were not readily available.

The longest gut retention time (measured in this chapter) in *Chironomus riparius* was associated with the highest tissue loading. However, there is a lack of a simple relationship between gut retention time and tissue loading across different species. It is possible that similar factors identified within the polychaete *Capitella* may be operating within the system under consideration here. Selck *et al.* (1999) performed experiments utilising cadmium-spiked sediment particles that were experimentally coated with a range of materials of variable digestibility. It was concluded that for substrates that were successfully attacked and solubilised by the gut enzymes of *Capitella sp.* I, assimilation efficiency varied in direct proportion to gut retention time. Conversely, where no suitable enzymatic attack could be made (e.g. in the case of humic acid coated particles) there was no relationship between gut retention time and assimilation efficiency. In other words, where there was no capacity to solubilise the substrate – extending the residence time within the gut could not enable additional absorption to take place. The most obvious deviation from the putative relationship between gut retention time and tissue loading, is the overprediction of tissue loading that would be accorded to *Lumbriculus variegatus* based on the retention times illustrated in Fig. 4.9. The experimental verification of the relative digestive capabilities of each species (with reference to sediment components and test compound) would enable verification of digestive capability combined with gut retention time (after Selck *et al.* 1999) as a potential mechanism in this system. Additionally, the potential for gut passage time of ingested artificial sediment (as measured via organism transplantation to sandy substrate: section 4.2.6) to differ from the ingestion behaviour exhibited within actual exposures must be acknowledged.

The degree of investment into gut structural, digestive symbiont, gut fluid enzymatic and surfactant adaptations made by each species was highlighted as a potential means by which extractive competence may be mediated. Further, the potential for the periodic variation in lipophilic contaminant assimilation to exist within certain study species (at particular developmental stages) also merits consideration. A lower investment into such features requires verification in relation to the apparent lesser efficiency exhibited in *Lumbriculus variegatus*.

4.4.1 Conclusions

The assessment of selective feeding, total tissue lipid/water content and biotransformation within each of the test species suggests that these factors are unlikely to be responsible for interspecific variation in DODMAC assimilation. However, a number of ways in which the organisms could potentially achieve such differential assimilation have been identified. These factors appear to influence, not only the efficiency of contaminant extraction from ingested sediment for the specific exposure duration, but also the ultimate tissue capacity for a given contaminant. Factors are therefore separated into those that influence uptake rate and those that influence ultimate tissue capacity. Firstly, in consideration of factors that influence uptake rate differences, the determination of assimilation efficiency by a combination of substrate-specific extractive competence for the contaminant and gut retention time (as proposed by Selck *et al.* 1999) requires further investigation. The factors suggested to influence this extractive competence include gut structural adaptations to rapid absorbtion, gut fluid surfactancy and substrate solubilisation (i.e. cellulase activity/cellulose-rich artificial sediment). There also appears, from comparisons between artificial and field-collected sediment, to be potential for the interaction between gut conditions and substrate-type to influence rate of uptake. However, extensive further characterisation of such interactions is required, such that relative digestibility of substrate components and ease of desorption from particles via gut fluids are quantified. Secondly, with reference to *Chironomus riparius* tissue loadings, there is clear evidence that species can vary (irrespective of lipid content) in their overall maximum tissue-loading capacity for a specific compound. Thus, variation in tissue loadings appears to depend upon a combination of case-specific extraction efficiency acting over a specific exposure duration as well as overall maximum tissue capacity.

At present there is a fundamental lack of understanding relating to the chemical interactions controlling DODMAC extraction from sediment by invertebrate gut fluids and the determinants of maximum tissue capacity. Further experimentation to quantify these phenomena is therefore greatly desirable. Similarly, studies reported here have, also, only considered biotransformation as a means of compound elimination. There is evidence of alternative processes controlling elimination of substances of minimal water solubility (i.e. those effected by coelomocytes in annelids; Dales 1961, Dales

1964, Marsden 1966). Therefore, in order to provide the most basic information required by toxicokinetic models (e.g. Lee 1992), there must also be additional consideration directed toward the existence, and extent, of all elimination processes. This combined characterisation of uptake efficiency/capacity and elimination would afford a much greater understanding of the factors determining realised body-burdens of sediment-sorbed DODMAC. In turn this would broaden understanding of the mechanisms that control general bioavailability of hydrophobic organic sediment contaminants to benthic invertebrates.

Chapter 5: Measuring proportional contribution to uptake from aqueous sources

5.1 Introduction

5.1.1 Bioavailability of contaminants in aqueous solution

Uptake and assimilation of sediment-sorbed contaminants has been suggested to occur via desorption into pore water and subsequent passage over respiratory exchange surfaces into biota (e.g. Landrum and Robbins 1990). The idea of pore water acting as the exchange-determining medium between sediment particles and biota has been formalised, without specific identification of the route of uptake, in equilibrium partitioning (EqP) models (Section 1.1.2). Additionally, it is clear from standard "water-only" ecotoxicological exposure methodologies (e.g. OECD 1981, 1984 and 1992) and studies of uptake across gill membranes (e.g. McKim and Erickson 1991) that compounds in aqueous solution are generally bioavailable. There are also many studies that demonstrate the toxicity of aqueous fractions (both pore waters and "elutriates" prepared via suspension of sediments in water) of contaminated sediments (Section 1.1.3). Further, the assimilation from pore water has been suggested to be the predominant route of exposure to sediment contaminants (Connell *et al.* 1988, Ankley *et al.* 1991) as well as studies explicitly linking the aqueous desorption of sediment contaminants to bioavailability (Section 1.1.4). It is, therefore, apparent that compounds can be assimilated from aqueous sediment fractions. However, the general bioavailability of freely dissolved compounds does not necessarily preclude uptake of compounds sorbed to sediment particles. In fact, the potential of ingestive uptake to explain anomalies observed in the relationship between compound desorption and bioavailability has been expressly cited (Landrum 1989). The use of aqueous desorption kinetics to infer bioavailability is stated to be equally applicable to dietary uptake of particle-sorbed compounds (Section 1.1.4).

5.1.2 Bioavailability of particle-sorbed compounds via invertebrate digestion

Particle ingestion is commonly implicated as a route of assimilation of sediment contaminants. For example, the results described in Chapter 2 and Chapter 3 highlight the potential importance of ingestive uptake in a desorption-resistant compound. It is especially apparent from these results that, in *Lumbriculus variegatus* and *Asellus aquaticus*, the act of ingestion is crucial in achieving uptake of such a compound from sediment (Section 3.3.1). There are also studies that specifically measure compound depletion from ingested sediment during gut passage (Section 1.1.5). This potential for uptake via ingestion is further implicated by the efficacy of isolated gut fluids taken from benthic invertebrates in extracting sediment-sorbed compounds (Section 1.1.5).

In addition to measured factors, the importance of digestive assimilation of highly lipophilic organic contaminants from sediment particles is predicted in modelling approaches (Section 1.1.5 and e.g. Thomann *et al.* 1992). In these studies it is suggested that, as compound hydrophobicity increases, ingestion must make an increasing contribution to overall uptake. Digestive assimilation of highly lipophilic compounds is also predicted from the interpretation of uptake observed in compounds that would not be supported by aqueous concentrations (Landrum 1989).

Thus, the conclusions of numerous direct measurements, as well as some predictive modelling approaches, suggest that ingestive uptake of contaminants sorbed to sediment particles is viable, particularly for more lipophilic compounds. Therefore, in addition to the widely accepted notion of uptake of free aqueous solute (Section 5.1.1), it appears that there is good reason to believe compounds can be de-sorbed from sediment particles during digestive processing. This highlights the potential contribution from both of these uptake routes to overall assimilation from contaminated sediment.

5.1.3 Relative contribution from each uptake route

5.1.3.1 Modelling

Wang and Fisher (1999) highlight the need to understand assimilation from particle ingestion as well as uptake from the aqueous phase. The extent that each of

these uptake routes contribute to overall assimilation has been investigated by a number of approaches. These approaches are considered in this, and the following, sections (Sections 5.1.3.1 to 5.1.3.3). As noted above, modelling approaches that assign the probable proportion of uptake for a range of chemicals, sediments and organisms have been forwarded (e.g. Landrum and Robbins 1990, Thomann *et al.* 1992, Forbes *et al.* 1998). These models, necessarily, acknowledge uptake from both aqueous solute and sediment particulate routes. In general, the cited models suggest an increase in the proportional contribution to uptake from particle ingestion with increasing octanol/water partition coefficient (K_{ow}) of the model compound. The predictions of these models, therefore, implicate the potential for the partitioning behaviour of compounds to influence the proportional importance of aqueous and particulate routes of uptake. Compounds of lower log K_{ow} are suggested to be accumulated in greater proportion from the aqueous phase than compounds that are more hydrophobic (and vice versa).

5.1.3.2 Experimental manipulation of uptake route

A range of methodologies have been used to compare the uptake or effects of compounds in exposure to "aqueous" relative to "whole sediment" phases. Perhaps the simplest involve comparisons between effects observed in "whole-sediment" exposures to those evident in separate, "water phase" exposures utilising aqueous solutions prepared at comparable concentrations to those measured in pore water (e.g. Kane-Driscoll *et al.* 1997a, Kane-Driscoll *et al.* 1997b, Lawrence and Mason 2001). Refinements of such an approach are various methods of "forced exposure" to a particular uptake route. For example, cadmium uptake in *Asellus aquaticus* from aqueous exposure to the metal both with and without simultaneous ingestion of cadmium-contaminated plant material (van Hattum *et al.* 1989). However, these methodologies do not achieve a direct separation of the contributions to overall uptake made within a single sediment sample.

Attempted measurement of in-situ contributions from sediment ingestion versus aqueous ventilation is evident in the use of enclosures. Their purpose is to delimit the solid and aqueous sediment phases to which particular organisms are exposed. In this

way, uptake achieved in segregated sediment fractions by constrained groups of organisms can be compared. Comparisons are therefore made between the contributions of actual fractions belonging to that specific sediment. Sediment ingestion and aqueous uptake in *Asellus racovitzai* has been measured via exposure of free ranging organisms to cadmium-contaminated sediment with the simultaneous exposure of organisms enclosed in dialysis membrane cylinders suspended in the overlying water (Eimers *et al.* 2001). It is noted by Eimers *et al.* (2001) that exposure to isolated pore water is not possible due to clogging of the dialysis membrane so that exposure is limited to overlying water concentrations of the metal. Even so, as perhaps would be expected from the relatively soluble nature of metals, the uptake of cadmium could be explained in terms of the aqueous exposure to the metal (Eimers *et al.* 2001).

Though there has been examination of cadmium uptake in the above-cited example, there is an apparent lack of experimental measurements of uptake-route contributions for organic compounds via "constrained exposure" apparatus. Further, the functional characteristics of dialysis membrane enclosures mean that, for any study compound, contributions from sediment pore water cannot be examined using this method. Therefore, if in-situ contributions of pore water to overall uptake are to be measured by apparatus that manipulates exposure route, a novel test system must be implemented.

5.1.3.3 Biological determination of uptake route

Aspects of organism feeding and ventilation biology have also been utilised to discern uptake route of sediment contaminants. For example, the exposure of feeding and non-feeding oligochaete worms to contaminated sediment in order to distinguish between ingestive uptake and cutaneous assimilation of aqueous contaminant (Leppannen 1999, Conrad *et al.* 2000). The technique of apical segment ablation in *Lumbriculus variegatus* after Conrad *et al.* (2000) was used, alongside a novel manipulation of *Asellus aquaticus*, to compare feeding and non-feeding organism tissue loadings in Chapter 3. These manipulations distinguish between whole sediment ingestion and contact of aqueous phase contaminant with the gas exchange surfaces of the organism, but do not account for ingestion of pore water in the non-feeding

organisms. However, it is apparent from these direct manipulations of organism biology (rather than experimental exposure vessel manipulation), that contributions from different uptake routes can be measured in certain instances. However, as highlighted in Chapter 3, many study organisms cannot be suitably manipulated. Consideration must also be given to the fact that comprehensively removing ingestion behaviour also removes uptake contribution from the ingestion of aqueous solute (if such solute is present).

In marine sediment exposures, the facultative filter-feeding and deposit-feeding modes of the tellenid clam *Macoma nasuta* have been used to elucidate contributions to uptake from aqueous and particle ingestion sources (Boese *et al.* 1990). From this study it is apparent that *M. nasuta* largely accumulates hexachlorobenzene via sediment ingestion, rather than ventilation of overlying water. The plasticity of feeding mode (both filter and deposit-feeding) in *M. nasuta* has, therefore, been utilised to discern the proportional uptake of sediment contaminants from each route. This technique, using a similar species *Macoma balthica*, is also successfully extended to include comparison to uptake in obligate filter-feeding and obligate deposit-feeding organisms (Kaag *et al.* 1997). Uptake of lipophilic polychlorinated biphenyl (PCB) and polyaromatic hydrocarbon (PAH) compounds was greatest in the obligate deposit-feeding worm *Arenicola marina* (Kaag *et al.* 1997). Conversely, the lowest uptake of this suite of lipophilic sediment contaminants was evident in the obligate filter-feeding mussel *Mytilus edulis* (Kaag *et al.* 1997). In the absence of filter-feeding competition from *M. edulis*, the clam (*M. balthica*) achieved similarly low accumulation of sediment contaminants whilst exhibiting filter-feeding behaviour. However, *M. balthica* was observed to switch to a deposit-feeding mode when experiencing feeding competition exerted by the presence of *M. edulis* in the exposure vessel. This lead to a concurrent dramatic increase in the amount of contaminant assimilation to levels approaching that of the obligate deposit-feeding *A. marina* (Kaag *et al.* 1997). Thus, not only is this an example of measurement of in-situ uptake route contributions, but it also highlights the importance of biological factors in determining the route and overall extent of uptake. In specific circumstances, therefore, organism biology can be utilised to make measurements of proportional uptake route contribution. However, the work by Kaag *et al.* (1997) also shows that organism biology, in all circumstances, exerts a

fundamental control over the uptake routes that are experienced. There is, therefore, potential for substantial interspecific variation in the extent to which each exposure route is processed.

In summary, it is clear that both aqueous and particulate ingestion uptake pathways are likely to operate. It is also apparent that the partitioning properties of contaminants, between aqueous and particulate sediment phases, are likely to influence the proportional uptake from each phase. Additionally, the feeding and ventilation biology of study organisms will fundamentally influence the volume of each phase that is encountered by a particular species. However, only a few studies have been able to directly measure the relative contributions of these uptake routes. Therefore, the expected decrease in contributions from aqueous solute (c.f. particulate uptake) with increasing compound log K_{ow} generally lacks practical investigation. Similarly, very few studies examine whether increased ventilatory efficiency is linked to increased proportional uptake from aqueous sources.

5.1.3.4 Use of acute exposures in assessing uptake route contribution

It was suggested in Chapter 4, that experimentally determined tissue loadings could be the result of a combination of an organism's storage capacity for that compound, coupled with the rate of uptake of that compound from a particular substrate or uptake route. Therefore, there is potential for the exposure duration to influence tissue loadings achieved in both whole sediment and isolated aqueous exposure. Equilibrium partitioning theory, for example, predicts ultimate tissue loading at equilibrium (DiToro *et al.* 1991). In contrast toxicokinetic models predict tissue-loading change through time using instantaneous contributions to uptake. In the experiments described in this chapter, how near a given species is to achieving maximum tissue loading of a compound may influence measured contributions for different exposure routes. Given chronic exposure durations, it is possible that tissue loadings (that may previously be observed to vary between uptake route in acute exposure) ultimately converge to a similar asymptote. However, depending upon context, both "instantaneous contributions" of uptake route as well as "ultimate achievable assimilation" from a given uptake route could be equally important. This

notwithstanding, it is still clearly desirable to discern the likely influence that exposure duration may have on measured proportional uptake from each route.

Equilibrium partitioning models apply specifically to non-polar organic compounds (DiToro *et al.* 1991). In this chapter the test compounds utilised (Section 5.1.4) fulfil this requirement and can be appropriately subjected to EqP-based predictions of ultimate tissue loading. Estimates of the likely ultimate tissue loading can therefore be made and subsequently compared with the tissue loadings achieved by study organisms under varying exposure route and duration. This approach gives an estimation of how effective a particular exposure duration is in producing "steady state" tissue loading. Additionally, it allows comparison of uptake routes for their efficacy in realising estimated ultimate tissue loading.

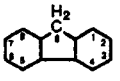
5.1.4 Test Organisms and model compounds

The potential influences that i.) chemical partitioning and ii.) organism ventilatory biology have upon the proportional uptake from aqueous sediment phases are investigated in this chapter. Four species of freshwater benthic macroinvertebrate, previously observed to ingest artificial sediment particles (Chapter 2), were chosen to examine the influence of ventilatory biology. *Gammarus pulex*, *Asellus aquaticus*, larval *Chironomus riparius* and *Lumbriculus variegatus* were utilised as the invertebrate models for these investigations. Both *G. pulex* and *A. aquaticus* have specialised ventilatory posterior appendages (pleopods); their rhythmic beating facilitating respiratory gas exchange (Schram 1986). In contrast both *L. variegatus* and larval *C. riparius* lack such ventilatory structures (Brinkhurst and Jamieson 1971 and Armitage *et al.* 1995 respectively). The presence of these specialised structures was used to infer an investment into an increased efficiency of processing the aqueous phase for respiratory exchange. However, no direct estimation of the volume of water processed for respiratory exchange was formally undertaken between the four model species. An assumption was made that the modification of these appendages conferred a ventilatory advantage compared to reliance upon simple trans-cutaneous diffusion (as in *C. riparius* and *L. variegatus*; Brinkhurst and Jamieson (1971) and Armitage *et al.* (1995)). Therefore, the turnover volume of aqueous phase processed for respiratory

exchange was assumed to be greater in *A. aquaticus* and *G. pulex* than in *L. variegatus* and *C. riparius*.

Polyaromatic hydrocarbons (PAHs) are non-polar lipophilic organic compounds commonly found sorbed to aquatic sediment particles (Lotufo 1998). They are derived from the combustion of recent (wood) and fossil (petrochemical and coal) fuels (Borchert *et al.* 1997, Lotufo 1998). The effect of compound log K_{ow} upon the proportional contribution of aqueous uptake was examined using three, structurally related, PAH compounds that vary in their octanol/water partitioning characteristics (Table 5.1). The ease of desorption into aqueous sediment phases for each of these PAHs would be expected to vary such that naphthalene>fluorene>pyrene. All three compounds were used in exposures to *G. pulex* investigating the influence of log K_{ow} upon the proportional contribution of aqueous uptake to overall assimilation (see hypotheses and predictions, Section 5.1.5). Conversely, only naphthalene (the compound expected to desorb most readily into the aqueous phase) was used in exposures to all four species in order to investigate the influence of ventilatory appendages (see hypotheses and predictions, Section 5.1.5).

Table 5.1: Physical properties of three PAH compounds used to investigate the variation in route of uptake

Compound	Structure	Log K_{ow}	Water solubility
Pyrene		4.88[†]	0.135 mgl^{-1}[†]
Fluorene		4.18[†]	1.89 mgl^{-1} [††]
Naphthalene		3.30[†]	31 mgl^{-1}[†††]

[†] **Hansch (1995)**

[††] **Wauchope and Getzen (1972)**

[†††] **Pearlman *et al.* (1984)**

5.1.5 Hypotheses and Predictions

5.1.5.1 Hypotheses:

The studies detailed in this chapter utilise a novel test system to investigate the following hypotheses:

5.1 That a higher affinity for sediment particles (inferred from increasing log K_{ow}) decreases the proportional contribution of uptake from aqueous solute sources, by increasing the proportion of dietary uptake.

5.2 That organisms with specialised ventilatory appendages could more efficiently process aqueous phases for respiratory exchange and concurrently enhance the proportion of aqueous uptake of a compound relative to organisms that lack such adaptations.

Two alternative hypotheses were also tested:

5.3 That differences in uptake from aqueous and particulate routes measured in acute (kinetically driven) exposures do not converge to identical (fugacity driven) values if dietary desorption kinetics substantially differ from aqueous desorption.

Or alternatively:

5.4 That the EqP-estimated ultimate tissue loadings should be independent of exposure route due to equivalent fugacity in both aqueous and particulate fractions.

The subsequent predicted results (Predictions 5i-5iv respectively) are expected if each of the Hypotheses 5.1-5.4 are correct.

5.1.5.2 Predictions:

5i. Ranked order of compound log K_{ow} (pyrene>fluorene>naphthalene) would be mirrored by decreasing ranked order of proportional uptake attributable to aqueous uptake in *Gammarus pulex*; such that proportion of aqueous uptake in

pyrene< proportion of aqueous uptake in fluorene< proportion of aqeous uptake naphthalene.

5ii. Both *Gammarus pulex* and *Asellus aquaticus* would achieve a greater proportion of their total naphthalene uptake via ventilation of the aqueous phase than either larval *Chironomus riparius* or *Lumbriculus variegatus.*

5iii. For the most lipophilic compound tested (pyrene), both 48-h and 14-day exposures to whole sediment and isolated overlying water would result in significantly greater uptake from whole sediment.

5iv. Tissue loadings in all study species, independent of exposure route, would be significantly positively correlated with the theoretical predicted values derived by EqP modelling.

5.1.6 General approach

A novel test system was utilised that enabled the contribution to overall uptake attributable to the aqueous route to be measured against the uptake achieved in whole sediment exposure (Section 5.2.4, Figs. 5.1, 5.3 and 5.4). This test system was used to address Predictions 5i and 5ii in a series of short-term exposures, 48 h in duration (Section 5.2). A separate test system (Section 5.2.5, Fig. 5.2) was utilised to address the longer (14-day) exposure duration required in Prediction 5iii.

To test whether log K_{ow} influences the proportion of aqueous uptake (Prediction 5i) the freshwater amphipod *Gammarus pulex* was exposed, in three separate studies, to sediment dosed with pyrene, fluorene and naphthalene. The test species had previously been observed (Chapter 2) to ingest the components of the artificial sediment utilised in these studies. In addition, this species actively ventilates the aqueous phase in order to effect respiratory gas exchange (Schram 1986). Therefore, both routes of uptake under investigation are potentially available to *Gammarus pulex.*

Prediction 5ii was addressed by utilising sediment dosed with naphthalene to expose *Gammarus pulex, Asellus aquaticus, Lumbriculus variegatus* and *Chironomus riparius* concurrently. The expected higher propensity of naphthalene (c.f. pyrene or

fluorene) to desorb into the aqueous phase was deemed to give the best opportunity to study the influence of ventilatory appendages upon contributions from aqueous uptake.

Finally Prediction 5iv was tested by deriving EqP predicted tissue loadings. Such derivations utilised aqueous data collected for Predictions 5i-5iii and organism lipid content measured in Chapter 4. The EqP-modelled values would then be compared to the tissue loadings of organisms exposed in the experiments addressing Predictions 5i-5iii.

Each of the above experimental approaches represents an individual objective in addressing each of Predictions 5i-5iv. Therefore, these practical objectives are numbered, from 5i-5iv, according to the prediction to which they pertain.

5.2 Materials and Methods

5.2.1 Test species, test compound and overlying water

Organism collection and husbandry is detailed for *Gammarus pulex* and *Asellus aquaticus* in Section 2.2.1. The culturing procedures for *Chironomus riparius* and *Lumbriculus variegatus* are detailed in Section 2.2.1. Radiolabelled (^{14}C) pyrene, fluorene and naphthalene samples were supplied by Sigma-Aldrich (Sigma-Aldrich Company Ltd., Dorset, England). Overlying water used in all experiments was artificial pond water (APW, HSE 1982 and Naylor *et al.* 1989).

5.2.2 Sediment composition and preparation

All experiments utilised artificial sediment made up using the same clay and cellulose detailed in Section 2.2.2.1. Due to the large mass of sediment required, a source of sand that was readily available in bulk quantities was utilised ("SUPAMIX" play pit sand, Pioneer supamix Ltd, Griff Lane, Griff Clara, Nuneaton, Warwickshire, CV10 7PP). Artificial sediment was mixed on a dry-weight basis of constituents in the following proportions: 88% play sand (Section 5.2.2, above), 10% powdered cellulose (Section 2.2.2.1) and 2% Kaolin clay (Section 2.2.2.1).

For Objectives 5i and 5ii artificial sediment was made up in 17.5-kg batches with 15 kg allocated to a 30-l plastic bucket for dosing (see Section 5.2.3) and 2.5 kg directly allocated in 500-g aliquots to five "non-dosed" test vessels (Section 5.2.4). Following bulk dosing within the 30-l plastic bucket (Section 5.2.3) 500-g aliquots of dosed sediment were added to each of 30 "dosed" test vessels (Section 5.2.4). Conversely, for Objective 5iii, artificial sediment was added in 50-g batches to each of 20 non-dosed vessels and each of 80 dosed vessels (Section 5.2.5) whereupon dosing occurred within the test vessels (Section 5.2.3).

5.2.3 Sediment dosing

For Objectives 5i and 5ii, the 15-kg batches of dry artificial sediment (one batch per compound) were spiked with 500 ml of an ethanol stock solution (dosing stock). The dosing stock solution for each compound was prepared via the transfer of the appropriate volume of concentrated refrigerated ethanol stock to 500 ml of ethanol (2000 µl of 0.45mg/ml pyrene, 92µl of 9.78 mg/ml fluorene and 563µl of 1.6mg/ml napthalene refrigerated stock respectively). The transfer of concentrated refrigerated stock to a 1-litre glass beaker, containing approximately half of the final 500 ml ethanol, was performed using a 1-ml Gilson pipette for pyrene and naphthalene and a 200-µl Gilson pipette for fluorene. Subsequently, for each dosing stock, the remaining ethanol (approx. 250 ml) was added to the 1-l beaker to promote mixing of the dosing stock solution. Each dosing stock solution was added to a separate 15-kg batch of artificial sediment that was subsequently thoroughly mixed using a metal trowel. Sediment loadings were, therefore, prepared at 60 ng of each PAH per g dry weight of sediment. Sediment batches were retained within a fume cupboard to control dust evolution due to mixing. Fume cupboard storage also promoted the evaporation of dosing ethanol via the maintenance of a constant air-flow across the mouth of the sediment container. The ethanol was allowed to evaporate over a 7-day period; during which time the sediment was re-mixed daily.

For Objective 5iii, the dosing stock was made up via the addition of 534 µl of 0.45 mg/ml pyrene to 80 ml of ethanol. One millilitre of this dosing stock was added to each 50-g sediment sample within each of 80 dose vessels (vessels described in Section 5.2.5). The dosing, as well as the dosing stock solution preparation, proceeded using

the same method described in Section 2.2.2.2. Again, this resulted in sediments that were dosed at 60 ng pyrene per g dry weight of sediment.

5.2.4 Test system components (Objectives 5i and 5ii)

For Objectives 5i and 5ii, the test vessels consisted of 6-cm diameter, 30-cm tall glass columns fused at one end to the mouth of a glass funnel with a maximum diameter also of 6 cm ("sediment columns" Fig. 5.1). In all exposures this funnel was lined with a 90 mm diameter filter paper folded into a cone (Whatman number one qualitative circles 90 mm, Whatman International Ltd., Maidstone, England). Exposures using sediment dosed with fluorene and naphthalene included a 4-cm deep layer of small glass beads within the filter paper cone (1mm -1.25 mm diameter glass beads, Jencons Scientific Ltd., Cherrycourt Way, Stanbridge Road, Leighton Buzzard, Bedfordshire, LU7 4UA, England). This layer prevented sediment particles from clogging the fine glass tube at the base of the funnel. The use of glass beads was prompted by the incidence of clogging evident in their absence during the first exposure to be performed (48-h exposure of *Gammarus pulex* to pyrene-dosed sediment). Therefore, whilst filter paper cones were used in all exposures for Objectives 5i and 5ii, the glass beads were present in all but the pyrene exposure.

The basal tube of the glass funnel was connected via silicone tubing (3.35-mm internal diameter, 0.635-mm wall thickness, SF medical grade tubing, SF medical, P.O. box 450, Hudson, Ma 01749, USA) to a syringe filter (32-mm acrodisk, 0.45-μm pore size supor membrane, ref 4645, Pall Corporation, 600 S. Wagner Rd, Ann Arbor, MI 48103-9019). The length of this tubing varied between approximately 20 and 30 cm. Plastic clamps were used 4-5 cm above the syringe filter to dictate water flow to the syringe filter as required during system assembly and use. These "pre-filter" clamps were screwed closed for sediment addition. The "effluent" stem of the syringe filter was capped with a blunted hypodermic needle (21-Guage x 38.1-mm length Microlance hypodermic needle, BD 1 Becton Drive, Franklin Lakes, NJ USA). The needle was inserted into fine-bore manifold tubing at the "influent" side of a multichannel peristaltic pump (PVC manifold tubing "Orange/Green" with internal diameter of 0.38 mm and Watson-Marlow 202U (multichannel) peristaltic pump respectively; Watson-Marlow

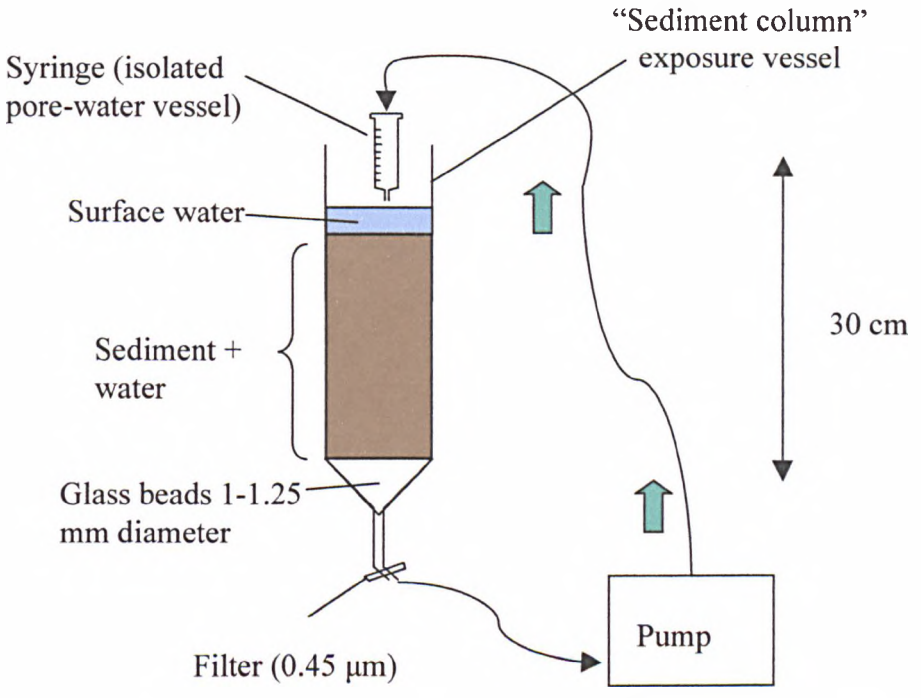


Fig. 5.1 Forty eight-hour exposure vessels used for pairwise whole sediment versus isolated pore-water exposure. Organisms were exposed simultaneously in the isolated pore-water syringe and at the overlying water/sediment interface.

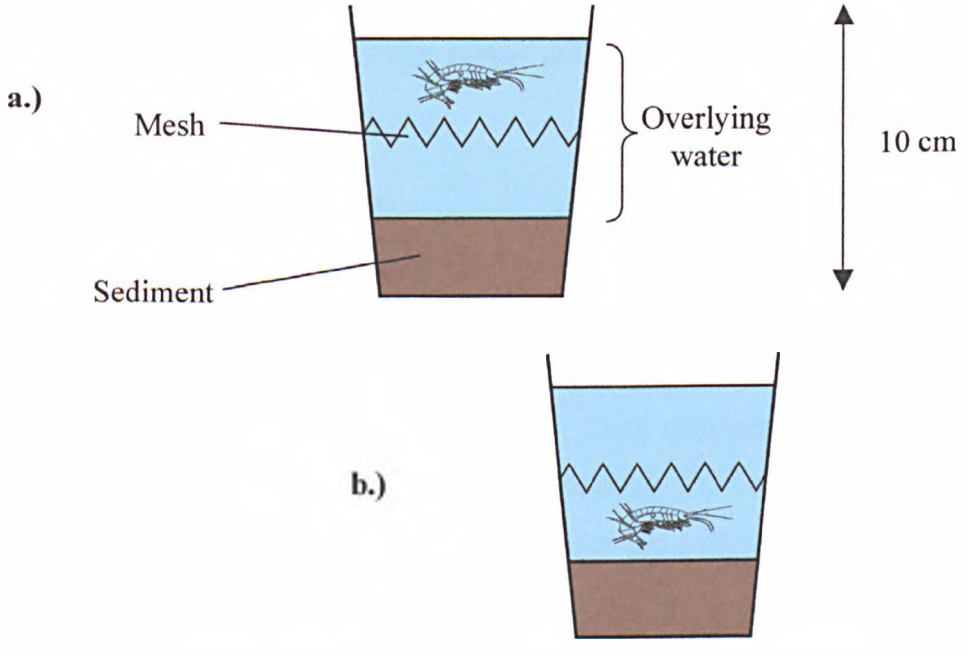


Fig. 5.2 Fourteen-day mesh exclosure exposure vessels **(a)** "sediment excluded" vessel **(b)** "sediment contact" vessel

Bredel Pumps Ltd., Falmouth, Cornwall TR11 4RU, England). At the effluent side the manifold tubing was connected directly to 50-70-cm length of fine silicone tubing (1.575-mm internal diameter x 0.432-mm wall thickness SF medical grade tubing, SF medical, P.O. Box 450, Hudson, Ma 01749). This tubing was connected to a blunted hypodermic needle (25 guage 15.9-mm length Microlance hypodermic needle, BD 1 Becton Drive, Franklin Lakes, NJ USA) that longitudinally pierced a rubber bung used to stopper a 5-ml glass syringe. A hermetic seal was effected by fitting a 5-mm length of vinyl manifold tubing as a sheath to the hypodermic needle. The fine silicone tubing was then fitted over this sheath so that the sheath and silicone tube terminus abutted the broad face of the rubber bung. In this way, the bung was secured between the plastic hub of the hypodermic needle and the tubing terminus. Therefore, when the bung was inserted into the 5-ml syringe, the plastic collar of the hypodermic needle projected into the syringe barrel (Fig. 5.4). Each 5-ml syringe, so stoppered, was secured by means of adhesive tape to the inner surface of the upper rim in each large sediment column (Fig. 5.3). All sediment columns were held in plastic racks (Fig. 5.3) manufactured at The University of Sheffield (Western Bank, Sheffield, S10 2TN). During exposure, the flow-rate was set to 3.6 ml per hour in all cases. The system is designed to maintain equivalent concentrations of PAH within overlying water, syringe water and pore water.

5.2.5 Test system components (Objective 5iii)

The test vessels for comparing tissue loadings achieved in overlying water and whole sediment at both 48-h and fourteen-day exposure durations were much simpler than the sediment columns described above. They consisted of 200-ml plastic cups with 2-mm pore-size plastic mesh partitions (Plastok® Meshes and filtration Ltd. 75-77 Market Street, Birkenhead, CH41 6AN) at half-depth within the overlying water (Fig. 5.2). Animals were either added above the mesh partition (Fig. 5.2a) or below the mesh partition (Fig 5.2b).

5.2.6 Pre-exposure "conditioning" of test vessels with test compound

For Objectives 5i and 5ii, overlying water (500 ml) was added to each sediment column. The pre-filter plastic clamps (Section 5.2.4) were opened to enable the water

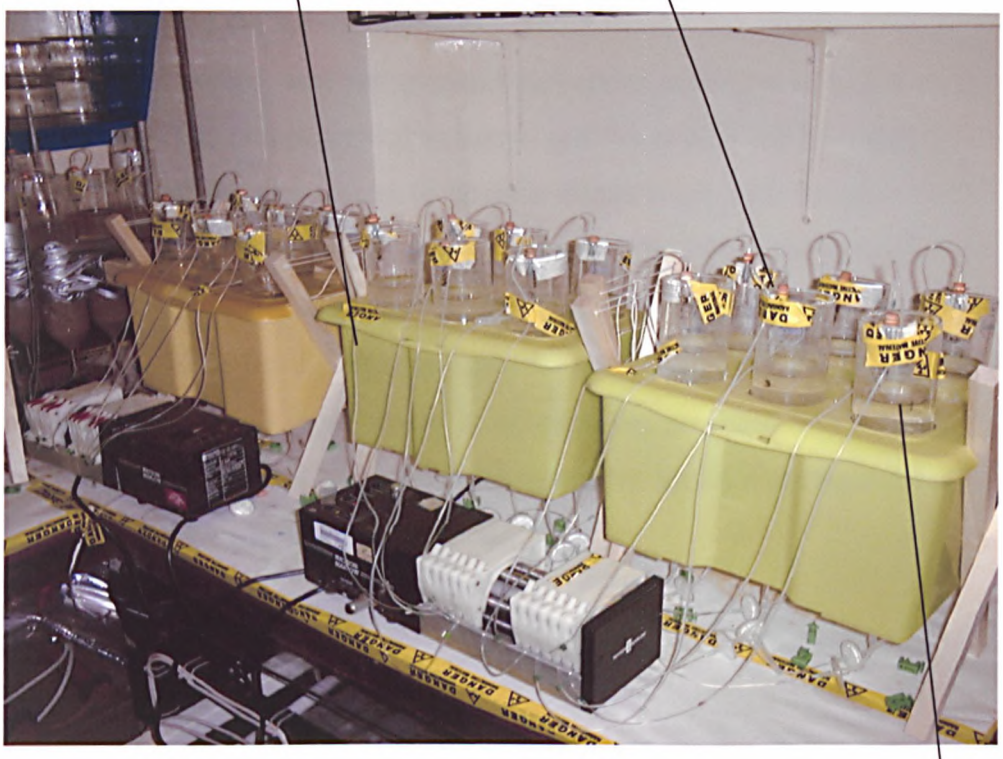


Fig. 5.3 In-situ 48-h pairwise exposure vessels for assessment of isolated pore-water uptake vs. whole sediment uptake

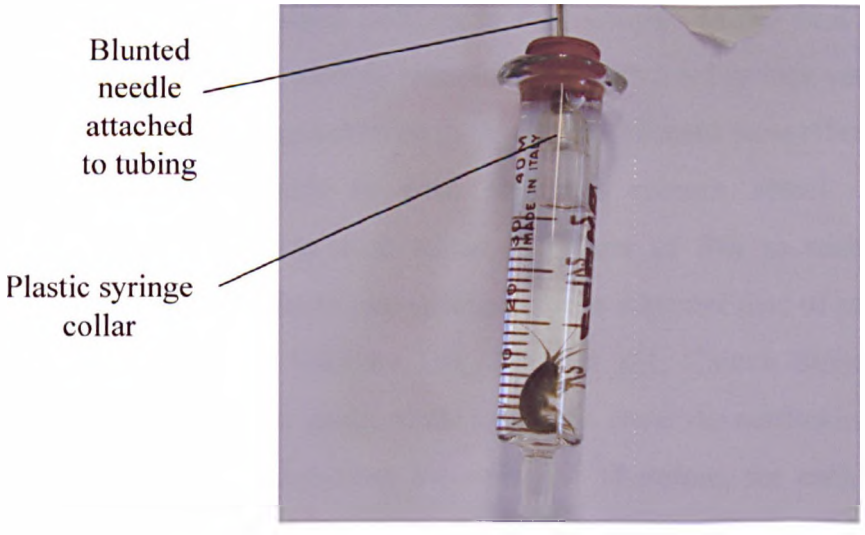


Fig. 5.4 *Gammarus pulex* individual inside isolated pore-water vessel (N.B. lack of gaseous headspace)

to percolate down through the sediment. This was effected by allowing air to escape from the open end of the wide silicone tubing by temporarily disconnecting the syringe filters. Clamps were re-secured and the tubing re-connected to the syringe filter when the water had reached the tubing terminus. Subsequently the clamps were removed completely and the pumps were run at maximum speed to fill the tubing ascending to the 5-ml syringes. The pumps were then halted and the system left for 2 days to allow the inner surfaces of the test system to become conditioned with the test compound. For Objective 5iii, 75 ml of overlying water was added directly to the sediment surface. The sediment was allowed to settle for two days before the mesh partitions were added. These were left in place for a further day prior to the initiation of the test. The initial overlying water level in all vessels used in all exposure protocols was marked on the vessel wall in waterproof pen. In all cases evaporative losses were replaced with de-ionised water added via plastic pasteur pipette.

5.2.7 Animal additions

Objectives 5i and 5ii

At the start of the test each syringe vessel was filled to capacity with overlying water from the sediment column using a plastic pasteur pipette. Syringes were sealed with the bung only after animal addition to the syringe. In the case of crustacean species, single gammarids or asellids were added to each 5-ml syringe vessel. Pairwise comparisons to tissue loadings achieved in the whole sediment were effected by adding single gammarids or asellids to each sediment column vessel. Conversely, lumbriculids and chironomids were added in groups of five to each syringe and sediment vessel. For these latter two species a 1-cm diameter disc of steel mesh (36-µm pore-size, Locker Wire Weavers Ltd., PO Box 161, Church Street, Warrington WA1 2SU) was placed on the inside of the syringe to cover the outflowing nozzle; thus preventing organism expulsion from the syringe. Therefore, for each experimental unit, organisms were exposed to both whole sediment (including pore water and overlying water at equal toxicant concentration) and the isolated pore water derived from that same sediment (within syringe vessels).

Objective 5iii

Half of the gammarids added to dosed sediment vessels were added beneath the mesh screen and therefore had access to the whole sediment ("sediment contact vessels") whilst the remaining gammarids were added above the mesh screen ("sediment excluded vessels") and were exposed only to overlying water. Only one gammarid was added to each vessel. Therefore, half of the gammarids were exposed to whole sediment and half experienced only the overlying water. At no time were "overlying water" organisms exposed within the same vessel as "whole sediment" organisms. Gammarids added to non-dosed vessels were all allowed access to the sediment since their purpose was to provide background radioactive counts achieved in tissues exposed to non-dosed sediment.

5.2.8 Exposure regime

5.2.8.1 Pyrene-dosed sediment and fluorene-dosed sediment exposures to Gammarus pulex for Objective 5i.

Thirty dosed sediment vessels were prepared and used alongside five non-dosed sediment vessels. Each vessel was numbered (from one to thirty five). The exposure duration was 48 h followed by 24-h gut-purging (Section 2.2.2.5). Each gut-purge vessel was labelled with the exposure vessel number as well as whether the animal within was exposed via the syringe or the whole-sediment column. One-millilitre samples of overlying water and syringe water were taken directly from the vessels at the start and end of the 48-h exposure period. One-millilitre samples of pore water were produced by taking 20-ml (approx.) samples of whole-sediment and centrifuging at 3000 rpm for one hour. One millilitre samples of the supernatant were then analysed as pore water for sediment sampled at T0 and T48. Samples of sediment weighing approximately 10 g were taken at T48 and air-dried for a week. Dried sediment sub-samples weighing between approximately 0.05 and 0.15 g were taken for analysis. Similarly, tissue samples were removed from gut purge vessels, blotted and weighed prior to analysis. All samples were analysed for ^{14}C activity as described in Sections 2.2.2.5 and 2.2.3.

5.2.8.2 Naphthalene-dosed sediment exposure to all species for Objective 5i and 5ii

The exposure of a single batch of naphthalene-dosed sediment to *Gammarus pulex* (to fulfill Objective 5i) was combined with the exposure of *Asellus aquaticus*, *Lumbriculus variegatus* and larval *Chironomus riparius* (to fulfill Objective 5ii). This was achieved by preparing 28 dosed-sediment vessels and five non-dosed sediment vessels. Seven dosed-sediment test units (sediment column connected to isolated pore water syringe, Fig. 5.1) were allocated to each species in four 48-h blocks. However, no single species was re-exposed using the same group of seven test units in a subsequent 48-h block. Similarly, one of the five non-dosed test units was allocated to each of the four species for a 48-h exposure period, with an additional test unit allocated to one species at each new 48-h exposure period. This additional test unit was allocated to each of the species in turn at each new 48-h exposure block. Thus a total of 28 dosed-sediment units (seven units in each of four 48-h exposures) and a total of 5 non-dosed sediment units were occupied by each species for 48 h during the 8-day exposure phase. For all test units, samples of overlying water, pore water and syringe water were taken at the start and at the end of the 8-day exposure as described in Section 5.2.8.1. Additionally at the end of each 48-h exposure block, samples of overlying water were taken in the same manner. Tissue samples were taken, following 24-h gut purges (Section 5.2.8.1), at the end of each of the four 48-h exposure periods and analysed as indicated in Section 5.2.8.1.

5.2.8.3 14-day duration pyrene-dosed sediment exposure to Gammarus pulex for Objective 5iii

Ten non-dosed mesh exclosure vessels and forty dosed mesh exclosure vessels were used in a 48-h exposure of *Gammarus pulex* to pyrene-dosed sediment. Simultaneously, another ten non-dosed and forty dosed mesh exclosure vessels were employed in a 14-day exposure. Each of the two groups of 40 dosed vessels (sampled at 48 h and 14 days respectively) consisted, half of vessels in which organisms had access to whole sediment ("sediment contact" vessels) and half of vessels in which organisms were exposed only to overlying water ("sediment excluded" vessels). One-

millilitre samples of overlying water (from the depth of the mesh partition) and pore water were taken from all vessels at 48 h and also 14 day timepoints. Pore water was obtained by sucking approximately 5 ml of sediment slurry into a plastic pasteur pipette plunged at ¾ depth into the sediment. Following centrifugation for 1-h at 3000 rpm, 1-mm samples of supernatant were removed for analysis. Such samples were taken from all vessels (both sediment contact and sediment excluded) after the removal of mesh partitions at the end of each specific exposure period (48 h or 14 days). Sediment samples of between approximately 0.05 and 0.15 g were taken after seven day air-drying of sediment within each of the vessels. Tissue samples taken after 48 h and 14 days were analysed following gut-purging for 24 h (Section 5.2.8.1). All sediment and aqueous samples, once obtained, were analysed by the method indicated in Section 5.2.8.1.

5.2.9 Data analysis

5.2.9.1 Aqueous samples

When the data conformed to the appropriate assumptions, parametric Analysis of variance (ANOVA) with subsequent Tukey's pairwise comparisons at $p<0.05$ was utilised. In other instances, non-parametric approaches were taken since no suitable transformations could be found to improve the compliance of such datasets (Kruskall-Wallace analysis, followed by pairwise differences determined by Bonferroni "z" values). For Objectives 5i and 5ii, all water samples from all timepoints were compared. This enabled comparison between the type of sample (i.e. syringe, pore and overlying water). Additionally, comparisons could be made between sampling times. Thus, compound concentration in different aqueous phases through time was assessed for the repeated 48-h exposures of Objective 5ii. For Objective 5iii, aqueous concentrations were analysed separately for the distinct 48-h and 14-day exposures. Critical Bonferroni z_5 value for $p<0.05 = 2.475$, z_3 value for $p<0.05 = 2.128$ and z_8 value for $p<0.05 = 2.773$. In addition, critical values for Tukey pairwise comparisons for d.f. 5, 160 at $p<0.05 = 4.08$ and d.f. 3, 76 at $p<0.05 = 3.72$.

5.2.9.2 Tissue samples

Objective 5i

The absolute tissue loadings (ngg^{-1}) achieved in each individual isolated pore water vessel (Fig. 5.1 and Fig. 5.4) were divided by the tissue loading achieved in the attached sediment exposure vessel (Fig. 5.1). Subsequently, these replicate "measured proportional aqueous uptake" values were converted to percentages. Arcsine square route transformation of the original proportional data was not observed to improve the fit of the dataset to assumptions of parametric testing. Therefore, differences in percentage aqueous uptake between the three PAH compounds were investigated via a Kruskall-Wallace analysis, followed by pairwise differences determined by Bonferroni "z" values (critical z_2 value = 1.834 at $p<0.05$).

Statistical analysis was, therefore, carried out upon the proportional contribution of aqueous uptake. However, data for absolute tissue loadings were also derived for each compound. These data were used in interpretation of the uptake route driving proportional differences and also to address Prediction 5iv (Section 5.2.9.2, below). The gammarid tissue loadings achieved in isolated aqueous exposure to each compound were compared via ANOVA. Conversely, due to dependence of residual values upon fitted values, Kruskall Wallace analysis (followed by Bonferroni "z" value analysis of pairwise differences) was used to assess variation in sediment uptake. As above, critical z_2 value = 1.834 at $p<0.05$.

Objective 5ii

Paired t-tests were used to analyse the amount of naphthalene uptake achieved in aqueous ventilation versus whole sediment exposure for each species. This was facilitated by the replicated pairwise exposure vessel design (Fig. 5.1). The tissue loading (ngg^{-1}) achieved in each isolated pore water vessel (Fig. 5.1, Fig. 5.4) being paired with the tissue loading achieved in the whole sediment vessel from which the pore water was derived (Fig. 5.1).

Objective 5iii

The tissue loadings achieved in isolated aqueous (overlying water) and whole-sediment exposed organisms after 48 h were compared by Mann-Whitney "U" test (vessels as in Fig. 5.2). The same analysis was used to test whether, after exposure for 14 days, the tissue loadings achieved equivalence between each phase.

Objective 5iv

Tissue loading at equilibrium was estimated via Equation 5.1 (after e.g. Mackay 1982, Connell and Markwell 1990 and Jager 1998).

$$C_{org} = C_{wat} \times F_{lip} \times K_{ow} \qquad \textbf{Eq. 5.1}$$

Where C_{org} is tissue loading within the organism, C_{wat} is the concentration within pore water, F_{lip} is the proportional lipid content of the organism and K_{ow} is the octanol/water partition coefficient. Mean or median (according to frequency distribution) pore water concentrations (C_{wat}) were taken (at the end of each study) from forty eight-hour pyrene, fluorene and naphthalene exposures to *Gammarus pulex*, as well as 48-h naphthalene exposures to *Lumbriculus variegatus*, *Chironomus riparius* and *Asellus aquaticus* (vessels as in Fig. 5.1). Mean pore water concentrations of pyrene during exposure of *G. pulex* for 48 h and 14 days using vessels shown in Fig. 5.2 were taken after 48 h and 14 days respectively. The highest pore water concentration from within 14-day exposure vessels was measured within vessels where the organisms had access to the sediment. Therefore, this value was used for organisms exposed for 14 days as a maximum estimate of tissue loading. Organism lipid contents (F_{lip}) were those measured in Chapter 4 (Section 4.3), converted from percentage values to proportional values. Both the lipid proportional values and PAH tissue loading values were expressed on a wet weight basis. The mean or median PAH tissue loadings (according to frequency distribution) measured in each of the experiments cited above (both isolated aqueous and whole sediment exposed) were then compared to their predicted values by Spearman rank correlation (r_s). This analysis was performed separately for tissue loadings measured in aqueous-exposed and whole sediment-exposed organisms. Additionally, correlation (r_s) analyses (for both aqueous and whole sediment exposures)

were performed with the exclusion of all pyrene data to enable more detailed examination of the lower K_{ow} compounds fluorene and naphthalene.

5.3 Results

5.3.1 Confirmation of nominal sediment loadings

The measured PAH loading within sediment at the end of each exposure conformed to the nominal 60 ngg^{-1} dosed prior to exposure (Fig. 5.5).

5.3.2 Aqueous samples (Objective 5i and 5ii)

5.3.2.1 Syringe water comparison with other aqueous phases

The measured aqueous concentration of each PAH within the syringe vessels was never lower than that measured in pore water. During naphthalene exposure, no significant difference was detected between pore water and syringe water at either the beginning or the end of the 8-day exposure duration ($H_8 = 27.56$, $z < 2.773$, $p > 0.05$). Median syringe-vessel concentrations of fluorene of 0.39 $ngml^{-1}$ were greater than both overlying-water (0.25 $ngml^{-1}$) and pore water (0.21 $ngml^{-1}$) samples at the start of the study ($H_5 = 33$, $z > 2.475$, $p < 0.05$; Fig. 5.6). Similarly, at the end of the 48-h exposure, median fluorene concentrations in syringe vessels of 0.31 $ngml^{-1}$ exceeded overlying water (0.20 $ngml^{-1}$) and pore water (0.24 $ngml^{-1}$) concentrations ($H_5 = 33$, $z > 2.475$, $p < 0.05$).

Mean syringe concentrations of 0.57 $ngml^{-1}$ of pyrene (Fig. 5.7) at the end of the 48-h exposure exceeded concentrations measured in all other samples at the beginning and end of the exposure ($F_{5, 160} = 5.90$, Tukey's pairwise comparison value >4.08, $p < 0.05$), except for 48-h pore water (Tukey's pairwise comparison value <4.08, $p > 0.05$). The mean concentration in 48-h pore water was 0.44 $ngml^{-1}$, whilst the highest remaining concentrations were found in 48-h overlying water; 0.39 $ngml^{-1}$. Mean syringe-vessel concentrations of pyrene at the beginning of the exposure of 0.36 $ngml^{-1}$ differed only from those of above-mentioned 48-h syringe vessel concentrations (Tukey's pairwise comparison value >4.08, $p < 0.05$ versus Tukey's pairwise comparison values of <4.08, $p > 0.05$ for all remaining comparisons).

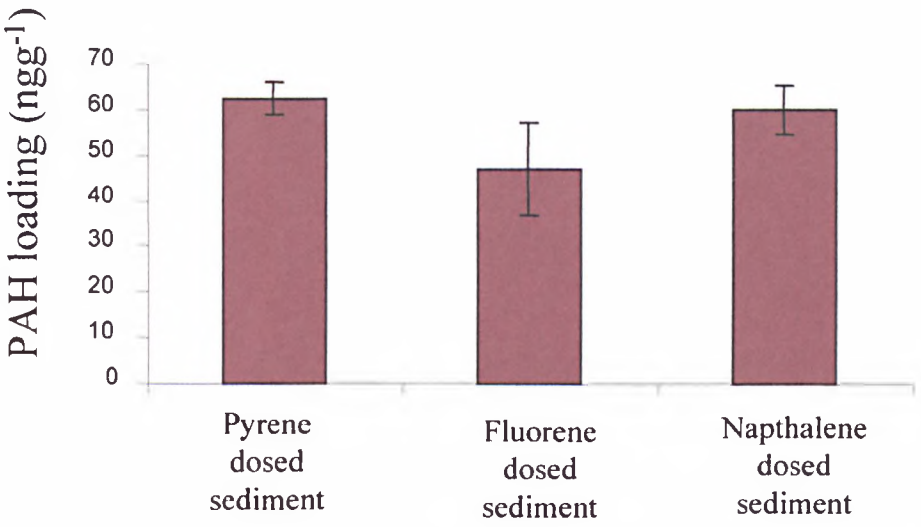


Fig. 5.5 Mean(± SE) loadings of 3 PAH compounds measured within sediment columns depicted in Fig. 5.1.

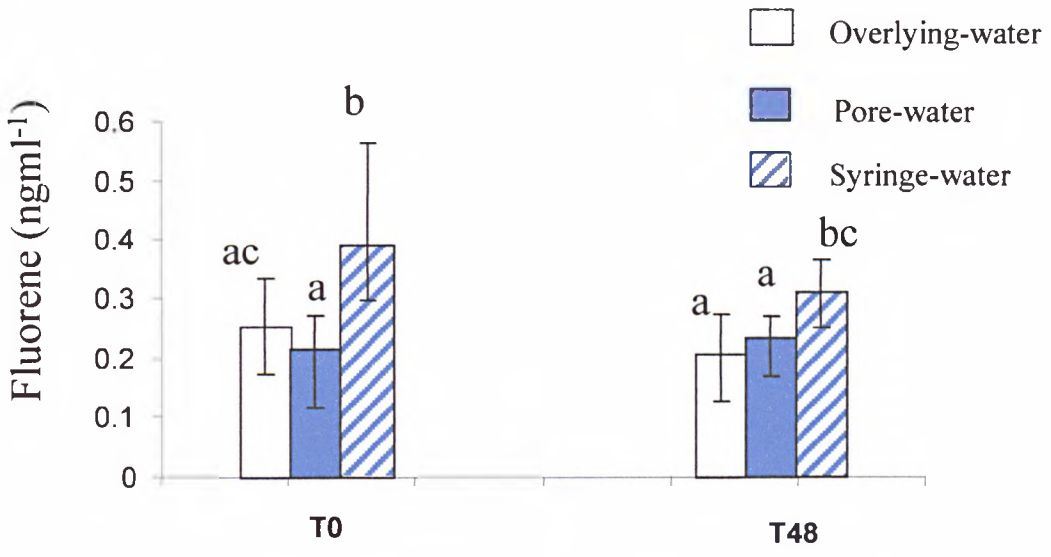


Fig. 5.6 Median(± interquartile range) fluorene 48-h exposure water sample concentrations. Columns sharing the same letter do not differ significantly. Comparisons made across all timepoints and sample types (Bonferroni "z" value comparisons at $p<0.05$)

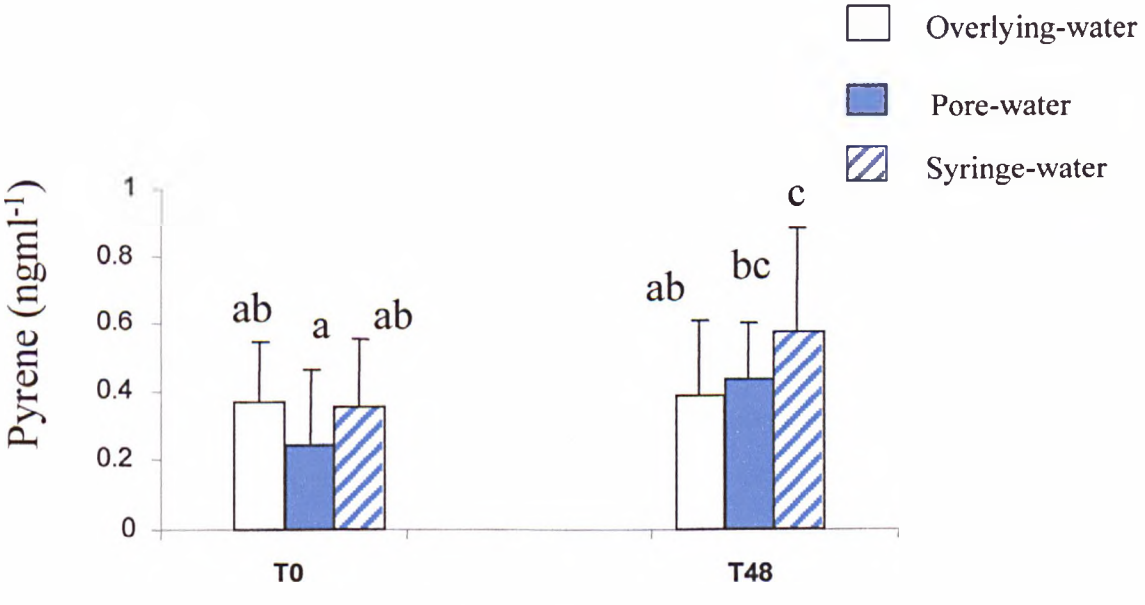


Fig. 5.7 Mean (+SD) pyrene 48-h exposure water sample concentrations. Columns sharing the same letter do not differ significantly. Comparisons made between all sample types and timepoints by Tukey's pairwise comparison values at $p<0.05$

5.3.2.2 Naphthalene aqueous concentrations during 8-day exposure

Out of 33 pairwise comparisons of samples from each aqueous phase and timepoint, five indicated statistical differences (Fig. 5.8). The median overlying water concentration at the beginning of the study (0.27 $ngml^{-1}$) was significantly higher than samples of overlying-water (0.12 $ngml^{-1}$), pore water (0.15 $ngml^{-1}$) and syringe water (0.11 $ngml^{-1}$) at day 8 (H_8 = 27.56, $z > 2.773$, $p<0.05$). The median naphthalene concentration in overlying water at Day 4 of 0.23 $ngml^{-1}$ exceeded that measured in both overlying water; 0.12 $ngml^{-1}$ and syringe water; 0.11 $ngml^{-1}$ at Day 8 ($H_8 = 27.56$, $z > 2.773$, $p<0.05$). The upper interquartile limit of 0.69 $ngml^{-1}$ is large compared to the sample median of 0.23 $ngml^{-1}$ for Day 4 overlying water.

For all PAH exposures, the largest mean or median aqueous concentration value never exceeded the smallest by more than a factor of 2.5 within each specific exposure. Additionally, syringe-vessel concentrations of the test compound were never statistically lower than those experienced by the organisms in the pore water.

5.3.3 Aqueous samples (Objective 5iii)

5.3.3.1 Variation between aqueous sample phase and organism/sediment access

The concentration of pyrene within the aqueous samples of mesh exclosure vessels (Figs. 5.9 and 5.10) was found to vary after 48 h and 14 days of exposure ($F_{3, 76}$ = 84.93, $p<0.001$ and H_3 = 63.48, $p<0.001$). After 48 h there was no statistical difference between the pore water and overlying water either within "sediment contact" vessels or within "sediment excluded" vessels ($F_{3, 76}$ = 84.93, Tukeys pairwise comparison value <3.72, $p>0.05$). However, in "sediment contact" vessels, both mean pore water and overlying water concentrations exceeded the concentrations of both pore water and overlying water in "sediment excluded" vessels at 48 h ($F_{3, 76}$ = 84.93, Tukey's pairwise comparison values >3.72, $p<0.05$).

At 14 days, median aqueous pyrene concentrations (Fig. 5.10) in each aqueous phase and vessel type differed from all others such that "sediment excluded" overlying water < "sediment excluded" pore water < "sediment contact" overlying water <

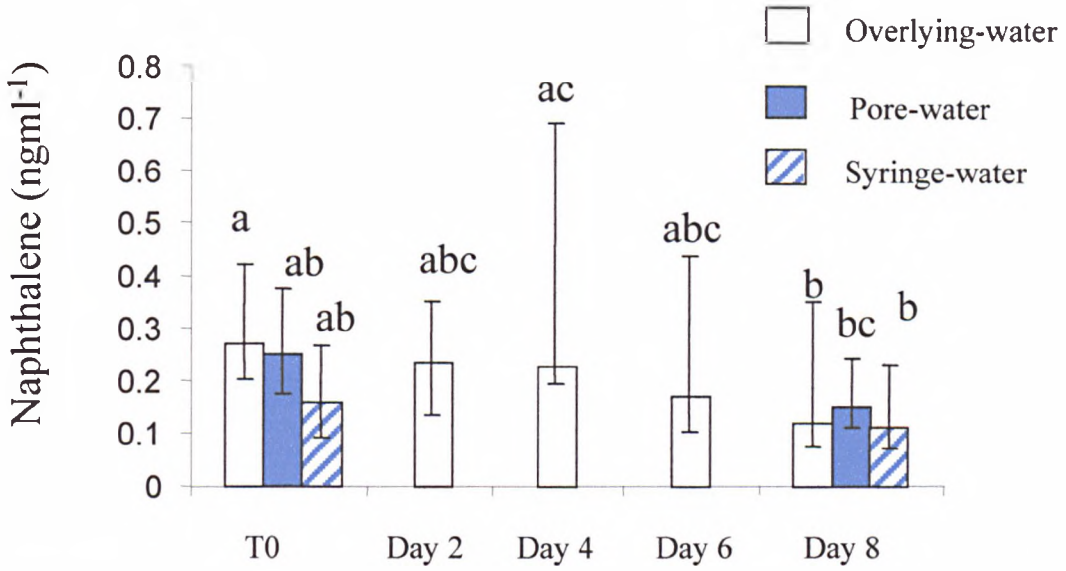


Fig. 5.8 Median(± interquartile range) napthalene aqueous concentration in 4 x 48-h (8-day) exposure. Columns sharing the same letter do not differ at $p<0.05$ determined by Bonferroni "z" value comparisons.

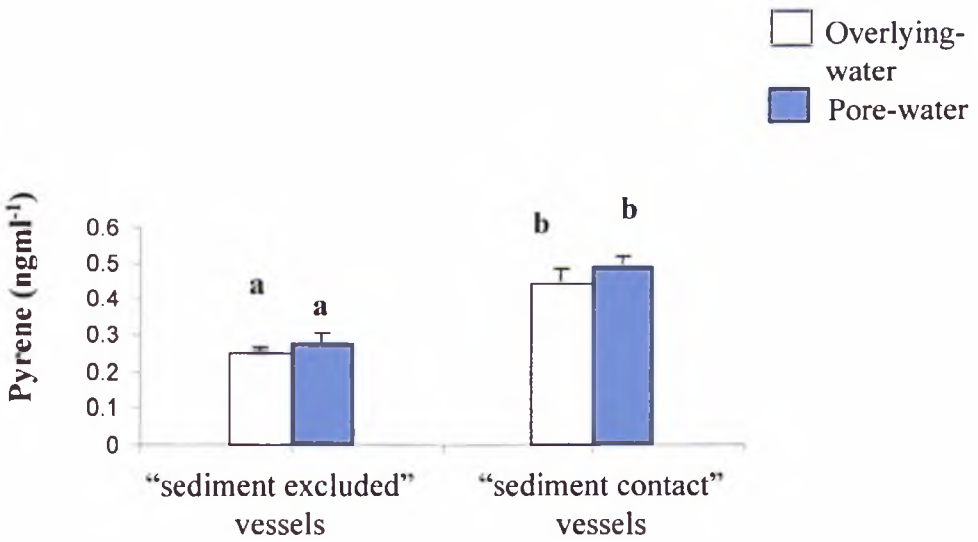


Fig. 5.9 Mean (+SE) pyrene 48-h exposure water sample concentrations of pyrene in mesh exclosure vessels used in exposures of objective 5iii. Columns sharing the same letter do not differ at $p<0.05$ (determined by Tukey's pairwise comparisons).

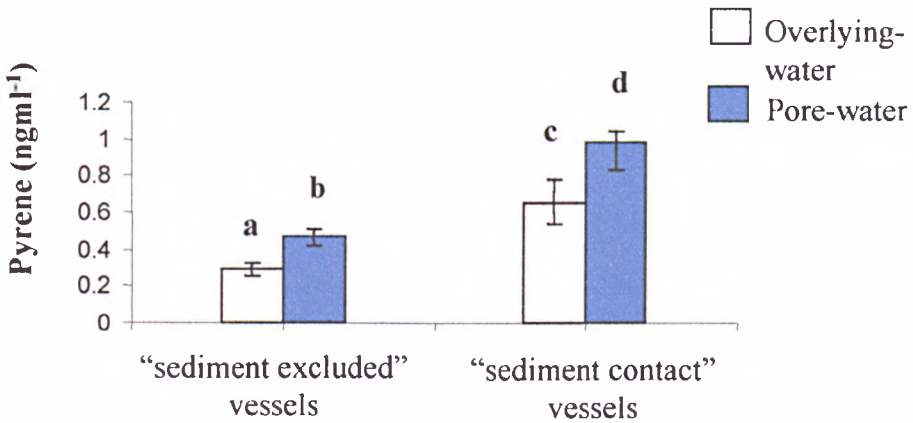


Fig. 5.10 Median (± interquartile range) pyrene 14-day exposure water sample concentrations of pyrene in mesh exclosure vessels used for exposures of objective 5iii. Columns sharing the same letter do not differ at $p<0.05$ (determined by Bonferroni "z" value comparisons).

"sediment contact" pore water (H_3=63.48, z>2.128, p<0.05). The lowest to highest median of concentrations varied between 0.30 $ngml^{-1}$ and 0.99 $ngml^{-1}$.

In summary, pore water concentrations of pyrene in mesh exclosure vessels tended to become higher than the overlying water concentrations at 14 days, but not after 48 h. The contact of animals with sediment appeared to elevate overlying and pore water concentrations of pyrene in both 48-h and 14-day exposures.

5.3.4 Tissue samples

5.3.4.1 Objective 5i: Prediction "Ranked order of compound log K_{ow} (pyrene>fluorene>naphthalene) would predict the hierarchy of proportional uptake attributable to aqueous uptake in Gammarus pulex, such that pyrene<fluorene<naphthalene."

The tissue loadings achieved in pairwise exposures to whole sediment and pore water isolated from that same sediment revealed that the proportion of aqueous uptake varied between the three test compounds (Fig. 5.11, H_2 = 33.35, p<0.001). Specifically, the proportion of aqueous uptake in the highest log K_{ow} compound, pyrene, was significantly lower than in the compound with the next highest log K_{ow} value, fluorene (z > 1.834, p<0.001). Further, fluorene showed a significantly lower proportion of aqueous uptake than the compound of the lowest log K_{ow} value, naphthalene (z >1.834, p<0.01). Therefore, for each PAH, the median (interquartile range) uptake achieved in isolated pore water expressed as a percentage of uptake achieved in whole sediment varied such that pyrene; 1.51 % < fluorene; 34.7 % < naphthalene; 125.3 %.

Gammarus pulex tissue loadings achieved in isolated pore water (Fig. 5.12) did not vary statistically between exposures to pyrene, fluorene and naphthalene ($F_{2,51}$=1.51, p>0.05). This is mirrored by similar bioconcentration factor (BCF) values for each PAH (Fig. 5.12). The BCF values are an expression of the ratio between tissue loading achieved in exposure to isolated pore water and the aqueous concentration of PAH. In contrast, following exposure to whole sediment, median PAH tissue loading varied such that pyrene (565.9 ngg^{-1}) > fluorene (26.85 ngg^{-1}) > naphthalene (5.43 ngg^{-1}) (Fig. 5.13, H_2 = 42.43, z>1.834, p<0.01).

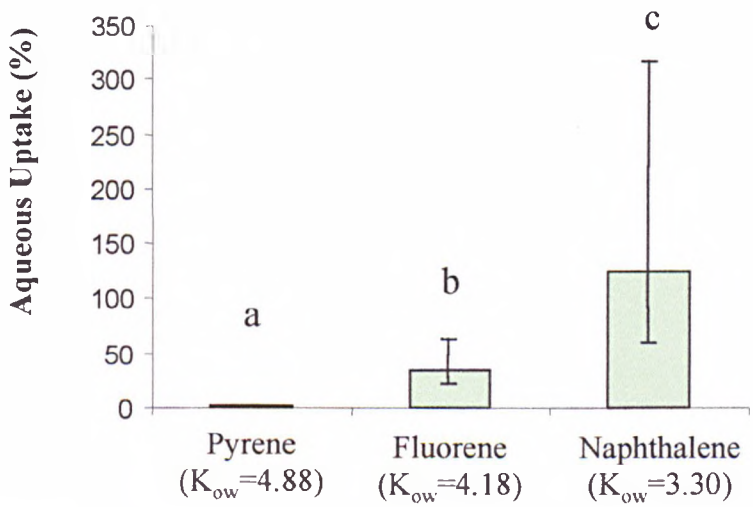


Fig. 5.11 Median (± interquartile range) percentage aqueous uptake (relative to whole sediment uptake) in 48-h exposures of *Gammarus pulex* to 3 PAH compounds. Columns that do not share the same alphabetic label differ at $p<0.05$ (determined by Bonferroni "z" value comparison)

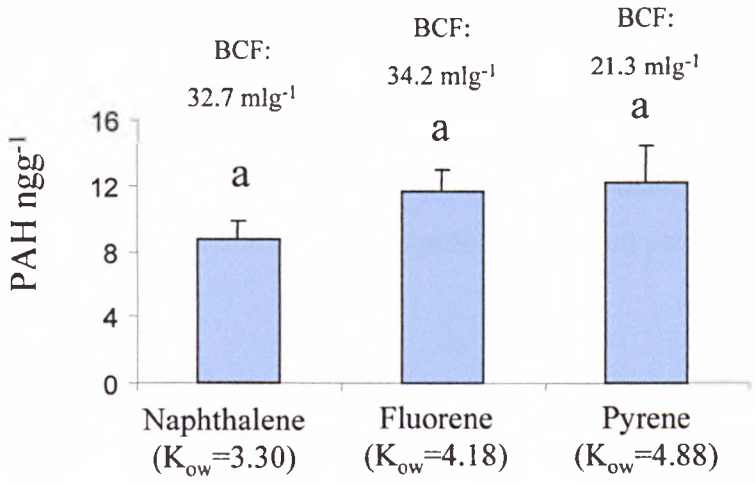


Fig. 5.12 Mean (+SE) tissue loadings (ngg^{-1}) in 48-h exposures of *Gammarus pulex* to 3 PAH compounds in isolated pore water. Analysis of variance did not indicate statistical differences ($p>0.05$). The lack of significant difference is denoted by a shared alphabetical label for each column. Bioconcentration factors (BCF; ratio of tissue loading:water concentration) are also displayed for each PAH.

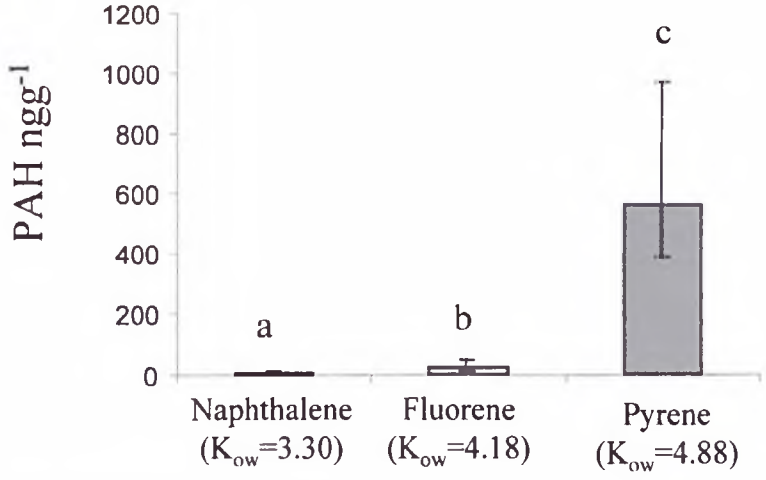


Fig. 5.13 Median(± interquartile range) tissue loadings (ngg^{-1}) in 48-h exposures of *Gammarus pulex* to 3 PAH compounds in whole sediment. Columns that do not share the same alphabetic label differ at $p<0.05$ (determined by Bonferroni "z" value comparison)

Therefore, proportion of whole sediment uptake achieved in isolated pore water matched the predicted hierarchy based upon compound K_{ow}. Further, the proportional variation was effected by large changes in tissue loadings achieved in whole sediment, rather than by changes in tissue loadings exposed to isolated pore water.

5.3.4.2 Objective 5ii: Prediction "Both Gammarus pulex and Asellus aquaticus would achieve a greater proportion of their total naphthalene uptake via ventilation of the aqueous phase than either larval Chironomus riparius or Lumbriculus variegatus".

The two species possessing specialised ventilatory appendages, *Gammarus pulex* and *Asellus aquaticus*, achieved tissue loadings via aqueous exposure that were not significantly different from those achieved in whole sediment (Fig. 5.14, $t_{>20} \leq 1.33$, $p>0.05$). In contrast, both *Chironomus riparius* and *Lumbriculus variegatus*, lacking such physical adaptations to respiratory ventilation, achieved significantly lower tissue loadings via aqueous exposure alone when compared to the uptake apparent from whole sediment (Fig. 5.14, $t_{>22} > 2.33$, $p \leq 0.001$).

5.3.4.3 Objective 5iii: Prediction "For the most lipophilic compound tested (pyrene), both 48-h and 14-day exposures to whole sediment and isolated overlying water would result in significantly greater uptake from whole sediment."

Gammarus pulex individuals that had access to whole sediment for 48 h achieved significantly higher tissue loadings than organisms held in overlying water (Fig. 5.15, Mann- Whitney U test, $U_{20, 20} = 6$, $p<0.0001$). Similarly, in exposures lasting 14 days, sediment ingesting organisms achieved significantly greater uptake than those exposed only to overlying water (Fig. 5.15, Mann-Whitney U test, $U_{15, 14} = 0$, $p<0.0001$).

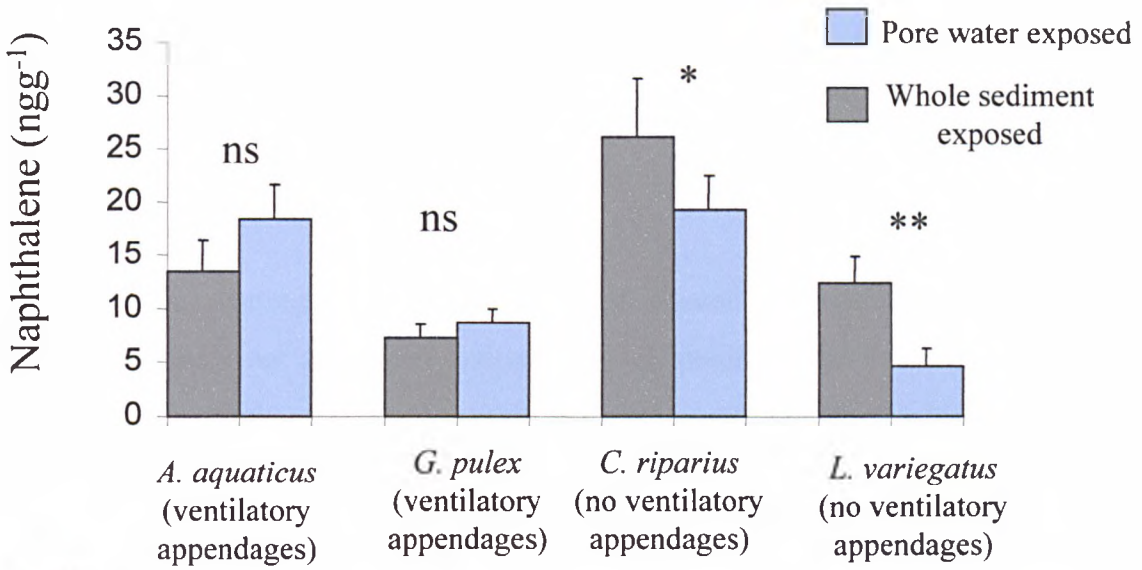


Fig. 5.14 Mean (+ SD) Naphthalene tissue loadings in four benthic invertebrate species exposed for 48 h. Statistical differences determined by paired t-tests. Paired columns bearing "*" notation differ at $p<0.05$, whilst columns annotated "**" differ at $p<0.001$; "ns" denotes no significant difference between pore water and whole sediment exposed tissue loading

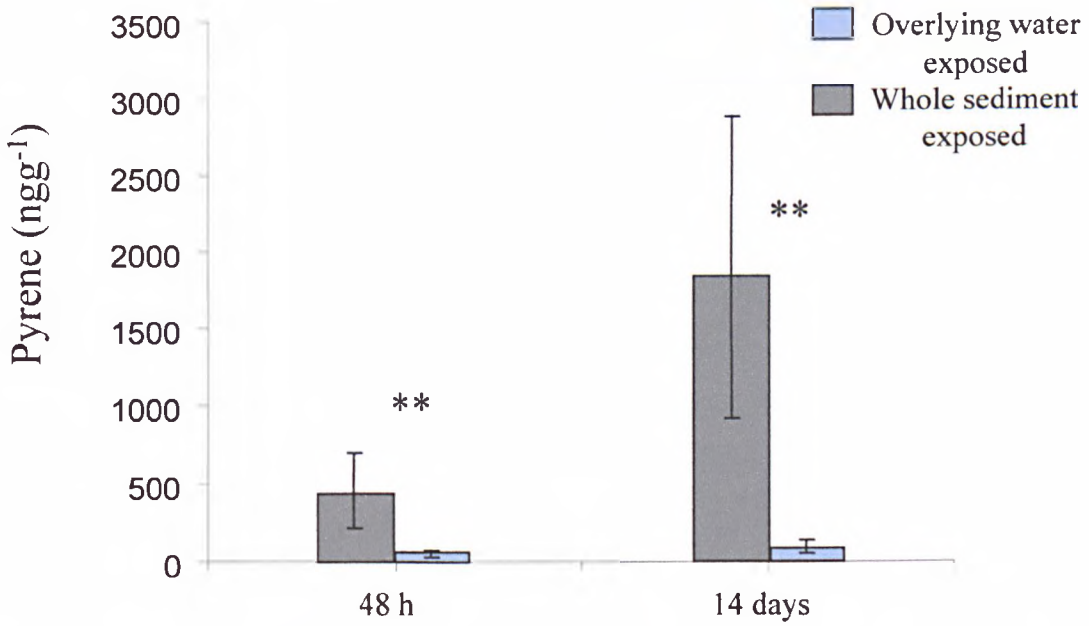


Fig. 5.15 Median (± interquartile range) tissue loadings achieved in 48-h and 14-day exposures to pyrene dosed sediment in overlying water and whole sediment. Sediment exposed tissue loadings were compared to overlying water exposed tissue loadings separately for 48-h and 14-day timepoints using Mann-Whitney U tests. The notation ** denotes statistical differences between paired columns at $p<0.001$

5.3.4.4 Objective 5iv: Prediction "Tissue loadings in all study species, independent of exposure route or duration, would be significantly positively correlated with the theoretical predicted values derived by EqP modelling."

Tissue loadings achieved in isolated aqueous exposures of all 3 PAH compounds were not statistically correlated with predicted EqP values (r_s = 0.500, $p>0.05$). Conversely, exposure to whole sediment resulted in observed tissue loadings that were significantly correlated to EqP predicted values (r_s = 0.762, $p<0.05$). Additionally, it was noted that the highest predicted value, belonging to 14-day pyrene exposure, corresponded to a measured aqueous value that deviated further below the EqP model than 48-h measured aqueous exposure tissue loading (Fig. 5.16). In contrast, the measured "sediment exposed" tissue loading for 14-day pyrene exposure lies above the EqP model line (Fig. 5.16). Therefore, there is no evidence that the increased exposure duration, as in Objective 5iii, enabled convergence of aqueous exposed tissue loadings to either EqP predicted values or measured tissue loadings achieved in whole sediment exposure.

With the exclusion of the pyrene data (Fig. 5.17), no statistical correlation between either aqueous or sediment exposed organisms was observed with EqP predicted values (r_s = -0.381 and r_s = 0.100 respectively, $p>0.05$). Tissue loadings measured in *C. riparius* exposed to naphthalene appear to be high relative to the EqP model in both aqueous and whole sediment exposures (Fig. 5.17). The measured fluorene loadings for sediment and aqueous exposed *G. pulex* tissues were apparently lower than modelled by EqP (Fig. 5.17).

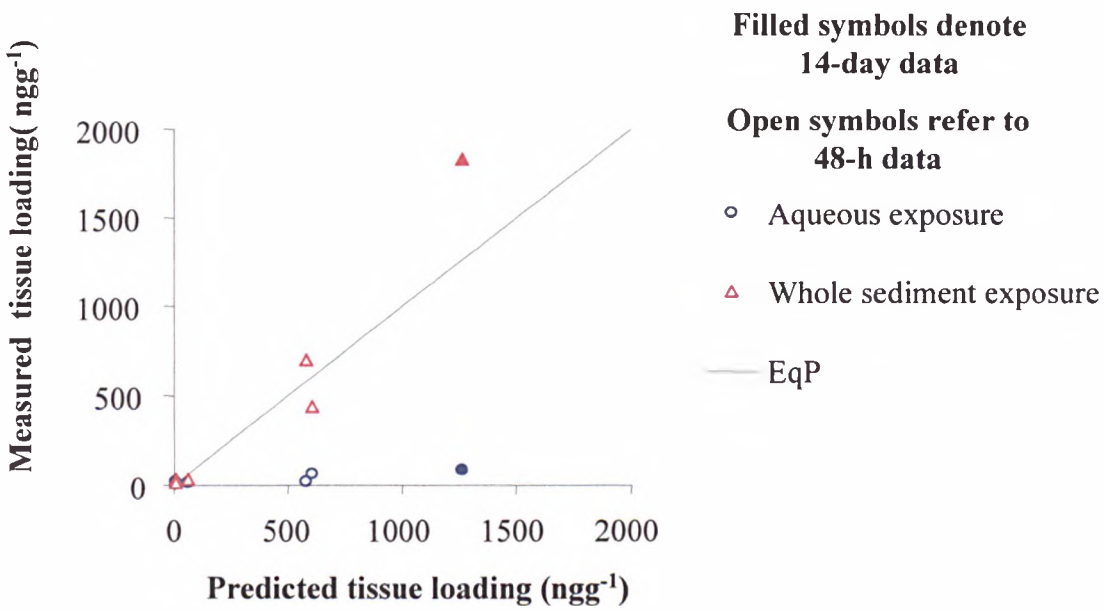


Fig. 5.16 PAH tissue loadings predicted by an EqP approach plotted against measured loadings for pyrene, fluorene and naphthalene. Measured loadings that exactly match predicted loadings would lie on the line "EqP". Measured values are separated into aqueous and whole sediment exposure.

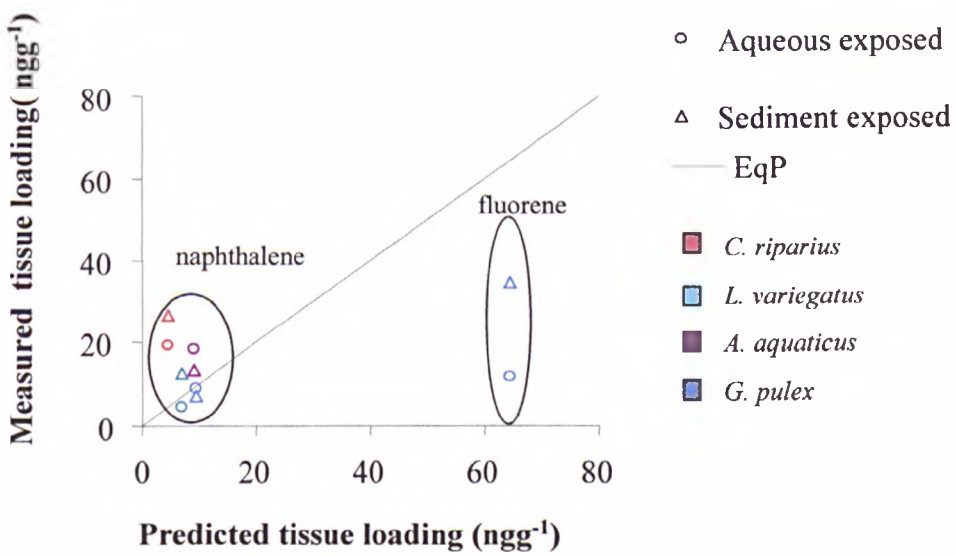


Fig. 5.17 Predicted tissue loadings plotted against measured loadings with exclusion of pyrene data. Measured values that match predicted values lie on the line "EqP". Symbols for each species are colour coded as indicated.

5.4 Discussion

The overall aim of this chapter was to investigate whether compound partitioning behaviour, as well as the degree to which contaminated aqueous phases are processed by organisms, can influence the proportional contribution of aqueous uptake. To this end, investigation was also made into the validity of comparing proportional contributions from aqueous and sediment ingestion on a 48-h timescale. The results obtained strongly suggest that, for naphthalene, fluorene and pyrene, increasing K_{ow} values result in a reduction in the proportional contribution of aqueous uptake. Further, it appears that for fluorene (K_{ow} 4.14) and pyrene (K_{ow} 4.88), exposure to whole sediment resulted in *Gammarus pulex* tissue loadings significantly greater than those that could be achieved in isolated water exposure. In contrast, *G. pulex* tissue loadings of naphthalene (K_{ow} 3.30) did not differ between whole sediment and isolated aqueous exposure. Naphthalene uptake from sediment by *G. pulex*, that can be achieved solely by exposure to pore water, mirrors the uptake of cadmium by *Asellus racovitzai* that is supported by aqueous uptake (Eimers *et al.* 2001).

Aqueous contributions to uptake measured in naphthalene, fluorene and pyrene exposures, varied from ≥100% of whole sediment uptake, to approximately 1.5% of whole sediment uptake. Further, comparison with EqP predicted ultimate values suggests that the proportional contributions measured on 48-h timescales are unlikely to be a function of exposure duration. It is stressed that the use of EqP to generate predictions of what ultimate tissue loadings might be does not imply that the test system was designed to mimic equilibrium conditions. Rather, EqP was used as a tool to examine how closely the different exposure regimes yielded tissue loadings to a theoretical steady-state value. Only whole sediment exposure (rather than aqueous exposure) tissue loadings were significantly correlated with EqP predicted values. Increasing the exposure duration did not improve the fit of aqueous uptake to predicted values or values measured in sediment-exposed organisms. These findings are in accordance with modelling approaches that predict the increased importance of ingestion as a route of uptake with increasing log K_{ow} of compound (e.g. Landrum and Robbins 1990 and Thomann *et al.* 1992).

Landrum and Robbins (1990) predicted that, of two compounds, the one with the lower affinity for sediment particles would be assimilated in greater proportion from the aqueous phase. For example, 88% of phenanthrene uptake was modelled to be due to aqueous uptake, compared to 0% of a more hydrophobic compound, benzo[a]pyrene (Landrum and Robbins 1990). Benzo[a]pyrene was, therefore, predicted to be accumulated entirely by particle ingestion.

Landrum and Robbins (1990) estimate that overall pyrene uptake would be attributable to 74% from the aqueous route and 26% from particle ingestion. Similarly, modelling of an aquatic food web incorporating benthic invertebrates predicts that compounds with log K_{ow} values of 5.5-7.0 are transferred solely by ingestive uptake, rather than potential uptake from pore water or overlying water (Thomann *et al.* 1992). Again, this implies potentially variable contributions to overall uptake from aqueous routes across compounds of varying K_{ow} values. However, for *Gammarus pulex* exposed to pyrene in this chapter, only approximately 1.5% of overall uptake resulted from the aqueous route. Overall, the results reported in this chapter support the view that aqueous and ingestive uptake routes of PAH compounds can be influenced by compound partitioning. Increasing K_{ow} values between naphthalene, fluorene and pyrene tends to reduce the proportion of aqueous uptake in a similar manner to that suggested by e.g. Landrum and Robbins (1990) and Thomann *et al.* (1992).

The observed changes in proportional uptake with varying compound K_{ow} could be caused in three ways. Firstly, variation in uptake achieved from aqueous solute could define the remaining proportion of overall uptake accounted for by particulate ingestion. Alternatively, the reverse could be true, with variation in particulate uptake delimiting the remaining proportion of uptake accounted for by aqueous solute. Finally, there could be simultaneous changes in both routes that result in a new overall balance of proportional contributions.

In this chapter, variation in proportional uptake appears to be mediated by changes in the contribution from sediment ingestion (Figs. 5.11 and 5.12). Variation in gut assimilation leading to variation in proportional uptake route contribution is also noted by Thomann and Komlos (1999). A differential decline in biota/sediment accumulation factor of PAH compounds with increasing K_{ow} was found between an unspecified crayfish species and *Lepomis sp.* sunfish (Thomann and Komlos 1999).

This interspecific variation resulted from a reduction in gut assimilation in sunfish (compared to largely invariant gut assimilation in crayfish) with increasing compound K_{ow} (Thomann and Komlos 1999). Further, this reduction in gut assimilation efficiency was observed to elevate the relative contribution of aqueous uptake (Thomann and Komlos 1999). Therefore, where uptake is largely the result of the ingestion of sediment particles, potential control of bioavailability lies in variations in digestive extraction efficiency. Digestive physiology is further implicated in studies that, using sediment-sorbed contaminants, demonstrate differences in the extraction efficiency of gut fluids isolated from different species (Mayer *et al.* 2001). Mayer *et al.* (2001), in common with Ahrens *et al.* (2001a) and Ahrens *et al.* (2001b), highlight the importance of gut fluid biochemistry (including pH and surfactancy/micelle concentration) in setting upper and lower limits to contaminant absorption efficiency. That solubilisation of sediment-bound organic compounds is much greater within gut fluids than solubilisation in environmental aqueous phases is also highlighted (Ahrens *et al.* 2001b, Mayer *et al.* 2001).

The variation, observed in this chapter, in proportional uptake route measured for naphthalene, fluorene and pyrene (driven by variation in sediment assimilation) could be influenced by gut fluid solubilisation. As stated above, compound/gut fluid interactions potentially enable greater compound absorption than that achieved from aqueous solute present in overlying or pore water. However, the degree to which this operates depends upon the specific interaction between compound and target organism (Mayer *et al.* 2001). With increasing hydrophobicity of study compound, the solubilisation achieved by surfactant gut fluids is expected to diverge more markedly from solubilisation achieved in water alone (Ahrens *et al.* 2001b, Mayer *et al.* 2001). The greater uptake from whole sediment (c.f. isolated water) in pyrene and fluorene exposures appears to support the notion that compound desorption from particles may differ between digestive and aqueous environments. Additionally, the range of compound hydrophobicity (indicated by K_{ow}) exhibited by naphthalene, fluorene and pyrene was matched by the extent of uptake by *Gammarus pulex* from whole sediment sources rather than aqueous sources. Again, this may be expected, following the findings of gut fluid extraction studies (e.g. Ahrens *et al.* 2001a, Ahrens *et al.* 2001b, Mayer *et al.* 2001).

Whereas gut physiology may determine the uptake of sediment-sorbed contaminants, respiratory physiology may determine the uptake of aqueous contaminants (Section 1.1.8 and Section 5.1.3.3). In this chapter, greater proportional aqueous phase uptake of naphthalene was measured in species that possessed physical adaptations to increase the efficiency of respiratory gas exchange. The beating pleopods of both *Gammarus pulex* and *Asellus aquaticus* appeared to be related to the occurrence of aqueous uptake that was indistinguishable from uptake in whole sediment. In contrast, the reliance upon simple respiratory diffusion across cutaneous surfaces in *Lumbriculus variegatus* and *Chironomus riparius* larvae (Brinkhurst and Jamieson 1971 and Armitage *et al.* 1995 respectively) appears to result in significantly reduced aqueous uptake when compared to uptake achieved in whole sediment. Studies conducted in marine species also suggest that the degree to which the aqueous phase is processed influences the uptake achieved by that route. For example Boese (1990) and Kaag *et al.* (1997) cite the facultative control that tellenid clams (*Macoma nasuta* and *Macoma balthica*) exert over the volume of ventilated water as a fundamental determinant of the contribution that aqueous uptake makes to overall assimilation.

The apparent enhanced contaminant uptake from aqueous uptake in the two species possessing ventilatory appendages was not obviously related to available comparisons of relative oxygen uptake rate. For example, an oxygen uptake rate of 1.4 µl of oxygen mg^{-1} dry weight h^{-1} was estimated by converting from a fresh-weight measurement of *Asellus aquaticus* at 11 °C (Hervant *et al.* 1997). The fresh weight to dry weight conversion *of A. aquaticus* was based upon the approximate 80% water content measured in Chapter 4 (Section 4.3.3). The estimated oxygen uptake in *Asellus aquaticus* is apparently similar to examples of measurements in *Chironomus riparius*. Values of between 1.8 – 2.4 µl of oxygen mg^{-1} dry weight h^{-1} have been measured at 7 and 22 °C respectively in larval *C. riparius* (Barker and Wilhm 1982). In comparison, rates of between 5-10 µl of oxygen mg^{-1} dry weight h^{-1} have been measured in *Gammarus pulex* at 10 °C (Rumpus and Kennedy 1974). Barker and Wilhm (1982) cite a lack of precision in the measurements of oxygen uptake (ranging from 1.2 – 4.0 µl of oxygen mg^{-1} dry weight h^{-1}) of *C. riparius* between five replicate samplings. Therefore, comparisons to the values determined in crustacean species should be

treated with caution. Also, comparisons could not be made with all four species since measurements of oxygen uptake in *Lumbriculus variegatus* were not readily available.

In addition to the uncertainty of measurement precision, it is unclear what influence oxygen uptake mechanism may exert upon contaminant uptake. Changes in haemoglobin content within larval *Chironomus thummi-thummi* have been linked to changes in oxygen acquisition from the environment (e.g. Leyko and Osmulski 1985). Conversely, increases in oxygen uptake rate in *Asellus aquaticus* and *Gammarus fossarum* have been linked to increases in pleopod beating rate (Hervant *et al.* 1997). Thus, it is possible that the manner by which increased respiratory uptake is effected may influence the degree to which uptake of contaminant solute is enhanced. The increased volume of aqueous phase processed by increasing ventilatory activity is more readily linked to contaminant uptake than increased carrier-mediated uptake of oxygen. Unfortunately, it proved outside the scope of this project to conduct individual calorimetric determinations of metabolism/oxygen uptake. The potential relationship between ventilatory efficiency and PAH uptake via the aqueous route is, therefore limited, to a qualitative judgement based upon external physical characteristics.

It was also evident in extended pyrene exposures that, where organisms had access to the sediment, the action of bioturbation and ingestive processes appeared to be related to an approximate two-fold increase in the aqueous concentrations of pyrene. This finding is in agreement with studies that have found enhanced release of sediment contaminants due to deposit feeder ingestion and bioturbation (e.g. McCall and Tevesz 1982, Clements *et al.* 1994). Therefore, in addition to the important contribution to uptake within deposit-feeding species, there is potential for the process of sediment ingestion to enhance the exposure of other species that do not, themselves, ingest sediment particles. A similar process is exemplified in the enhanced transfer of sediment-bound fluoranthene to the mussel *Mytilus edulis* due to *Corophium volutator* (amphipoda) bioturbation (Ciarelli *et al.* 1999). Filter-feeding mussel tissue loadings of fluoranthene were found to increase linearly with bioturbating amphipods (Ciarelli *et al.* 1999). This underscores the potential importance of sediment-ingestive processes in the transport of sediment-bound contaminants into aquatic food-webs, both by direct and indirect routes.

5.4.1 Conclusions

The results of this chapter suggest that the proportion of overall uptake accounted for by aqueous uptake in PAH compounds, may be inversely related to K_{ow}. The reduction in proportional contribution from aqueous uptake appears to be controlled by large increases in the amount of dietary uptake. A large disparity between aqueous uptake of pyrene and the uptake of pyrene achieved in whole sediment was observed in 48-h exposure. This disparity was also evident after 14 days of exposure. Increasing the exposure duration of sediment-bound pyrene did not improve the fit of tissue loadings achieved in aqueous uptake to the values predicted by EqP models. The lack of convergence between aqueous and particulate uptake with increased exposure duration underscores the likely importance of digestive desorption kinetics in controlling the observed variation in dietary uptake.

Organism ventilatory physiology may potentially influence the proportional assimilation of compounds from the aqueous phase. The presence of adapted ventilatory appendages was apparently linked to increased proportional uptake of the aqueous fraction of naphthalene. However, the direct comparison of the rates of respiratory gas exchange and contaminant assimilation is complicated by the various physiological mechanisms that may be involved in oxygen uptake. For example, differences in ventilatory behaviour can be offset by variations in respiratory pigment physiology.

It is also apparent that the activities of benthic invertebrates may lead to enhanced exposure of pelagic organisms to sediment contaminants. In addition to established potential for food-chain transfer of assimilated sediment contaminants (Section 1.1.1), bioturbation of contaminated sediment was observed to enhance concentrations of pyrene within the water column. Benthic invertebrates may, therefore, enhance the transport of sediment contaminants into pelagic biota by both direct and indirect pathways.

Chapter 6: General Discussion

6.1 Main findings

The potential long-term risk posed by sediment associated hydrophobic organic compounds to aquatic food-chains was highlighted in the first chapter of this thesis (Section 1.1.1). Chronic persistence of compounds at levels greater than experienced in the water column, coupled with unpredictable transfer from sediment to biota (Section 1.1.1), prompted the experimental work detailed in Chapters 2-5. The overall aim of such experimental work was to examine whether sediment-sorbed contaminants could be bioavailable to benthic invertebrates through ingestive uptake.

It was hypothesised that exposure of sediment-ingesting species to a compound that was sorbed to sediment particles, and that was resistant to aqueous desorption, would result in dietary uptake of that compound. Within this main hypothesis, it was suggested that dietary uptake would vary between species in proportion with the amount of sediment processed by invertebrate digestion. Further, the hypothesis suggested that compounds associated with sediment would be assimilated in decreasing proportion from the aqueous route as compound hydrophobicity increased. Similarly, for compounds that can be assimilated from both aqueous and particulate sources, the presence of specifically adapted ventilatory appendages was hypothesised to increase the proportion of total uptake achieved via the aqueous route. The main findings of experimental work carried out to address this main hypothesis are detailed subsequently.

Four benthic macroinvertebrate species, *Lumbriculus variegatus*, *Chironomus riparius*, *Asellus aquaticus* and *Gammarus pulex*, were found to achieve measurable tissue loadings of a sediment contaminant known to resist aqueous desorption: Dioctadecyldimethylammoniumchloride (DODMAC). This apparent uptake varied between species exposed to compound dosed at a constant loading into sediment. *Chironomus riparius* achieved the greatest tissue loading of all species via exposure to artificial sediment and field-collected sediment dosed in the laboratory with DODMAC. The type of sediment also appeared to influence the variation in tissue loadings achieved by the remaining three species. *Asellus aquaticus* and *Gammarus*

pulex both achieved greater apparent uptake in artificial sediment than *Lumbriculus variegatus*. In contrast, *Lumbriculus variegatus* and *Gammarus pulex* achieved equivalent uptake from field-collected sediment, whereas *Asellus aquaticus* apparently achieved greater uptake in the field-collected sediment. Contrary to a priori predictions (Section 2.1.2.1), both *Asellus aquaticus* and *Gammarus pulex* ingested sediment. Substrate ingestion behaviour in the two crustacean species agrees with the omnivorous nature of *Asellus aquaticus* recorded by Marcus *et al.* (1978).

The assumption that DODMAC measured within the entire organism homogenate is representative of genuine assimilation required confirmation. This was prompted by the predicted non-bioavailable nature of DODMAC from sediment particles based upon a lack of aqueous desorption (Section 1.1.4). Feeding individuals of *Asellus aquaticus* and *Lumbriculus variegatus* achieved much higher tissue loadings than non-feeding individuals of the same species. Additionally, moulted *Asellus aquaticus*, *Gammarus pulex* and *Chironomus riparius* individuals did not rid their tissues of assimilated DODMAC. The visualisation of DODMAC distribution within sections of exposed organisms also suggested that the compound was genuinely assimilated. The concentration of DODMAC upon the surface of the midgut gland lobes in *Asellus aquaticus* was particularly noted.

When exposure to DODMAC within artificial sediment was extended from 48 h to 14 days, three of the species achieved equivalent tissue loadings. *Gammarus pulex*, *Asellus aquaticus* and *Lumbriculus variegatus* tissue loadings all converged to the same asymptote. However, *Chironomus riparius* larvae still achieved a significantly higher 14-day tissue loading than the remaining three species investigated. Differences in tissue loading measured during acute exposure can, therefore, be the result of variable short-term rates of uptake. However, the persistence of greater uptake in *Chironomus riparius* indicates that not all uptake trajectories converge to a comparable maximal loading. Therefore, the overall capacity of tissue to accumulate a compound may also be an important determinant of tissue loadings.

The experimental work reported in Chapter 4 generally revealed factors that could not explain variation in tissue loadings of DODMAC. Selective feeding, biotransformation, tissue lipid content, tissue water and gut retention time were all found to be unlikely to control variation in uptake of DODMAC. The review of

literature, whilst not providing definitive causal mechanisms, provided valuable insights into further potential mechanisms. Most notable amongst possible mechanisms were solubilisation of powdered cellulose, the surfactancy of gut fluids and the potential for enhanced fatty compound assimilation with specific developmental stages.

The same four study species exposed to DODMAC were used in exposures to polyaromatic hydrocarbon (PAH) compounds. All four species were exposed to naphthalene using an experimental system that allowed measurement of proportional aqueous contribution to uptake. Additionally, *Gammarus pulex* was exposed to pyrene and fluorene using the same exposure system as used for naphthalene.

A reduction in proportional contribution from aqueous sources with increasing compound K_{ow} was observed in *Gammarus pulex*. Pyrene was assimilated in the smallest proportion from the aqueous route. Fluorene showed the next smallest aqueous uptake contribution, followed by naphthalene. The change in proportional contribution appeared to be driven by variation in contributions from sediment ingestion. When uptake was restricted to the aqueous route, there was no statistical correlation between measured tissue loading and values predicted by an equilibrium partitioning model. However, when organisms had access to whole-sediment, there was a significant correlation between observed and predicted tissue loadings. Therefore, it is apparent that the theoretical maximum tissue loading (based upon tissue lipid content), was only achieved when both aqueous and particulate contaminant carrying fractions were processed.

In addition to the importance of dietary physiology, ventilatory physiology was also linked to the proportion of uptake achieved from aqueous versus dietary routes. The presence of specialised ventilatory appendages in two species (*Gammarus pulex* and *Asellus aquaticus*) appeared to enable greater proportional aqueous uptake of naphthalene than that achieved by two species lacking such appendages (*Lumbriculus variegatus* and *Chironomus riparius*).

Tissue loadings of pyrene achieved in 14-day exposure to *Gammarus pulex* exceeded equilibrium partitioning-predicted values by approximately 50%. Conversely, both *Gammarus pulex* and *Asellus aquaticus* achieved uptake of naphthalene that did not differ between exposure to isolated pore water and exposure to whole sediment. It

was apparent, therefore, that uptake of naphthalene by *Gammarus pulex* and *Asellus aquaticus* can be supported solely by processing of the aqueous phase. In contrast, *Gammarus pulex* must process sediment particles in order to support the maximal tissue loadings observed in fluorene and pyrene. It was also found that overall napthalene uptake in *Chironomus riparius* exceeded that of *Gammarus pulex* and *Asellus aquaticus*. In addition, both dietary and aqueous uptake of naphthalene in *Chironomus riparius* was much greater than the values predicted by equilibrium partitioning.

6.2 Implications of main thesis findings to risk assessment of contaminated sediment

Section 1.1.1 highlighted that organic contaminants of sediments pose a potential long term hazard to aquatic ecosystems. The degree to which that hazard is realised depends upon the toxic action of such contaminants and the extent of exposure that sensitive organisms experience. The quantification of such risks forms the basis of ecological risk assessments.

Risk assessments of environmental contaminants consist of three fundamental processes. Firstly, the identification the inherent harmful properties of a particular contaminant (i.e. hazard identification). This is followed by assessments of the likely exposure (and effects of such exposure) of organisms to a contaminant. Finally, the predicted incidence and extent of harmful effects are estimated based upon the combination of the hazard and likely exposure (after e.g. van Leeuwen and Hermens 1995, Chapman 2001). O'Connor and Paul (2000) concluded that chemical measures of sediment contaminants were a poor assessment of biological exposure. The notion that chemical analysis may be a poor predictor of biological effects of contaminated sediment has recently been endorsed by the statement "toxicity currently is defined as a biological response that is best measured directly" (Chapman *et al.* 2002). Therefore, this prompts investigation of the biological determinants of exposure to sediment contaminants.

The established unpredictable nature of contaminant transfer from sediments into biota (Section 1.1.1) makes ecological risk assessments of sediment contaminants

difficult to perform (Solomon and Sibley 2002). In contrast, ecological risk assessments relating to water column pollutants have benefited from improved levels of sophistication due to the relative ease of quantifying exposure to contaminant solute. For example, probabilistic assessments of water-column contaminant effects using statistical distributions of species sensitivity have been applied to field and microcosm communities (reviewed in Solomon and Sibley 2002). The use of the distribution of species sensitivity, where carefully applied, may (potentially) enable improved accuracy and relevance in ecological risk assessment (Forbes and Calow 2002). Solomon and Sibley (2002) specifically cite the unpredictable nature of contaminant bioavailability as the main obstacle to implementing more sophisticated risk assessments of contaminated sediment. Previous attempts to derive simple predictive measures of contaminant bioavailability show the complexity of factors controlling exposure to sediment contaminants. For example, within the compounds that it was designed for, equilibrium partitioning modelling can be a useful broad estimation of potential bioavailability (Section 1.1.2). However, in addition to application that is restricted to non-polar organic compounds, the limitations of equilibrium partitioning lie in the observation of substantial departures from predicted bioavailability and the assumption that systems are at equilibrium (Section 1.1.2). Therefore, improved quantification of the mechanisms of contaminant transfer from sediment into biota would allow more accurate prediction of bioavailability, and hence, exposure.

Desorption from substrate within the gut is strongly implicated as being a powerful determinant of bioavailability in hydrophobic organic compounds within sediment. The importance of gut-desorption in determining bioavailability was supported by the uptake of DODMAC observed in Chapter 2 and Chapter 3. Therefore, the ability to quantify or predict desorption of compounds in the gut is likely to be a significant boon to exposure assessments of sediment contaminants. Where the combination of gut environment and compound chemistry results in solubilisation that is comparable to simple aqueous partitioning, aqueous desorption kinetics (Section 1.1.4) may give a suitable indication of bioavailability. Similarly, where compound chemistry and organism ventilatory biology result in uptake that can be supported entirely by aqueous ventilation, aqueous desorption kinetics may, again, give an appropriate indication of bioavailability. Conversely, where organism gut physiology combines with hydrophobic compounds to produce solubilisation that is not well

described by simple aqueous partitioning, quantification of gut solubilisation is required. Desorption in the gut has been described in terms of the alimentary canal acting like a chromatography column, with the sediment particles as the solid phase and the gut juices as the mobile/solvent phase (Simkiss *et al.* 2001). Simkiss *et al.* (2001) suggest that for compounds of differing lipophilicity, both the attraction for the sorbant particles and the nature of the gut-juice solvent phase are important determinants of bioavailability. The apparent importance of interspecific variation in gut environments is exemplified by large differences in uptake of phenolic compounds in *Lumbriculus variegatus* and *Chironomus riparius*, despite identical compounds and particles (Davies *et al.* 1999b). However, in order to discern when simple aqueous desorption cannot describe bioavailability (i.e. when gut physiology produces departures from simple aqueous kinetics) requires a great deal of knowledge of the species' ecology and physiology as well as properties of the contaminant. At present, there is a shortfall in our understanding of the biological parameters controlling such bioavailability.

As well as the importance of the nature of gut-juices acting as a solvent phase, the nature of sorbant material appears to be important. The pattern of tissue loadings achieved in four species exposed to DODMAC in Chapter 2 was clearly different between artificial and field-collected sediment. Similarly, variation in *Lumbriculus variegatus* bioaccumulation factors is apparent during exposures to compounds sorbed to different artificial particles and natural sediments (Simkiss *et al.* 2001). Changes in both toxicity and tissue loadings of e.g. prochloraz and permethrin have also previously been observed between different substrates in *Chironomus riparius* (Barrett 1995, Fleming *et al.* 1998 respectively). One apparently important variable in substrate composition is the quality of the organic carbon. Gunnarsson *et al.* (1999) found that assimilation of tetrachlorobiphenyl by *Amphiura filiformis* was greatest in sediments amended with labile organic material. Conversely, amending sediment with an identical proportion of refractory organic material supported much lower contaminant assimilation (Gunnarsson *et al.* 1999). A similar process was highlighted in the potential for differences in cellulose digestion between the four species exposed to DODMAC in artificial sediment (Section 4.3.6). Substrate susceptibility to digestion as well as particle-surface/compound attraction (after e.g. Davies *et al.* 1999a, Lawrence *et al.* 2000, Simkiss *et al.* 2000), therefore, appears to have the potential to

influence bioavailability. Thus, in addition to the interaction of aqueous fractions of contaminants and ventilatory physiology, the complex interplay between dietary physiology, compound chemistry and substrate properties appears to control uptake from sediment particles.

The work performed in this thesis, therefore, prompts quantification of the ecological and physicochemical factors that control exposure route and compound-uptake. Specific processes that are highlighted to be important in efficacy of contaminant assimilation are substrate solubilisation capacity, gut surfactancy (Section 4.3.6) and aqueous ventilation biology (Section 5.3.3.2, Section 5.4).

In addition to contaminant extraction efficiency, tissue morphological features controlling total contaminant storage capacity (i.e. lipid content or protein-content Section 4.4) also appear to influence contaminant uptake. Such morphology has the potential to change with developmental stage. Peaks in lipid assimilation and lipid content within tissues have been noted prior to moulting and metamorphosis stages in arthropods (e.g. Dadd 1973, Gibson and Barker 1979, Cargill *et al.* 1985; cited in Calow and Petts 1992). West *et al.* (1997) recorded an increase in tissue lipid in 4th instar *Chironomus tentans* to 1.08 % (±0.12 %) compared to the 0.82 % (±0.01 %) measured in 3rd instar larvae. In this project, *Chironomus riparius* had the lowest measured lipid content of the four species exposed to DODMAC. However, accumulation of DODMAC may have resulted in increased lipid content of DODMAC exposed chironomids. A single sample of homogenised chironomid tissues exposed to DODMAC was found to contain more than double the lipid content measured in non-dosed tissue homogenate (S. Harding Pers. Comm.). It is currently unclear whether intact DODMAC molecules can elevate the lipid content measured by the assay used in this project (Appendix 4.2). The existence of enhanced lipid assimilation in 4th instar *Chironomus riparius*, and the potential knock-on effect upon enhanced DODMAC assimilation requires further investigation. Similarly, whether the fatty components of intact DODMAC molecules can elevate measured tissue lipid also requires experimental validation.

The notion of characterising the range of ecological and physiological parameters that influence bioavailability is in agreement with cited desirability of greater incorporation of ecology within ecotoxicological risk assesment (e.g. Chapman

2002). Factors that may influence assimilation efficiency and uptake capacity, identified above, provide an indication of some characteristics that could be used to generate more accurate ecological risk assessments of sediment contaminants. The prime obstacle of uncertainty in exposure (bioavailability) that makes risk assessment of contaminated sediments difficult (Solomon and Sibley 2002) is highlighted in this project. However, areas of study that will aid quantification of bioavailability have also been identified.

The immediate benefit of characterising uptake into benthic invertebrates is to allow better modelling of toxicity to sediment biota resulting from sediment-associated contaminants. For example, Lee *et al.* (2002a) and Lee *et al.* (2002b) combined improved characterisation of compound uptake into organism tissues with cumulative toxicity through exposure duration to describe and predict PAH toxicity in *Hyallela azteca.* Clearly, in order to describe toxicity through time as a function of tissue loading, the ability to understand what controls uptake and tissue loading is paramount. Although invertebrate toxicity is beginning to be described in terms of internal loadings of contaminants, it is presently unclear whether the different routes of uptake within a single toxicant can elicit different toxicity. This project highlighted enhanced aqueous-phase assimilation of naphthalene in response to gill-ventilation. It is not yet apparent whether the increased importance of ventilatory uptake route has implications for the mode of toxic action of a compound. Currently there are too few datasets to enable meaningful comparisons of uptake route and toxicity within gilled and non-gilled species to sediment-associated organic compounds. Generally, the characterisation of proportional contributions from different uptake routes is lacking for the majority of compounds. Also, the mode of toxicity is not routinely determined for sediment biota. For example, differences in phenanthrene toxicity to *Chironomus riparius* and *Hyalella azteca* (Verrhiest *et al.* 2001) are potentially explicable in terms of internal tissue loading, mode of action controlled by route of uptake or a combination of both. However, present knowledge is insufficient to allow identification of the causal factors of such variation.

As well as direct exposure of sediment-ingesting benthic invertebrates, uptake of sediment contaminants has implications for the exposure of disparate sections of the food-web. Benthic invertebrates are an important conduit for food-chain transfer of sediment contaminants to benthivorous fish (Section 1.1.1, and e.g. Chapman 2001,

Egeler *et al.* 2001). However, two other factors that can be exhibited by benthic invertebrates make the transfer of contaminants from sediment to invertebrates especially important in ecological risk assessment. Firstly, although they can be sensitive to pollutants (e.g. Chapman *et al.* 2001), benthic invertebrates can exhibit physiological characteristics that make them relatively insensitive to pollutants that are toxic to vertebrates. For example, the lack of the aryl hydrocarbon receptor in aquatic invertebrates renders them insensitive to dioxin toxicity (West *et al.* 1997). The observation that *Lumbriculus variegatus* and larval *Chironomus tentans* readily accumulated a dioxin compound represents a significant risk to their vertebrate (fish) predators. The significance of this risk is inferred from the observation that vertebrates are more sensitive to dioxin toxicity by approximately four orders of magnitude (West *et al.* 1997).

In addition to the potential to bioaccumulate levels of pollutants that can be toxic to benthivorous predators, some invertebrates can act as a direct vector to terrestrial foodwebs. For example, Baron *et al.* (1999) assessed the risk to aerial insectivorous predators (two bat species, *Myotis lucifugus* and *Myotis grisescens* and the rough winged swallow *Stelgidopteryx serripennis*) to contaminated sediment. Sediment contaminants that were accumulated by benthic larval stages of mayflies (identified to order Ephemeroptera) were measured in captured adult mayflies. Baron *et al.* (1999) concluded that the foraging insectivorous species were unlikely to suffer adverse population-level effects in this system. However, it was noted that toxicity to individual aerial predators was likely (Baron *et al.* 1999). The potential for larval benthic invertebrates to transport sediment contaminants to terrestrial predators is, however, well illustrated by this example.

Out of the four species studies in this project, *Chironomus riparius* larvae achieved the greatest uptake of DODMAC in artificial and field-collected sediment during both 48-h and 14-day exposures. No acute deleterious effects were observed in response to the higher tissue loadings in *Chironomus riparius*, as there were no apparent toxic effects of accumulated DODMAC in any of the four study-species. Further, *Chironomus riparius* also achieved the greatest uptake of naphthalene when allowed to ingest sediment particles. Again, no toxicity was apparent in exposures of the four species at the loadings of PAH experienced. Given that *Chironomus riparius* also has a flying adult developmental stage (Armitage *et al.* 1995, Oliver 1971),

accumulation of hydrophobic organic compounds by *Chironomus riparius* is an important consideration for the ecological risk assessment of contaminated sediment. In compounds that are tolerated by *Chironomus riparius*, the importance of this consideration is underscored by the observed transfer of accumulated DODMAC from larval stages to post-exposure pupated forms (Section 3.3.2). Therefore, compounds assimilated from sediment can be carried over between developmental stages that include substantial metamorphosis.

It is apparent that chironomid larvae will not always achieve greater uptake of organic compounds from sediments than alternative benthic species. For example, greater dietary accumulation of tetrachlorodibenzo[p]dioxin was achieved by *Lumbriculus variegatus* than *Chironomus tentans* (West *et al.* 1997). Greater uptake of dichlorophenyl in *Lumbriculus variegatus* than *Chironomus riparius* has also been observed and tentatively attributed to differential desorption resulting from differing gut environments (Davies *et al.* 1999b). In another instance, greater tissue loadings of organic compounds within *Lumbriculus variegatus* have been specifically attributed to greater catabolic capacity of *Chironomus riparius* (Simkiss *et al.* 2001). The consistently greater accumulation of DODMAC and naphthalene by *Chironomus riparius* within the substrates and exposure regimes used in this project are somewhat contradictory to the above findings. Similarly, Bremle and Ewalde (1995) found that chironomids achieved more than double the bioaccumulation of polychlorinatedbiphenyls than oligochaetes via exposure to the same sediment. Once again, the complex interaction of sorbant particle, gut environment and compound chemistry is suggested to be a powerful determinant of dietary uptake. Understanding such factors resulting in differential uptake between sediment ingesting species would allow better assessment of the ultimate exposure of both pelagic and terrestrial predators. Here, it is stressed that species differences in uptake can influence direct toxicity to benthic invertebrates as well as the ultimate exposure pathway to predatory consumers. The potential for a lack of toxicity within invertebrate species at exposure levels that are toxic to vertebrate consumers is also noted.

6.3 Limitations of experimental approach

In this thesis, experimental objectives were addressed using laboratory systems with exposure periods shorter than 14 days. The majority of exposure durations were of 48 h. In addition, the exposure medium was largely artificial sediment. The exception to this was the observation that uptake of DODMAC appeared to be viable when field collected sediment was used as the substrate. This offers specific information on the processes of contaminant transfer into individual species as an example of what could happen in natural systems. However, the use of artificial sediment or homogenised field-collected sediment can affect the kinetics of contaminant uptake (e.g. Barrett 1995, Rossi and Beltrami 1998, van Hoof *et al.* 2001). In addition, the processes evident in single-species sediment exposures are difficult to extrapolate to fully integrated processes occurring in natural systems (e.g. Hall and Giddings 2000). Therefore, this thesis has concentrated upon initial examinations of some specific processes that are potentially experienced by organisms in natural systems. The actual iterations of such processes, in combination with myriad other biological and chemical processes, may be modified in natural systems.

The laboratory dosing of sediments 3-7 days prior to use within exposures takes very little account of changes in contaminant bioavailability occurring with increasing contact time between substrate and contaminant (Section 1.1.4 and e.g. Varanasi 1985). Kukkonen and Leppanen (2000), suggest that contaminant contact time should be considered when modelling or measuring bioaccumulation of contaminants from sediment. The reduction in bioavailability, postulated to be due to accretion within sediment particles (Section 1.1.4), appears to be difficult to quantify. Lamoureux and Brownawell (1999) estimated that contact time over centuries resulted in reduction in bioavailability of hydrophobic organic compounds of approximately four orders of magnitude. Whereas, for contact times ranging from one week to thirteen months in benzo[a]pyrene, there was an apparantly consistent (~40%) reduction in availability compared to benzo[a]pyrene added to the sediment just prior to organism exposure (Kukkonen and Landrum 1998). Therefore, the studies performed in this thesis may be representative of contaminants that have been recently sorbed to sediment (<12months). It is likely that compounds similar to DODMAC could be exposed to organisms in natural systems in a manner reasonably approximated by recently dosed

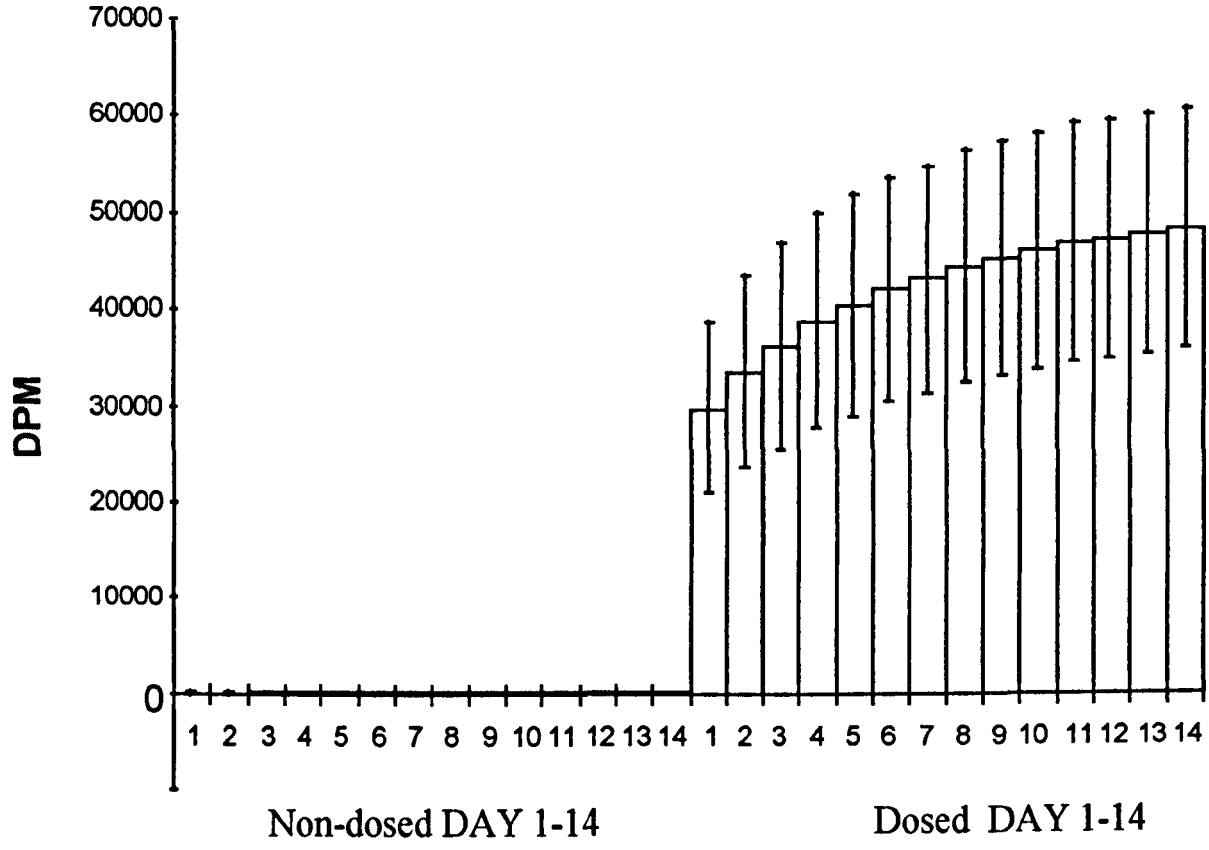
sediment. Cationic surfactants, when used in fabric conditioners, are likely to become sorbed to particulate matter during waste-water processing immediately prior to discharge into receiving water courses (ECETOC 1993). Similarly, polyaromatic hydrocarbons entering receiving waters as a result of road runoff pollution events (e.g. Krein and Schorer 2000) are likely to be recently-sorbed to sediment particles. However, recently-dosed laboratory studies are unlikely to take account of historically contaminated sediment particles that feature extremely long (>100 years) contact periods between the sorbant particle surfaces and the contaminant (e.g. Lamoureux and Brownawell 1999). The only potential exception to this is where organsisms can liberate accreted contaminant by possessing the capacity to solubilise the sediment particle itself (e.g. cellulose solubilisation). It is currently unclear whether particles that could be solubilised in this manner would be likely to persist for centuries within the sediment.

6.4 Conclusions

The main hypothesis addressed by this thesis was that uptake of particle-sorbed contaminants is a viable assimilation route: the relative importance of which may vary in response to both chemical partitioning and organism biology. The results of the experiments carried out in addressing this hypothesis suggest that sediment-sorbed contaminants can be bioavailable via dietary uptake. There was no evidence that observed variation in dietary uptake was due to selective feeding, biotransformation capacity, lipid content, water content or gut retention time. However, further research into gut fluid surfactancy, dietary solubilisation of cellulose and periodic enhancement of lipid assimilation was prompted. Sediment-associated PAH compounds were assimilated from both aqueous and particulate sources. The proportional importance of aqueous uptake was enhanced by reductions in compound K_{ow} and the presence of ventilatory appendages in the exposed organisms. The improved characterisation of processes controlling dietary assimilation and route of uptake, in conjunction with the resultant toxicity from each route, will allow more accurate risk assessments of contaminated sediments to be performed.

Appendix 2.1

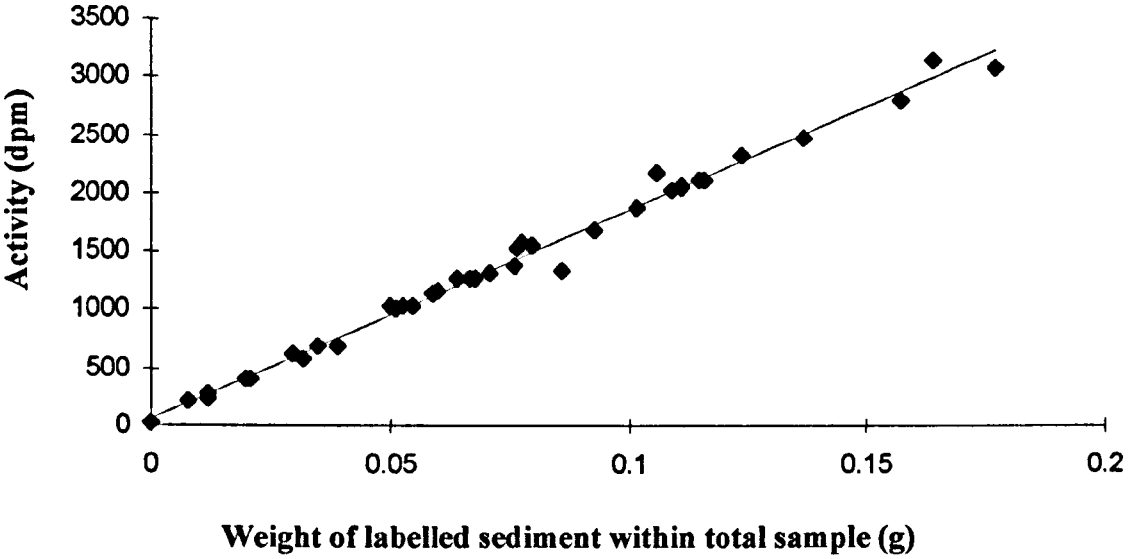
Attenuation of radioactive counts in sediment samples in scintillation cocktail over 2 weeks



Mean (±SE) radioisotope disintigration per minute (dpm) values in sediment samples subjected to daily recounts. Values not normalised to sample weight.

Appendix 2.2

Validation of sediment analysis by LSC: known weights of sediment-sorbed DODMAC vs. measured radioactive disintigrations



Weight of radiolabelled sediment within each total sediment sample (made up to between 0.073 and 0.188g with unlabelled sediment) against measured radioisotope disintigrations per minute (dpm); n=38, Pearson's r=0.995

Microautoradiography performed at Unilever Research Colworth (Unilver R&D Colworth, Sharnbrook, Bedford, MK44 1LQ) by Helen Minter.

Slide preparation Microscope slides were pre cleaned in 1% hydrochloric acid in 95% ethanol/water (v/v) ("acid alcohol"), rinsed well in Ultrapure water and then "subbed" by dipping them into a gelatin/alum solution (gelatin 2.6g, chromium potassium sulphate 0.5g in 500ml Ultrapure water). The slides were drained and dried in an oven at approximately 37°C. Once dried the slides in their racks were removed from the oven and cooled, placed in plastic bags and stored until required the following day.

Emulsion application

The application of emulsion took place under dark room lighting (Kodak No 1 filter). Ilford K5 emulsion, Batch number 56BK51753 (ILFORD Imaging UK Limited, Town Lane, Mobberley, Knutsford GB-Cheshire WA16 7JL). The emulsion was melted in a water bath at 43°C ± 2°C and decanted into a custom made dipping vessel containing 1% glycerol in Ultrapure water to give a 30/20ml dilution (v/v). The "subbed" slides were dipped individually into the emulsion, drained and the back of the slide wiped to remove excess emulsion. The "dipped" slides were placed onto an aluminium rack in a horizontal position and allowed to set and dry, then placed in autoradiography boxes, with the lids removed but sealed in a light proof box containing a sachet of silica gel to ensure complete drying of the emulsion. The following morning the lids were replaced, under dark room safe lighting and the autoradiography boxes sealed using black electrician's tape. The boxes were placed in a -20°C freezer until required.

Blocking and sectioning

The samples were 'blocked' by mounting onto specimen discs (supplied by Leica

Microsystems, UK., Ltd) using OCT (supplied by BDH) at -20oC and allowed to stabilise for approximately 30 minutes. The samples were trimmed on a Leica 3050 cryostat (supplied by Leica Microsystems Nussloch GmbH Heidelberger). Eight micron thickness sections (destined for histology) were mounted onto ordinary microscope slides and air dried prior to fixation and staining with haematoxylin and eosin. Eight micron thickness sections (destined for micro-autoradiography) were mounted onto the emulsion-coated slides and placed in sealed autoradiography boxes stored in a -20°C freezer.

Development and staining

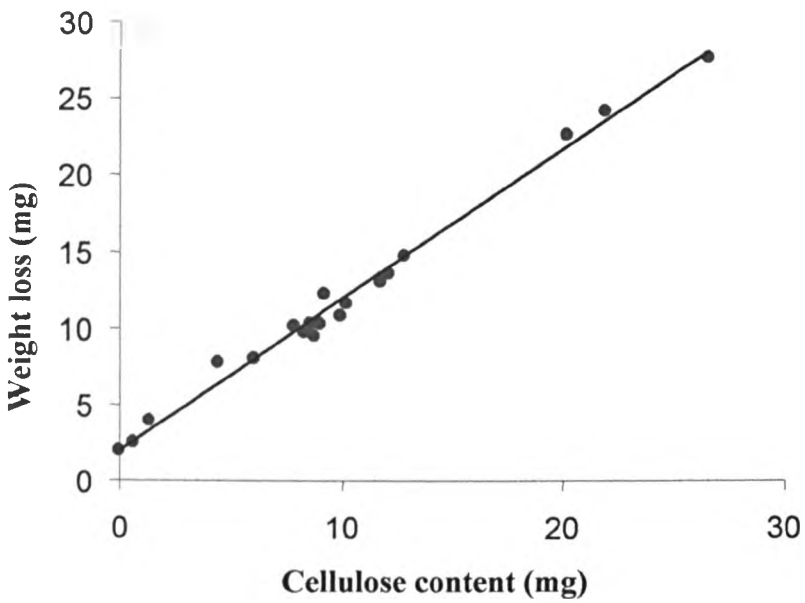
The micro-autoradiography sections were developed after 120 hour, 1 or 3 week exposure. Sections exposed to emulsion coated slides were air dried for a minimum of 30 minutes sealed in autoradiography boxes. The slides were transferred to histology staining racks and developed using a photographic processing procedure in phenisol developer diluted 1:4 (v/v) with Ultrapure water for 2 minutes. Development was stopped by immediate immersion in a "stop bath" of 1% acetic acid (v/v) in Ultrapure water and fixed in Hypam fixer diluted 1:4 (v/v) in Ultrapure water for 3 minutes. Following washing in tap water for approximately 10 minutes the sections were fixed in formal saline and stained with haematoxylin and eosin, dehydrated and mounted in DePeX. The sections were examined for areas of activity using a Leica DMRB microscope under dark field illumination. The histology of the sections was observed using bright field illumination.

Photomicroscopy

Photomicroscopy was carried out using a Leica DMRB microscope and a JVC KY-F55E 3CCD camera in conjunction with the OASIS 2000 image archive system (Unilver R&D Colworth, Sharnbrook, Bedford, MK44 1LQ). Photomicroscopy was carried out under dark and bright field illumination.

Appendix 4.1

Calibration curve of cellulose weight vs. weight loss on combustion



Calibration curve of artificial sediment weight loss after 48 h combustion at 550°C for manipulated cellulose contents. Regression equation is; Weight loss = 1.95 + 0.987 Cellulose weight, $p<0.001$

Lipid Extraction Report

Work conducted in support of a PhD by Paul Gaskell (Sheffield University)

Aim

The aim of this work was to quantify and compare the lipid content of four aquatic invertebrates. The animals were used as test species in a PhD project looking at the metabolism of a cationic surfactant by benthic (sediment dwelling) invertebrates. The four species investigated were *Gammarus pulex, Asellus aquaticus, Lumbriculus variegatus*, and *Chironomid riparius.*

Lipid Extraction Methods

Several extraction procedures were considered. A brief synopsis of those thought to have had the most potential for modification for the purposes of this work are listed below. The main criteria for selection were:

They allowed for the use of small sample sizes and small solvent volumes.
They were reasonably simple and quick, allowing for a number of samples to be extracted in a reasonable time.
The results obtained were consistently of comparable quality to a chosen "standard method" (Bligh & Dyer).

Erickson Method [1]

a) Use minced/homogenised samples of 0.5 or 1.0g
b) Chose $CHCl_3$:MeOH in a ratio of 2:1 as the solvent system
c) Use a solvent/tissue ratio of 20:1
d) Interaction of extraction solvents with the homogenised tissue initially achieved through mixing on a vortex mixer in a 16 x 125-mm screw-cap test tube for 2 minutes under a nitrogen atmosphere.
e) Subsequent separation of tissue residue from the extracted lipids achieved by filtration through a Whatman No.1 filter paper into a clean test tube. The filter paper and residue then being rinsed with additional solvent.
f) The extract was then evaporated to dryness, reconstituted in 2.0 ml $CHCl_3$ and filtered through sodium sulphate.
g) Both the tube and filter were then washed twice with 2-ml aliquots of $CHCl_3$.
h) The extract was then evaporated to dryness and weighed (or reconstituted with 1.0 ml $CHCl_3$).

Note:

i) Erickson found the protein contamination in the $CHCl_3$: MeOH extracts was of the order of 0.12%. If this level of contamination is assumed in the invertebrate extractions, then this will be considered acceptable.
j) To optimise extraction of lipids from the homogenised tissue in $CHCl_3$: MeOH, exposure of the tissue to methanol prior to chloroform is necessary. Erickson found that by penetrating the cellular membranes and forming a cloudy dispersion, methanol facilitated extraction of lipids on subsequent introduction of chloroform.
k) Advantages of the $CHCl_3$: MeOH method are that it only needs screw- cap test tubes in which to disperse the tissue and solvent, a vortex mixer and funnels for filtering the extractant into another set of test tubes. Consequently, a large number of samples may be extracted in a short period of time. Further, the minimal solvent volumes required represent another advantage, with multiple samples able to be evaporated on a nitrogen evaporation rack.

Lee, Trevino & Chaiyawat Method [2]

a) Homogenise sample in Waring Blender.
b) Weigh paste 1.0 – 2.0 ± 0.05g; record the exact amount (authors recommend 5.0±0.1g but this is too large for purpose of this exercise) and place in 250ml Eberbach homogeniser (model 8580).
c) Add 50 ml $CHCl_3$: MeOH in ratio 2:1 (high fat content; >6% mainly triacylglycerols) or 1:2 (for low fat content; <2% mainly phospholipids). Measure solvent as precisely as possible to give an accurate ratio.
d) Blend for 1.5 minutes at a constant (controlled) moderate speed.
e) Filter homogenate through course filter paper (fast speed) and funnel into a 100ml glass stoppered graduated cylinder (toward the end of draining, press the cake with the round tip of a spatula to moderately squeeze out remaining solvent; do not attempt to press all solvent out).
f) Add 20ml 0.5% NaCl and gently shake by tilting cylinder 4 times. Allow mixture to stand until visible separation occurs.
g) Using a 10ml pipette, remove an aliquot (5ml) of the chloroform layer and transfer 5ml into a pre-weighed (to 1 mg) 10ml beaker.
h) Evaporate the solvent on a hot plate (set at a low to moderate setting). Avoid excessive heating and drying. (This step will require about 30 minutes).
i) Reweigh the beaker (to 1mg) and calculate the lipid content using the following formula:

$$\text{Lipid content, \%} = \frac{\text{Lipid extracted (g)}}{\text{Sample weight (g)}} \times \frac{\text{chloroform volume (ml)}}{5\text{ml}} \times 100$$

Where chloroform volumes are 33 and 16.7 ml for 2:1 and 1:2 $CHCl_3$: MeOH ratios, respectively.

Hara and Radin Method [3]

a) To 1g of tissue add 18ml hexane: isopropanol (3:2 ratio) and homogenise using a polytron homogeniser for 30 seconds.
b) Filter the suspension through a sintered glass Buchner funnel (medium porosity) under vacuum.
c) Wash the homogeniser, funnel and residue three times with 2ml aliquots of hexane: isopropanol. Resuspend the residue each time and let the solvent soak for about 2 minutes before applying vacuum.
d) If desired, non-lipids in the extract can be removed by mixing the pooled filtrates for at least 1 minute with 12ml of aqueous sodium sulphate (prepared from 1g of anhydrous salt and 15 ml water). The two layers that form are each about 18ml in volume and the lipids are in the upper, hexane rich layer. No precipitate is visible at the interface.

Bligh and Dyer Method [4, 5]

a) For each 100g sample: homogenise in a Waring blender for 2 minutes with a mixture of 100ml chloroform and 200ml methanol.
b) Add 100ml chloroform to the mixture and blend for a further 30 seconds.
c) Add 100ml distilled water and blend for another 30 seconds.
d) Filter the homogenate through a Whatman No.1 filter paper on a Coors No.3 Buchner funnel with slight suction. Filtration is normally quite rapid and when the residue becomes dry, pressure is applied with the bottom of a beaker to ensure maximum recovery of the solvent.
e) Transfer the filtrate to a 500ml graduated cylinder, and, after allowing a few minutes for complete separation and clarification, the volume of the chloroform layer (at least 150ml) is recorded and the alcoholic layer removed by aspiration. A small volume of the chloroform layer is also removed to ensure complete removal of the top layer. The chloroform layer contains the purified lipid.

f) For quantitative lipid extraction the lipid withheld in the tissue residue is recovered by blending the residue and filter paper with 100ml chloroform. 165

g) The mixture is then filtered through the original Buchner funnel and the blender jar and residue washed with a total of 50ml chloroform. This filtrate is mixed with the original filtrate prior to removal of the alcoholic layer.

Determination of Lipid Content

A portion of the extract containing 100-200mg lipid is evaporated to dryness in a tared flask and the weight of the lipid residue determined.

h) Evaporation, facilitated by a stream of nitrogen, is carried out in a water bath at 40-50°C.

i) The residue is then dried over phosphoric anhydride in a vacuum desiccator.

j) After weighing, a small volume of chloroform is added to each flask to detect the presence of non-lipid material (insoluble).

k) If non-lipids are present, the chloroform is carefully decanted and the flask rinsed three times with chloroform. The dry weight of the residue is determined and subtracted from the initial weight.

l) The lipid content of the sample is calculated as follows:

$$\text{Total Lipid} = \frac{\text{weight of lipid in extract} \times \text{volume of chloroform layer}}{\text{Volume of aliquot}}$$

Note:
According to the authors, this extraction procedure applies to 100g of tissue containing about 80% ±1% water and about 1% lipid. Many alterations are permissible but it is imperative that volumes of chloroform, methanol and water, before and after dilution, be kept in the proportions 1:2:0.8 and 2:2:1.8 respectively. The size of sample can be reduced and the solvent quantities scaled down as appropriate. However, the use of very small sample sizes, such as is necessary in this exercise may make the small volumes of solvent impractical to work with. Further, since this procedure is a gravimetric one, the small initial sample size may represent a problem in terms of accuracy of weighing during lipid content determination.

The salient aspects of the methods were evaluated, theoretically or practically and it was concluded that a suitable procedure for determining the lipid content of the 4 invertebrates was a hybrid of the Bligh & Dyer method and the Erickson method.

Details of the extraction method employed are given below.

Lipid Extraction and Quantification

1. Weigh ~0.5g* tissue into glass mortar.
2. Chop\cut tissue into small pieces.
3. Add silica gel in a 4 parts silica to 1 part tissue ratio i.e. 0.5g tissue then need 2.0g silica gel
4. Grind tissue to an even consistency (takes ~5 minutes)
5. Carefully transfer mixture into a 40ml screw top vial
   Using a 20:1 solvent to tissue ratio (ignoring weight of silica) extract the tissue with methanol and chloroform (keeping the $CHCL_3$: MeOH:H_20 proportions before and after dilution as 1:2:0.8 and 2:2:1.8 respectively) as follows:

   i) Add 6.66ml MeOH and mix on a vortex mixer for 2 minutes.
   ii) Add 3.33ml $CHCL_3$ and 2.26ml H_2O and mix for a further 2 minutes. (take care with pressure build-up)
   iii) Add 3.33ml $CHCL_3$ and mix for 2 minutes.
   iv) Add 3.33ml H_2O and mix for 2 minutes.

6. Centrifuge for 5 minutes at 4000rpm. This assists in decanting of solvent without carrying over too much tissue\silca. 166
7. Carefully decant solvent mixture into a 25ml-separating funnel.
8. Wash tissue\silica with 3 x 3ml $CHCL_3$ and add solvent to separating funnel.
9. Using a 5-figure balance, accurately weigh a glass scintillation vial (without its cap).
10. Plug neck of a small glass filter funnel with small wad of non-absorbent cotton wool (not too compact)
11. Place the funnel in the vial and run bottom $CHCL_3$ layer carefully and slowly into the vial. Try to run the solvent directly onto the wad of wool to minimise loss of lipids onto glass.
12. Wash the separating funnel (alcoholic layer) with 3 x 2ml $CHCL_3$ and run $CHCL_3$ layer into vial.
13. Wash the funnel and cotton wool wad with 2ml $CHCL_3$ and add to vial.
14. Evaporate $CHCL_3$ to dryness using nitrogen and a sample concentrator.
15. Ensure vial is completely dry and free from dirt and weigh on a 5-figure balance. N.B. If necessary re-extract the tissue with more $MeOH\backslash CHCL_3\backslash H_2O$ and repeat procedure. This may be required if doubt that not all lipid has been stripped from the tissue\silca matrix.
16. Calculate mg of lipid per mg of tissue

e.g. If start with 0.5g tissue and weight of dried $CHCL_3$ extract = 10mg
then have 0.02mg lipid/mg tissue.

* Approximately 1g Lumbriculus tissue taken. Solvent and water volumes increased accordingly but kept in the same ratio.

Results

Vial No.	Tissue Type	Wt. Tissue (g)	Wt. Vial (g)	Vial + Lipid (g) 1st weight	Vial + Lipid (g) 2nd weight	Mg Lipid/Mg Tissue	% Lipid
PG-LIP-01							
1	Gammarus.	0.5044	11.97908	11.98683	11.98665	0.01501	1.50
2	Gammarus.	0.5034	11.88977	11.89992	11.89989	0.02010	2.01
3	Gammarus	0.5085	11.93920	11.94823	11.94815	0.01760	1.76
					Mean =	0.01757	1.76
4	Asellus	0.5160	11.86140	11.86778	11.86920	0.01512	1.51
5	Asellus	0.5224	11.94617	11.95182	11.95135	0.00992	0.99
6	Asellus	0.2169	11.90829	11.91538	11.91407	0.02665	2.66
					Mean =	0.01723	1.72
7	Triolein (v)	0.1036	11.86654	11.96312	11.96357	0.09703	93.66
8	Triolein (m)	0.0998	11.89216	11.98335	11.98305	0.09089	91.07
PG-LIP-02							
9	Lumbriculus	1.2186	11.86581	11.88461	11.88226	0.01350	1.35
10	Lumbriculus	1.2334	11.93510	11.95292	11.95129	0.01313	1.31
11	Lumbriculus	1.2403	11.93274	11.95161	11.95087	0.01271	1.27
					Mean =	0.01311	1.31
12	Chironomid	0.4877	11.89508	11.89871	11.89859	0.00720	0.72
13	Chironomid	0.4566	11.93053	11.93550	11.93522	0.01027	1.03
					Mean =	0.00874	0.87
15	Triolein (v)	0.1032	11.91914	12.00295	12.00344	0.08426	81.65
16	Triolein (m)	0.1055	11.91117	11.99879	11.99927	0.08810	83.51
N.B. No number 14 vial due to lack of sample							

Notes on Extraction Method

- Procedure has not been compared with other methods to provide comparisons on method suitability and extraction efficiency.

- Referenced methods referring specifically to the extraction of invertebrates were not found. Most papers referred to fish or rat tissue but there appears no reason why the methods cannot be successfully transferred to other types of tissue e.g. Gammarus, Lumbriculus etc.

- True "control tissue" was not extracted. Triolein was used as surrogate lipid in an attempt to gain an insight into extraction efficiency and to confirm that the lipid was successfully stripped from the silica gel grinding material. It should not be considered as a true control source and as such the extraction efficiency may be in some doubt, although the % lipid extracted from each test material was very consistent, which would indicate that the procedure was acceptable and the results suitable for comparison purposes. A better model for a representation of control tissue may be triolein homogenised with a non-lipid containing substance, such as filter paper. However, this also should be used with caution. The best way of checking extraction efficiency is probably by comparison with other methods.

- Due to the doubts about the triolein being representative of "true control" tissue the results have not been corrected for extraction efficiency. Doubts on the wisdom of applying a correction factor are compounded by the solvent to tissue ratio for the triolein extractions far exceeding 20:1, which may have enhanced extraction efficiency beyond that for the test tissue. If this factor is applied, then the differences in % lipid content between the two studies are brought closer by around 8%.

- On one occasion only approximately 0.2g of Asellus tissue was available. The solvent to tissue ratio was not altered accordingly and hence it exceeded the 20:1 ratio specified in the method. The lipid content for this sample was higher than for the other two samples in the series and it is not known if the greater solvent volumes had a bearing on this result.

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DODMAC Metabolites Report

Work conducted in support of a PhD by Paul Gaskell (Sheffield University)

Aim

The aim of this work was to qualitatively determine if labelled DODMAC ingested by four aquatic invertebrates remained intact as the parent species or was broken down into metabolites of the parent material. The animals were used as test species in a PhD project looking at the metabolism of a cationic surfactant by benthic (sediment dwelling) invertebrates. The four species investigated were *Gammarus pulex, Asellus aquaticus, Lumbriculus variegatus,* and *Chironomus riparius.*

In addition, this investigation also included further quantification and comparison of the lipid content of the four aquatic invertebrates.

Methods

The lipid of each of the four invertebrates was extracted into chloroform using the Bligh & Dyer\Erickson method detailed below (see Lipid Extraction Report). Radio-TLC (details given below) was then carried out on the lipid extracts to determine if any breakdown of the parent material had occurred.

Lipid Extraction and Quantification

1. ~0.5g* tissue was weighed into glass mortar.
2. The tissue was choped\cut into small pieces.
3. Silica gel was added in a 4 parts silica to 1 part tissue ratio i.e. if have 0.5g tissue then need 2.0g silica gel.
4. The tissue was ground to an even consistency (takes ~5 minutes)
5. The mixture was carefully transferred into a 40ml screw top vial.
   Using a 20:1† solvent to tissue ratio (ignoring weight of silica) the tissue was extracted with methanol and chloroform (keeping the $CHCl_3$: MeOH:H_2O proportions before and after dilution as 1:2:0.8 and 2:2:1.8 respectively) as follows:

   i) 6.66ml MeOH added and mixed on a vortex mixer for 2 minutes.
   ii) 3.33ml $CHCl_3$ and 2.26ml H_2O was added and mixed for a further 2 minutes. (take care with pressure build-up)
   iii) 3.33ml $CHCl_3$ added and mixed for 2 minutes.
   iv) 3.33ml H_2O added and mixed for 2 minutes.

6. The mixture was centrifuged for 5 minutes at 4000rpm. This assisted in decanting off solvent without carrying over too much tissue\silca.
7. The solvent mixture was carefully decanted into a 25ml-separating funnel.
8. The tissue\silica was washed with 3 x 3ml $CHCl_3$ and the solvent added to the separating funnel.
9. Using a 5-figure balance, a glass scintillation vial (without its cap) was accurately weighed.
10. The neck of a small glass filter funnel was plugged with small wad of non-absorbent cotton wool (not too compact)
11. The funnel was placed in the vial and the bottom $CHCl_3$ layer ran carefully and slowly into the vial. To minimise loss of lipids onto glass the solvent was ran directly onto the wad of wool
12. The separating funnel (alcoholic layer) was washed with 3 x 2ml $CHCl_3$ and the $CHCl_3$ layer ran into the vial.
13. The funnel and cotton wool wad was washed with 2ml $CHCl_3$, which was added to the vial.
14. The $CHCl_3$ was evaporated to dryness using nitrogen and a sample concentrator.
15. Ensuring it was completely dry and free from dirt, the vial was weighed on a 5-figure balance.
16. The mg of lipid per mg of tissue was calculated.

e.g. If start with 0.5g tissue and weight of dried $CHCl_3$ extract = 10mg then have 0.02mg lipid/mg tissue.

Notes on Extraction Method

- Procedure has not been compared with other methods to provide comparisons on method suitability and extraction efficiency.

- True "control tissue" was not extracted. Triolein was used as surrogate lipid in an attempt to gain an insight into extraction efficiency and to confirm that the lipid was successfully stripped from the silica gel grinding material. It should not be considered as a true control source and as such the extraction efficiencies can not be considered conclusive.

- Due to the doubts about the triolein being representative of "true control" tissue the results have not been corrected for extraction efficiency. In addition the solvent to tissue ratio for the triolein extractions far exceeded 20:1, which may have enhanced extraction efficiency beyond that for the test tissue.

- *The total amount of tissue available was taken, up to a maximum of approximately 0.5g. In some instances this was considerably less than 0.5g.

- †A decision was made to keep solvent and water volumes constant for all sample sizes e.g. the volumes used for a 0.5g sample were employed regardless of sample size. This meant that the 20:1 solvent to tissue ratio was greatly exceeded in some cases. This option was chosen because, in some instances, maintaining the 20:1 ratio would have meant using very small solvent volumes. This would have significantly effected the capability for stripping the lipid from the glassware e.g. mortars, and hence effected the extraction efficiency. The greater solvent volumes may have had a bearing on results.

Radio-TLC Method

1. The dried extracts were dissolved in Ca. 0.5 ml $CHCl_3$.
2. A 50µl aliquot of each dissolved extract was counted for 1 minute in a Packard scintillation counter using 10ml ESP scintillation cocktail.
3. Using the dpm obtained in "2", the size of aliquot to spot onto the TLC plate was determined (aimed for a minimum total activity spotted of 1500 dpm). Multiples of 20µl aliquots were spotted to build-up activity.
4. A TLC solvent system mix of 86 ml chloroform, 10 ml methanol, 3 ml acetic acid and 1 ml water was used. This solvent mixture was transferred to a TLC tank lined with filter paper were it was allowed to equilibrate for at least 30 minutes.
5. A quantity of the extracts was spotted onto the preadsorbant zone of a Whatman LK6D silica gel TLC plate and dried with a hair dryer. A control sample of radiolabelled DODMAC (X9901) was spotted at a similar radioconcentration as the test samples. A second control sample was spotted which had 20ul of Triolein (a lipid surrogate) spotted over it. This was used as a rough tool to ascertain the effect the lipid might have on how the DODMAC would run in the TLC system.
6. Extracts from control samples of each of the four species were also spotted on the plate to confirm that no contamination with radioactive material had occurred.
7. A line was scored across the TLC plate 10cm above the preadsorbant zone and the plate eluted in the solvent system until the solvent had reached the line. The plate was dried and the position of the labelled spots determined using a Packard Instant Imager.

Results of Lipid Quantification

Vial No.	Tissue Type	Wt. Tissue (g)	Wt. Vial (g)	Vial + Lipid (g) 1st weight	Vial + Lipid (g) 2nd weight	Mg Lipid/Mg Tissue	% Lipid
PG-LIP-03							
Dosed Samples							
1	Gammarus.	0.5196	11.31738	11.32312	N/A	0.01105	1.10
2	Asellus	0.4340	11.36439	11.36870	N/A	0.00993	0.99
3	Lumbriculus	0.1444	11.43356	11.43777	N/A	0.02916	2.92
4.	Chironomid	0.0845	11.29272	11.29443	N/A	0.02024	2.02
Control Samples							
5	Gammarus	0.4162	11.42058	11.42594	N/A	0.01288	1.29
6	Asellus	0.2056	11.38305	11.38845	N/A	0.02626	2.63
7	Lumbriculus	0.3403	11.26608	11.27453	N/A	0.02483	2.48
8	Chironomid	0.1907	11.34688	11.34816	N/A	0.00671	0.67
9	Triolein (v)	0.1017	11.33531	11.43054	N/A	0.09523*	93.64
10	Triolein (m)	0.1077	11.32226	11.41946	N/A	0.09720*	90.25

* Amount of Triolein recovered.
N/A = Due to time constraints on obtaining results, only one dry weight was taken.

Results of Radio-TLC

The printouts from the Instant Imager TLC , the scintillation counts of the extracts (including control samples) and the scintillation counts from the alcoholic layer (extraction solvent mix) are attached.

The TLC indicates that the compound has not been metabolised but has remained in its parent form. The X99/01 standard shows a small amount of impurities, as it could not be further purified. While the impurities are seen on the TLC scan, no indications of any breakdown products are evident in the test samples. This may indicate that the extraction procedure may not have extracted these slightly more polar components and they have remained in the alcoholic layer. However, this is highly unlikely as no radioactivity was detected in samples removed from the polar, alcoholic layer of the extraction mix. This activity mass balance proves that the extraction was fully efficient at pulling all lipid into the non-polar chloroform layer. Further, these later test samples had been exposed to a higher active, more radio-pure compound (X00/05) than animals used in earlier tests using X99/01* as the test material. It can be assumed that the integrity of the X00/05 test sample is unaltered since X99/01 has shown exactly the same TLC profile now as it did 12 months ago and X00/05 should act similarly. Therefore the extracted samples would not be expected to display the same impurities as the X99/01 standard.

*Note: X99/01 was used as the standard because all X00/05 had been sent to Sheffield University.

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