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Lipid Solubilization by Marine Benthic Invertebrates

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LIPID SOLUBILIZATION BY MARINE

BENTHIC INVERTEBRATES

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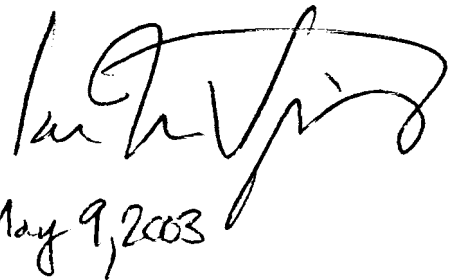Gary M. King, Professor of Microbiology and Marine Studies

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By Ian M. Voparil

Thesis Advisor: Dr. Lawrence M. Mayer

An Abstract of the Thesis Presented
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This work characterized the size of lipoidal colloids in benthic invertebrates' guts, using contact angle dilutions and imaging the fluorescence of the hydrophobic probe Nile Red. Deposit feeders and *Nereis virens* ingesting sediment were found to have micelles rather than emulsions in the gut. Gut fluids from *Arenicola marina* (a deposit-feeding polychaete) readily formed emulsions when incubated with mussel meat (*Mytilus edulis*; 80 g-mussel L^{-1}-gut fluid), suggesting that micelles form due to a paucity of emulsifying lipids in the gut. Lipid tracer contained in emulsion droplets was twice as likely to be captured by sediment than was tracer in micelles.

Solubilizate interactions among binary mixtures of nutritional and contaminant lipids occurred in gut fluids with micelles or high protein concentrations. In *Arenicola marina* gut fluid, benzo(a)pyrene (BaP) enhanced the solubilization of hexadecane (491% of single compound) and palmitic acid (130%), but hindered solubilization of cholesterol (83%). Addition of cholesterol, phenanthrene, lecithin, and hexadecanol modified BaP

solubilization (137%, 154%, 140%, and 232%, respectively, of BaP's concentration when alone).

Micellar gut fluids are much better than seawater at releasing polycyclic aromatic hydrocarbons (PAH) associated with anthropogenic particles. *A. marina* gut fluids dissolved significant concentrations of PAH from two tire treads, two diesel soots, and the urban particulate matter (SRM 1649). PAH in fly ashes and coal dusts were unavailable. Potential digestive exposure to PAH from these samples is much greater than that predicted to be available from these materials using equilibrium partitioning theory (EqP).

These impacts of digestive micelles make commonly used models of PAH bioavailability based upon sediment-water distribution, e.g., equilibrium partitioning theory, less accurate. A cocktail of commercially available compounds designed to mimic *A. marina* gut fluids was developed. Sodium taurocholate, a vertebrate bile salt, was found to be an excellent mimic for lipid solubilization; 13.0 mM sodium taurocholate and 5.0 g L^{-1} bovine serum albumin in artificial seawater released 12 PAH from four different contaminated sediments to similar extent as *A. marina* gut fluid (critical micelle dilution of 15%, protein content of 42.0 g L^{-1}). Ratios of release between cocktail and gut fluid ranged between 0.5 and 2.0 for 40 of 48 PAH-sediment combinations.

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Chapter 1 - GENERAL INTRODUCTION

In this thesis, I use the term "lipid" as shorthand to refer to a broad collection of compounds that are operationally defined, rather than according to their chemical structure. I define lipids as compounds that are more soluble in organic solvents than in water, following the functional definitions of Parrish (1988), Folch et al. (1957), and Bligh & Dyer (1959). Lipids are generally water-insoluble components, with aqueous solubilities ranging from 1×10^{-10} M for highly hydrophobic compounds like aliphatic hydrocarbons (Meylan et al., 1996) to 1×10^{-4} M (before aggregation) for compounds like phospholipids that have a hydrophilic group (Datta, 1987). There are more than 16 different subclasses of lipids (e.g., Fig. 1.1) having either a biogenic or an anthropogenic origin (Parrish 1988). Many types of lipids have a proximate biological origin; e.g., zooplankton store energy as wax esters and phospholipids are essential components of membranes of bacteria, plants, and animals. Other lipids, such as polycyclic aromatic hydrocarbons (PAH) often derive from anthropogenic sources and are considered contaminants. The common thread connecting these compounds is their method of isolation.

My definition of lipids is rather broad, and some readers may be more familiar with a more selective version. For example, Christie suggests that lipids be defined as "fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds" (www.lipid.co.uk/infores/Lipids/whatlip/, 4/2003). However, for this thesis dealing with the transport of lipids in digestive systems, an operational definition seems more appropriate as a wide variety of compounds behave similarly in
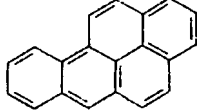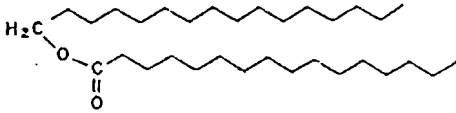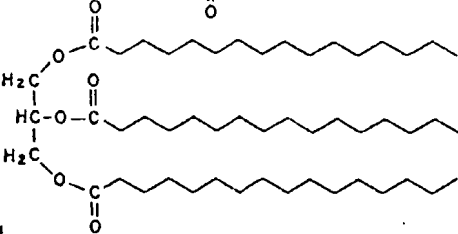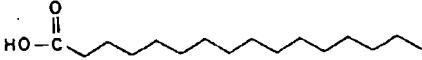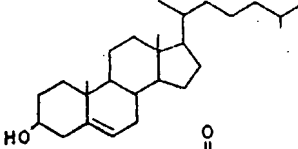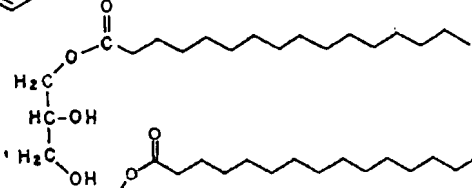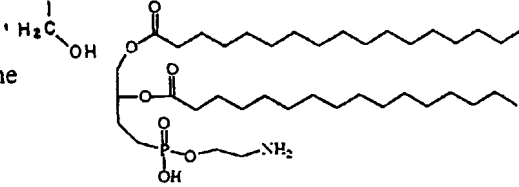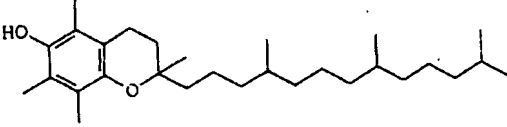
Lipid Class	Compound	Structure
Aliphatic hydrocarbons	nonadecane	

Polycyclic aromatic hydrocarbons	benzo(a)pyrene	

Wax esters	hexadecyl palmitate	

Triglycerides	tripalmitin	

Fatty acids	palmitic acid	

Sterols	cholesterol	

Monoglycerides	1-monopalmitin	

Phospholipids	phosphatidylethanolamine	

Lipophilic vitamins	α-tocopherol	

Figure 1.1: Lipid classes, representative compounds, and their structures.

digestive fluids. That is, fatty acid derivation is not necessarily the best indicator of solubility in digestive fluids of marine invertebrates.

Marine sediments serve as a repository for a host of lipids. Lipids tend to associate with particles because of their instabilities in an aqueous environment. Lipids have nonpolar regions with evenly spaced electrons that do not interact with water molecules' polar distribution of electrons. Adsorption onto mineral surfaces and absorption by sedimentary organic matter (SOM) are two mechanisms by which lipids escape the aqueous environment. If the associations among lipids, SOM, and mineral surfaces were unbreakable, then these compounds would be forever lost from biological systems, i.e., they would not be bioavailable. Their loss would be unexpected, as some lipids are potent sources of dietary energy, containing more energy per unit weight than either proteins or carbohydrates. Many organisms store energy as certain types of lipids, e.g., triglycerides and wax esters.

However, some marine metazoans have developed physiological mechanisms within their digestive tracts with which to mobilize lipids away from particles and then transport them to absorptive cells. Numerous potential solubilizers are present in invertebrate guts including proteins, dissolved organic material, and surfactants (Mayer et al., 1997). Surfactants have been found in mollusks, crustaceans (Lester et al., 1975; Tugwell & Branch, 1991), polychaetes, and holothuroids (Mayer et al., 1997; Smoot et al., in press). For the deposit-feeding polychaete *Arenicola marina*, surfactant micelles are responsible for 80-90% of the digestive mobilization of the contaminant benzo(a)pyrene (Voparil & Mayer, 2000). Surfactant molecules have both hydrophilic and hydrophobic regions. When present above a specific concentration, the critical

micelle concentration (CMC), surfactants exist as both monomers and spherical

aggregates (micelles) in aqueous solutions. In a micelle, monomers are arranged with

hydrophilic regions towards the aqueous exterior and hydrophobic regions composing the

interior. Lipids can be solubilized in digestive micelles at concentrations that are orders

of magnitude greater than their solubility in water (Fig. 1.2).

In this thesis, I set out to describe the mechanisms used by marine invertebrates to

transport dietary lipids and explore some ramifications of these mechanisms on the

bioavailability of lipids in the guts of deposit feeders. These goals were rather open-

ended, as the historical body of research on lipid mobilization in invertebrate guts is

small. Therefore, I turned to the vertebrate literature for guidance and insight to the

important processes involved, with hopes that analogies could be made between (mostly)

terrestrial vertebrate and marine, invertebrate systems.

1.1. Lipid mobilization by vertebrates

Vertebrate gastrointestinal lipid digestion comprises three sequential steps: (i) the

dispersion of bulk fat globules into finely divided emulsion particles; (ii) the enzymatic

hydrolysis of fatty acid esters at the emulsion-water interface; and (iii) the dispersion of

products into absorbable liposomes or micelles (Patton, 1981; Carey et al., 1983). First,

mastication in the mouth breaks ingested fat into smaller pieces that are emulsified in the

stomach by peristaltic action; these mechanical steps increase the surface area of the fat.

As food enters the duodenum (the beginning of the small intestine), bile salts are secreted

from the pancreas to further disperse the fat into smaller micelles. Some types of lipids

are directly solubilized by bile salt micelles; others need first be hydrolyzed into more
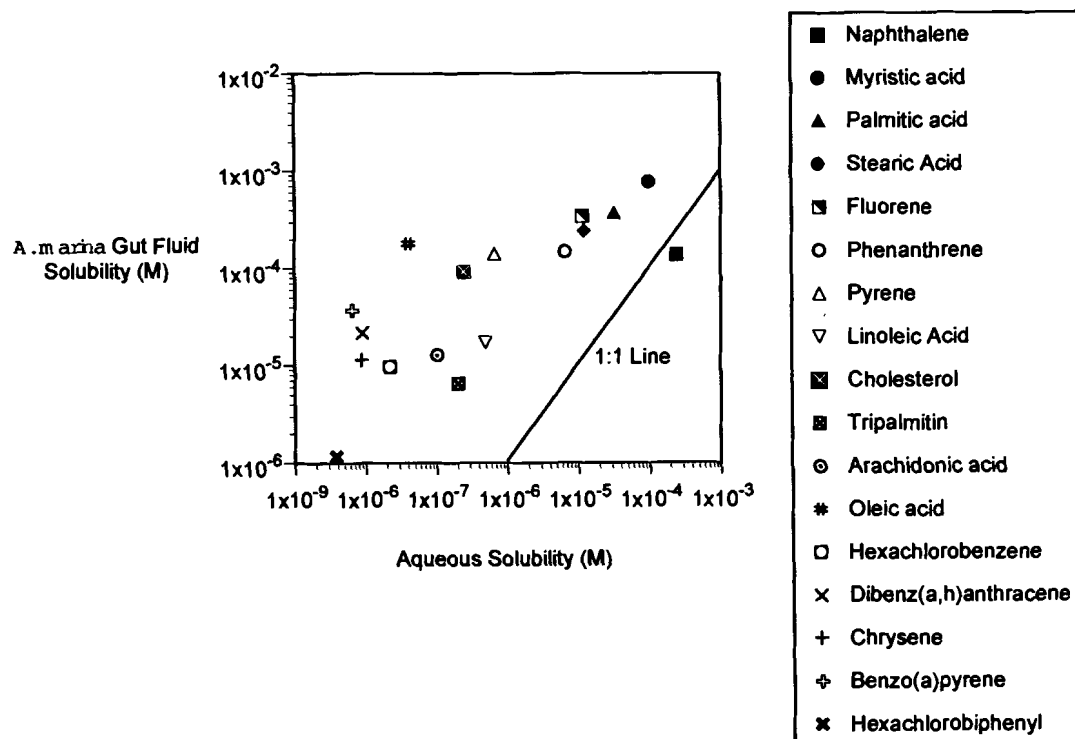
Figure 1.2: *Arenicola marina* gut fluids solubilize lipids to greater extent than does water.

soluble components. For example, fatty acids dissolve well in bile salt micelles, while triglycerides must first be cleaved into diglycerides, monoglycerides, and fatty acids by enzyme suites (lipases and colipases). Wax esters are also insoluble and often indigestible in mammals. However, some fishes (Patton et al., 1975) and marine birds (Jackson & Place, 1990; Place, 1992) can split wax esters into fatty acids and fatty alcohols using high gut concentrations of carboxyl-ester lipase, solubilize the products in bile salt micelles, and assimilate them with high efficiency.

Certain poorly soluble lipids are not broken down into more soluble components, due to inadequate enzymatic capability or the animal's nutritional requirement for the intact compound. In these cases, the compound may still be solubilized as the micelle's physical structure can be rearranged through a cooperative effect of other lipids. For example, ionized fatty acids cause bile salt micelles to expand, allowing the solubilization of larger molecules like cholesterol (Hofmann & Small, 1967). These are called mixed micelles –"mixed" in the sense that multiple lipids constitute the micelle.

Once in a micelle, lipids must be delivered to the gut epithelium in a form that can be absorbed within the digestive cells. Bile salt micelles cannot permeate the epithelium as intact structures (Wilson & Dietschy, 1972). Micelles shuttle lipids from the bulk aqueous solution to an acidic microenvironment extending a few hundred μm from the gut epithelium (Westergaard & Dietschy, 1974). The microenvironment's relative acidity (pH ~ 5.2; Shiau et al., 1985) causes dissociation of micelles and release of their lipid contents (Shiau & Levine, 1980). For examples, fatty acids, cholesterol (Shiau, 1987) and vitamin E (Hollander et al., 1975) are then absorbed passively as monomers. The rate of uptake depends on the amount of a lipid delivered to the microenvironment (in

colloids) and the permeability coefficient of the lipids. Permeability coefficients reflect the partitioning of products between the fluid phase and the strongly non-polar membrane; brush-border cell membranes have a polarity characteristic of a strong non-polar solvent like heptane or polyethylene (Sallee, 1978). Therefore, more hydrophobic compounds permeate the membrane faster than less hydrophobic compounds (Sallee, 1978; Sallee, 1979).

1.2. Lipid mobilization by marine invertebrates

In marine invertebrates, much less is known about lipid digestion and assimilation. Much of the research has focused on the enzymatic abilities of invertebrate digestive systems (see review in Vonk & Western, 1984). However, my review of vertebrate digestive physiology suggests that the ability to mobilize lipids in the fluid phase controls bioavailability. Therefore, I focus on lipid transport and its consequences for an animal's exposure to lipids in the diet.

Much of this thesis focuses on lipids that are also environmental contaminants – e.g., polycyclic aromatic hydrocarbons (PAH). The functional similarities of various lipids suggest that this work may also apply to compounds of more general ecological interest, e.g., nutritional lipids like cholesterol. The digestive bioavailability of PAH is particularly topical as PAH contamination of marine sediments surrounding urban areas is significant and worldwide (LaFlamme & Hites, 1978). Deposit-feeding animals typically ingest several times their own body weights in sediment each day. Digestive exposure to particle-associated contaminants is the primary pathway for the bioaccumulation of contaminant lipids in these animals (Fowler et al., 1978; Klump et al.,

1987; Boese et al., 1990; Leppanen, 1995; Weston et al., 2000). In this thesis, I often use the term bioavailability to refer to the potential, digestive exposure to lipids that would occur in the gut as modeled by *in vitro* incubations with digestive fluids extracted from animals. Bioavailability is not a synonym for bioaccumulation, for the latter term also incorporates the processes of assimilation (uptake into the cells of the body) and metabolic transformations that occur inside animals.

Starting in Chapter 2, I investigate the types of colloids used to mobilize ingested lipids in the guts of benthic, marine invertebrates – focusing on deposit feeders. Studies of lipid physical chemistry and digestion in vertebrates show that mobilization of lipids in an aqueous solution can be increased by orders of magnitude by the formation of colloids that range in size from surfactant micelles to emulsion droplets. I hypothesize that deposit feeders likely use micelles because of the general paucity of lipids in the diet and the potential that sediment grains in the gut could filter larger colloids.

In Chapter 3, I outline a consequence of the use of micelles to mobilize dietary lipids. Solubilizates (lipids solubilized within micelles) are concentrated in a micelle to the point where they can interact to influence one another's solubilities. Therefore, the digestive bioavailability of some lipids can be enhanced or antagonized by the presence of certain other solubilizates. I look at the solubilization interactions between a contaminant (benzo(a)pyrene) and a number of nutritional lipids.

In Chapter 4, I measure the digestive solubilization of an especially recalcitrant group of contaminant lipids (PAH associated with anthropogenic particles). PAH associated with anthropogenic particles are thought to be sequestered in an unavailable form. However, my results in the previous chapters suggested that gut fluids ought to be

far better extractants of hydrophobic organic chemicals than are aqueous solutions. My results offer new perspectives on the bioavailability of PAH in biologically relevant environmental matrices.

In Chapter 5, I use knowledge gleaned in the previous chapters on the mechanisms of lipid mobilization in guts to create a solution of commercially available compounds to mimic *Arenicola marina's* digestive solubilization of lipids. *In vitro* incubations of sediments with digestive fluids extracted from marine invertebrates have been shown to mimic digestive exposure to sediment-associated hydrophobic contaminants. However, collection of digestive fluid is time-consuming for me and fatal for the animals used. Creation of a commercially available "cocktail" would allow more widespread adoption of *in vitro* measures of bioavailability.

Chapter 2 - EMULSIONS VS. MICELLES IN THE DIGESTION OF LIPIDS BY BENTHIC MARINE INVERTEBRATES

2.1. Abstract

Using contact angle dilutions and imaging the fluorescence of the hydrophobic probe Nile Red, we investigated the size of lipoidal colloids used by various marine benthic invertebrates to mobilize ingested lipids within their guts. Animals can transfer food lipids from particles to the gut wall by forming colloidal suspensions. These colloids range in size from surfactant micelles to emulsion droplets, depending on the amounts of dietary lipids and digestive surfactants. With a cross-phyletic test of deposit feeders vs. animals using other feeding modes and a feeding experiment with the omnivore *Nereis virens*, we found that animals ingesting sediment have micelles rather than emulsions in the gut.

There is nothing inherent in the composition of deposit-feeder gut fluids that prohibits emulsification. *Arenicola marina* (a deposit-feeding polychaete) gut fluids formed an emulsion when incubated with mussel meat (*Mytilus edulis*; 80 g-mussel L^{-1}-gut fluid). Instead, deposit feeders may have to use micelles because of a paucity of emulsifying lipids in the gut. This paucity could result from low lipid content of ingested material and behavioral adaptations that limit the amount of lipid-rich food that enters the gut.

Emulsion droplets, rather than micelles, are captured more efficiently as they travel tortuous pathways among mineral grains. Using an *in vitro* system to mimic the gut of *A. marina*, ^{3}H-BaP tracer contained in emulsion droplets was captured with twice

the efficiency as tracer in micelles was. Filtration can provide an ecological pressure on lipid mobilization in the gut that forces deposit feeders to use surfactant micelles – perhaps explaining the widespread finding of micelles in all deposit feeders' gut fluids tested thus far.

2.2. Introduction

Nutritional lipids are a potent source of dietary energy, containing more energy per unit weight than either proteins or carbohydrates. However, animals have difficulty digesting and assimilating these compounds because lipids do not dissolve well in water. In general, metazoans must mobilize ingested lipids (which tend to associate with solids) for transport across the aqueous gut lumen to the gut wall for incorporation into the animal's tissues. Aqueous solubilities of lipids suggest that the delivery of freely-dissolved compounds would be low. For marine animals, this problem is exacerbated by the presence of dissolved salts that further decrease most lipids' solubilities (the "salting-out" effect; Setschenow, 1889).

Many marine invertebrates have solved this problem by secreting surfactants into the gut to form a water-soluble coating around these otherwise insoluble compounds (Van Den Oord et al., 1965; Lester et al., 1975; Vonk & Western, 1984; Mayer et al., 1997). Surfactants, having both hydrophobic and hydrophilic regions, stabilize the interface between lipids and an aqueous phase. When above a critical concentration and temperature, individual surfactant monomers aggregate to form micelles that have a hydrophilic exterior and hydrophobic interior able to solubilize other lipids (which are then called "solubilizates"; Carey & Small, 1970). The transfer of solubilizates into

micelles increases mobilization in the gut by orders of magnitude (Voparil & Mayer, 2000). Surfactant micelles are small (hydrodynamic radii of ≤ 4 nm for human bile salt micelles *ex vivo*; Hernell et al., 1990) and form without the presence of additional solubilizates.

Much larger colloids, such as vesicles and emulsion droplets, form when specific types of solubilizates surpass threshold concentrations in the fluid phases (Staggers et al., 1990). Particularly effective emulsifiers include ionized fatty acids, monoglycerides, and phospholipids (Carey et al., 1983). Emulsions are dispersions of droplets of one liquid in a second, immiscible liquid, e.g. oil in water (Barnes, 1994). Emulsion droplets, usually defined in practice to be greater than 1 μm, are much larger than micelles and their size distribution is controlled by the amount of available constituents and the energy and order of their mixing. Differences between micelles and emulsion droplets are also expressed in the relative ratio of solubilizates to surfactants; molar solubilization ratios (MSR; the number of solubilizate molecules divided by the number of surfactant molecules) are low for micelles (e.g., 10^{-3}) and higher for emulsions (>1) (Carey & Small, 1970). These MSR values indicate that micelles require a greater investment in chemical capital (surfactants) per solubilizate molecule transported.

Although micelles and emulsions consist of solubilizates and surfactants in a fluid state, they behave like particles in suspension. For example, colloids passing through tortuous media can be captured by constrictions of smaller sizes. Filtration effects on the mobilization of lipids are often seen when drilling for oil. When drilling fluids emulsify, the recovery of hydrocarbons can decrease by 55% (Castro Dantas & Sousa, 1995). Surfactant injection is a common strategy for clearing clogged oil wells. Greater

concentrations of surfactants relative to solubilizates "break" larger emulsion droplets into micelles that can flow more easily through the constrictions of the medium (Castro Dantos et al., 2001; Angle, 2001).

Within the gut of a deposit feeder, analogous to the situation in an oil well, fluidized material needs to be delivered through a complex of mineral grains. Deposit feeders may have to deal with the consequences of filtration when mobilizing ingested lipids within their digestive tracts. In some deposit feeders, sediment plugs traversing the digestive tract are not radially mixed (Penry, 1989). Lack of mixing forces digestible material to pass through the sediment for delivery to absorptive cells lining the gut (assimilation). Sediment may filter lipoidal colloids and decrease the efficiency of lipid assimilation for deposit feeders. *A priori*, one would expect filtration to have a greater effect on larger colloids. Thus, as potent sources of dietary energy, lipids are likely mobilized in deposit-feeder guts in a way to restrict these effects. Deposit feeders may, therefore, rely upon micellization – the use of the smallest colloids – rather than emulsification to transport lipids in the gut.

In this paper, we examined the potential for filtration to reduce the efficacy of lipid transportation in a deposit feeder's gut. We determined delivery through a plug of sediment of micelles and emulsion droplets suspended in a deposit feeder's gut fluid. We used a phyletically diverse collection of animals and a feeding study with *Nereis virens* to test our investigation of filtration for ecological relevance; if animals that ingest sediment freely use emulsion droplets rather than micelles as vehicles for digestive lipid transport, then there would not appear to be an *in vivo* problem. Finally, we tested the size of

lipoidal colloids formed under varying *in vitro* conditions of food lipids and digestive fluids.

2.3. Materials & Methods

2.3.1. Filtration impacts on colloids of different sizes

Arenicola marina gut fluid (containing micelles) and an aliquot of this fluid amended to have emulsion droplets were passed through a column of sediment under conditions designed to mimic this animal's gut. Individuals of *A. marina* (a deposit-feeding polychaete) and sediment from their feeding funnels were collected from Lubec, Maine, USA. Animals were stored in seawater up to 4 h and gut fluids were removed by carefully cutting open the body wall and inserting a pipette tip directly into the stomach. Individuals' fluids were pooled, passed through a 0.45-μm PTFE (Teflon) membrane filter, decanted into plastic containers, stored on ice until returned to the lab, and then stored at -80°C until used.

Direct from the animal, as indicated by contact angles of gut fluid titrations with artificial seawater, *A. marina* gut fluids contained micelles. 0.5 g L^{-1} of "Self Emulsified Liquid Concentrate" (SELCO, www.inve.be) was added and the mixture vortexed (30 s) to create an emulsion. SELCO is a proprietary mixture of lipids and proteins formulated to emulsify in seawater and commonly used to increase the delivery of nutritional lipids to aquacultured animals. Both fluids were spiked with hydrophobic ^{3}H-benzo(a)pyrene at ~5.0 x 10^{-3} μM, to serve as a tracer for the colloids suspended in solution. This concentration is well below the micellar capacity for benzo(a)pyrene (BaP) in this gut

fluid (Voparil & Mayer, 2000) thereby ensuring BaP solubilization rather than precipitation.

The gut environment was mimicked using sediment from *Arenicola's* feeding pits with a thickness to match the radius of their guts for our experiments. Both fluids were passed through columns of freeze-dried Lubec sediment supported by a 20-μm Nitex mesh (www.sefar.com). Sediments were packed at a height of 3 mm (the radius of the midgut), and overlying, labeled fluid was drawn through under a slight vacuum (5 in. of Hg). Vacuum was applied to decrease the time required for fluid permeation, but may have deformed larger droplets, allowing them to squeeze through restrictions. Both fluids were passed through Nitex mesh alone to adjust for BaP sorption to this polymer. Polycarbonate membrane filters of 3.0, 1.0, and 0.45 μm pore diameter (Nuclepore, Pleasanton, CA, USA) were also used to compare sediment filtration to a system of clearly defined pores. Permeability was calculated from measurements of ^{3}H-BaP activity of the gut fluid before and after filtration (corrected for sorption to Nytex). Radioisotopic activity was counted in ScintiVerse BD cocktail (www.fishersci.com) on a LKB Wallac 1217 RackBeta liquid scintillation counter.

2.3.2. *In vivo* tests of filtration as an ecological pressure

We looked for situations where animals ingesting sediment used emulsion droplets to mobilize lipids in the gut. Finding such conditions would be *prima facie* evidence that filtration was not a significant problem *in vivo* for deposit feeders. Absence of emulsions coexisting with sediment ingestion would be consistent with an ecological

cost of filtration. We tested for emulsions and sediment in guts with both a cross-phylum survey and a more restrictive feeding experiment.

2.3.2.1. Cross-phyletic survey

Seven species of benthic animals were collected from Washington, California, and Maine, USA, in both subtidal and intertidal environments (Table 2.1). Most animals were deposit feeders, except for one suspension feeder (*Cucumaria frondosa*) and an obligate carnivore (*Glycera dibranchiata*). Animals uninjured during collection were used for study and were dissected immediately for collection of fluid from the digestive tract. Gut fluids were removed by carefully cutting open the body wall and inserting a pipette tip directly into the midgut. This region of the gut usually has maximal enzyme activities and surfactant concentrations (Mayer et al., 1997). Fluids from individuals of each species were pooled, centrifuged (1200 g for 10 min) to remove mineral particles, and stored at −80°C until surfactancy measurements and emulsion droplet size analysis. Note that these gut fluids were not filtered, which would have removed emulsion droplets.

2.3.2.2. Feeding experiment

Individuals of the omnivorous polychaete, *Nereis virens*, were fed three different diets and then dissected to collect their digestive fluids to determine whether the sizes of lipoidal colloids in the gut are influenced by sediment and lipid in the diet. Worms and the surface sediments surrounding their burrows were collected from the intertidal mudflat in Lowes Cove, Walpole, ME, USA, rinsed in flowing seawater, and assigned to

Table 2.1: Marine invertebrate gut fluids investigated

Species (location)	Taxon	Feeding mode	CMD [A]	Emulsion? [B]
Cucumaria frondosa (ME)	Holothuroid	Suspension feeder	ND	No
Molpadia intermedia (WA)	Holothuroid	Deposit feeder	ND	No
Parastichopus californicus (WA)	Holothuroid	Deposit feeder	40%	No
Arenicola brasiliensis (CA)	Polychaete	Deposit feeder	22%	No
Arenicola marina (ME)	Polychaete	Deposit feeder	15%	No
Nereis virens (ME) -sediment diet	Polychaete	Omnivore	26% (avg.)	No
Nereis virens (ME) -mussel diet	Polychaete	"	22% (avg.)	Yes
Glycera dibranchiata (ME)	Polychaete	Carnivore	X	Yes

[A] CMD = critical micelle dilution; the dilution below which surfactant micelles are converted to monomers. ND indicates that micelles were not detected.

[B] Via Nile Red visualization

a dietary treatment (8 worms/treatment). Worms were housed individually in 15-cm diameter buckets and placed randomly in a large, flowing-seawater table.

The dietary treatments for this experiment were: (i) freeze-dried mussel tissue (*Mytilus edulis*); (ii) organic matter-free sediment amended with freeze-dried mussel tissue; and (iii) natural sediment. These treatments represent, respectively, a carnivorous diet (very high lipid, no mineral), a relatively rich deposit-feeder's diet (high lipid, high mineral), and a normal deposit-feeder's diet (low lipid, high mineral). Mussels were collected from the Darling Marine Center dock, and their flesh was removed and homogenized in a blender. The resulting mussel paste was freeze-dried in ice cube trays. For the OM-free sediment + mussel treatment, Lowes Cove sediments were baked at 400°C for 12 h to volatilize organic matter. Mussel tissue (the same as used in the first treatment) was added to sediment at 1% by weight. This fraction of mussel tissue was chosen to match the organic carbon content of unadulterated sediment. For the regular deposit-feeder diet, sediment was rinsed repeatedly with clean seawater to remove debris, passed through a 1-mm sieve, and freeze-dried in ice cube trays. The food treatments were measured for organic carbon and nitrogen on a Perkin Elmer 2400 elemental analyzer following vapor phase acidification to remove carbonate minerals. Food treatments were cleaned out and replaced with fresh material every 72 h; there was always excess food for the animals. On the 9th day, the experiments were terminated and the gut fluids were removed for surfactancy measurements and emulsion droplet size analysis.

2.3.2.3. Surfactancy measurements

We measured the change in the contact angle of gut fluid during titration with artificial seawater (ASW) on Parafilm following the methods of Mayer et al. (1997) to determine the presence of surfactant micelles in the gut fluids. The ASW recipe of Parsons et al. (1984) was used. If micelles are present, contact angles remain constant during initial dilution. Once diluted to below the surfactants' critical micelle concentrations, the contact angles increase to become more like those of water. This inflection point is termed the "critical micelle dilution" (CMD), with greater concentration of a particular surfactant leading to lower CMDs. A critical micelle concentration value is specific for a particular surfactant; thus, we cannot use CMD values to compare the concentration of surfactants in different species' gut fluids, as the identity of the surfactants used by these different animals is not known.

2.3.2.4. Emulsion droplet size analysis

200 μL of each animal's gut fluids were incubated with 10 μg of the fluorescent, lipophile Nile Red (www.probes.com) in order to visualize lipoidal colloids. Nile Red in chloroform was dispensed into glass test tubes and the solvent was evaporated under N_2 gas for 15 min. Gut fluids were incubated with Nile Red for 60 min. on a rotary shaker (30 rpm) in the dark. After incubation, Nile Red fluorescence in the fluids was visualized using a Zeiss Universal research microscope with epifluorescence attachment. The filters used for fluorescence were a 480 ± 60 nm for excitation, a 515 nm dichroic, and a 530 nm longpass for emission (all from www.chroma.com). Digital images were captured with an Apogee KX85 CCD camera with a CRI tunable RGB filter for full color images

(www.apogee-ccd.com). Camera controls were adjusted at onset for realistic color and intensity and standardized for all subsequent images. Because this camera setup captures red, green, and blue images in succession and then digitally combines them to form a full-color image, any movement of the droplets resulted in some blurring of the final images. Under the microscope, emulsion droplets appeared spherical with sharply defined borders while micelles exhibited an unresolved background haze.

Digital images of the fluids were analyzed using the "measure/count" function of Image-Pro Plus software (www.mediacy.com). The light intensity required to differentiate an emulsion droplet from the background was manually adjusted for each image to ensure recognition of all visible droplets. For images of mussel flesh and gut fluid (see below), emulsion droplets were visually distinguished from flesh particles. Each droplet's radius was calculated by Image Pro Plus after the software was calibrated with an external scale.

2.3.3. Controls of emulsification

We attempted to create an emulsion in *A. marina* gut fluids to test whether the paucity of lipids in the normal deposit feeder diet might preclude emulsion droplet formation. Gut fluid was incubated with freeze-dried mussel meat (the same as used in section 2.3.2.2.) at solid:fluid ratios from 5 to 180 [g mussel (L gut fluid)$^{-1}$]. Mussel was considered a realistic potential food substrate whose ingestion might lead to formation of large droplets of lipid. The solid:fluid ratio was varied to determine the threshold amount of mussel flesh (and, by proxy, constituent lipids) required for the onset of emulsification. Lipoidal colloids were stained with Nile Red and measured following the same procedure

outlined in Section 2.3.2.4. Incubations were performed once, but 3 different images of each resultant fluid were taken for statistical analysis.

2.4. Results

2.4.1. Filtration impacts on colloids of different sizes

Benzo(a)pyrene (BaP) in micelles passed through sediment columns and polycarbonate filters more efficiently than BaP in emulsions (Fig. 2.1). When using Lubec sediment columns as filters, both were retained by sediment with greater efficiency; 20% of the BaP in micelles and 40% of the BaP in an emulsion was retained. Contact angles of the micellar gut fluid before and after filtration are similar; contact angles of full-strength gut fluid after filtration are ~ 3° greater than before, but the CMD in both fluids is ~15% gut fluid (Fig. 2.2). Therefore, BaP was not an inert tracer as some of the BaP lost to sediment likely resulted from partitioning of BaP to sedimentary organic material (SOM), rather than filtration of micelles from gut fluid.

Micelles containing BaP were unaffected by even the smallest polycarbonate membrane filter (0.45 μm); there was no significant difference between the initial BaP concentration in micellar fluid and the concentration after passage through any of the polycarbonate membranes. Emulsion droplets were retained by all of the filters; as expected, the smaller the pore size of the filter, the greater the retention of emulsified BaP. The sediment column retained the same amount of emulsified BaP (63%) as the 3.0 μm polycarbonate filter (59%).

2.4.2. Tests of filtration as an ecological constraint

2.4.2.1. Cross-phyletic survey

Emulsion droplets were present only in the gut fluid of the carnivore *Glycera dibranchiata* (Table 2.1, Fig. 2.3C). No other species' gut fluid's contained emulsion droplets. However, all deposit feeders, except *M. intermedia*, had micelles in their guts according to contact angle titrations and Nile Red incubations. Incubations with Nile Red yielded a diffuse, background glow indicative of solubilization into micelles, which would be too small to resolve with a light microscope. Gut fluids from animals eating natural sediment also contained amorphous material that seemed to be detritus. These particles were not spherical, nor did they fluoresce strongly after incubation with Nile Red, which would be characteristic of lipids in a fluid state. A representative image for all deposit-feeder gut fluids is shown in Fig. 2.3B (from *A. marina*).

Molpadia and the suspension feeder *Cucumaria* did not have micelles in their gut fluids. These two species solubilize a variety of lipids including hexadecane, hexadecanol, palmitic acid, cholesterol, and BaP to an extent no greater than seawater (Mayer et al., 2001; Voparil, unpublished). *Cucumaria* and *Molpadia* fluids exhibited little Nile Red fluorescence and were almost completely dark (Fig. 2.3A), supporting CMD work that indicated no surfactant micelles.

We were unable to determine the presence of micelles in the gut fluid from *Glycera dibranchiata*. This fluid was too viscous to accurately transfer with an air displacement pipette and did not mix well with artificial seawater during contact angle titrations.
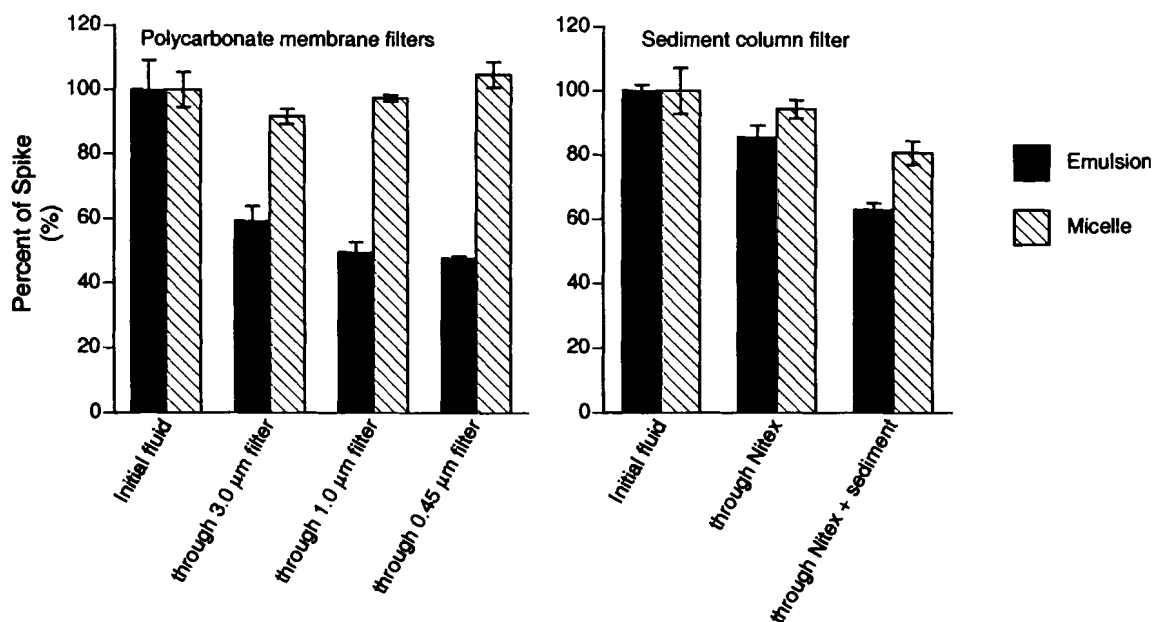
Figure 2.1: Filtration of ^{3}H-BaP-labeled lipoidal colloids by sediment and membrane filters.

Abscissas are the particular materials through which the fluids containing either emulsion droplets or micelles were passed. Ordinates are the percent of the ^{3}H-BaP spike in each fluid after filtration. Fluids containing emulsion droplets are in black, and fluids containing micelles are hatched.
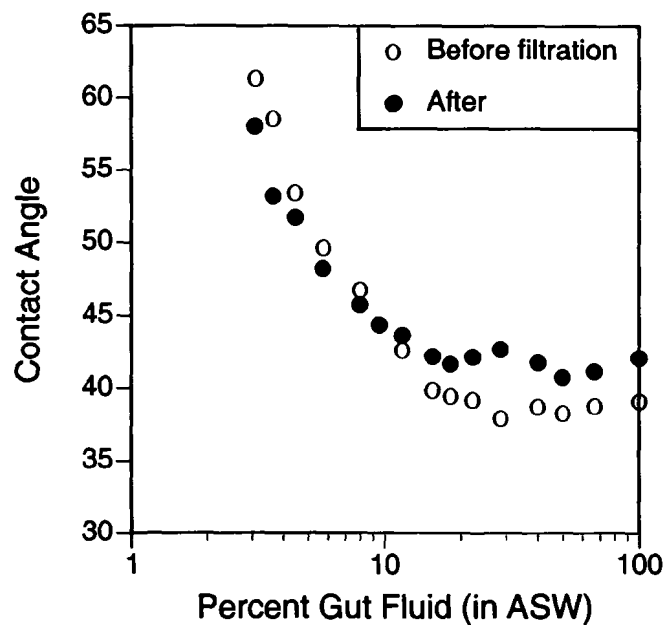
Figure 2.2: Contact angles of *A. marina* gut fluid containing micelles before and after
passage through Lubec sediment column.

Abscissa is the percent of gut fluid diluted in artificial seawater. Ordinate is the contact angle.titrations. In fact, the handling of this fluid was the original impetus for our attention to the differences between emulsions and micellar fluids in their abilities to flow.

2.4.2.2. Feeding experiment

Emulsion droplets were found only in the guts of *Nereis virens* eating pure mussel meat (Fig. 2.4). These droplets had a mean diameter of 7.7 ± 6.2 μm (Table 2.2). Individuals eating the natural sediment treatment or the OM-free, sediment + mussel treatment contained micelles but no emulsion droplets in their guts. Feeding treatments had no effect on the surfactancy of *Nereis virens* gut fluids (Fig. 2.5). All of the worms feeding on natural sediment, and all but one individual for each other treatment, had surfactant micelles in the gut as indicated by contact angle titrations and Nile Red incubations. However, the CMD varied to great extent among individuals within each treatment, as reflected by the large standard deviations of the data.

Food treatments varied in their content of organic carbon (OC) and nitrogen (Table 2.3). The mussel diet had much more OC than the diets containing sediment (> 100 times more OC) and lower C:N ratios than the natural sediment. Total levels of OC and nitrogen in the mussel-amended sediment were similar to natural Lowes Cove sediments (Table 2.3). However, organic carbon and nitrogen in mussel meat is likely in more labile forms, e.g., phospholipids and proteins, which would emulsify in the gut. (Indeed, in section 2.4.3, I note that mussel meat mixed with gut fluid forms an emulsion.) Previous analysis of this sediment and another batch of freeze-dried mussel tissue indicated that both samples had ~25% of total organic material as lipids (Bock & Mayer, 1999). This number seems suspiciously high for the sediment, but TOC values are extremely low, implying a coarse sand whose organic matter is often relatively labile (Dauwe et al., 1999).
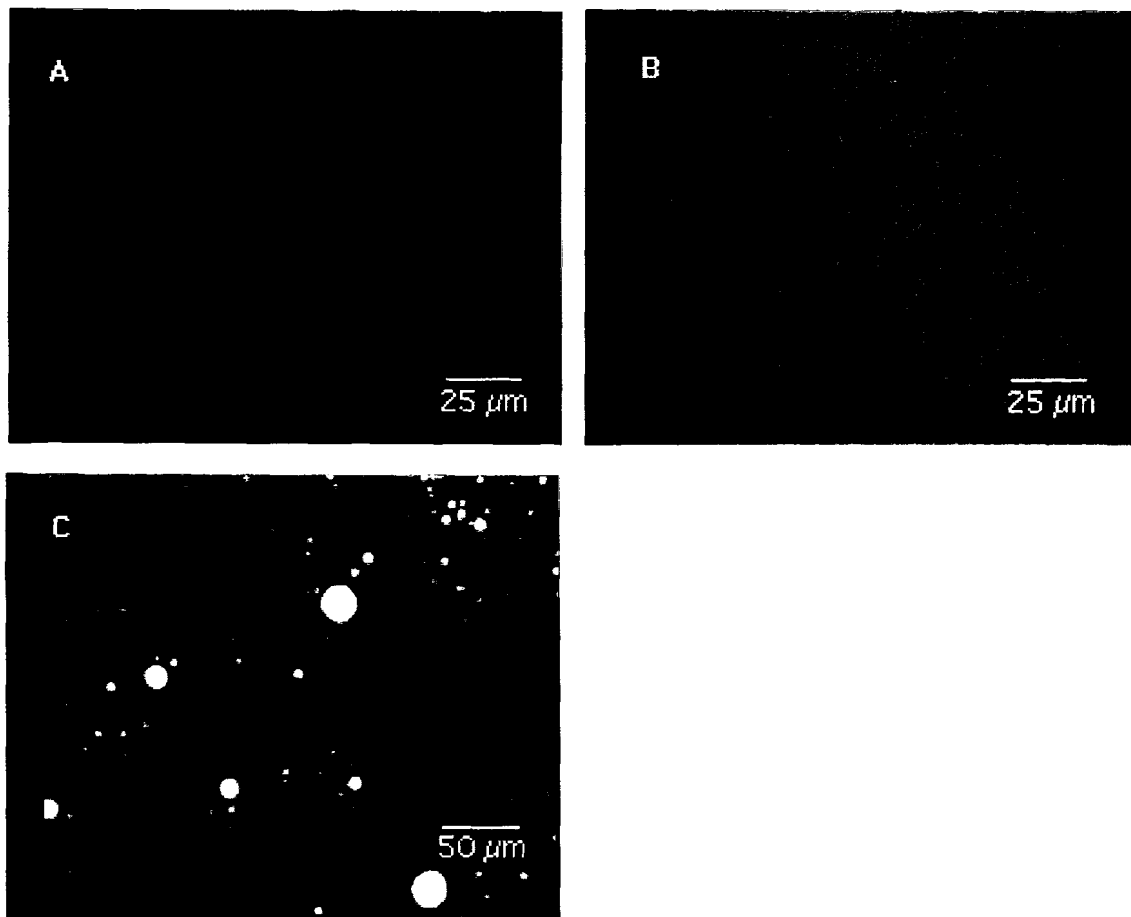
Figure 2.3: Nile Red solubilization by eight different animals' gut fluids indicates three different conditions: almost no lipid solubilization, micelles, and emulsions.

A) *Cucumaria frondosa* gut fluids were dark, with specks of fluorescence that did not appear to be spherical, thus indicating solid particles. B) *Arenicola marina* gut fluids have a diffuse orange glow in the background which indicates micellar solubilization of Nile Red. C) *Glycera dibranchiata* gut fluids show spherical lipid droplets (emulsion) ranging in diameter up to 25 μm. The golden tint of the droplets suggests a relatively more hydrophilic environment, for example an emulsion of phospholipids. The background color is almost perfectly black. These gut fluids showed no red or orange autofluorescence.

Mussel Diet Mussel + OM-free Sediment Sediment Diet
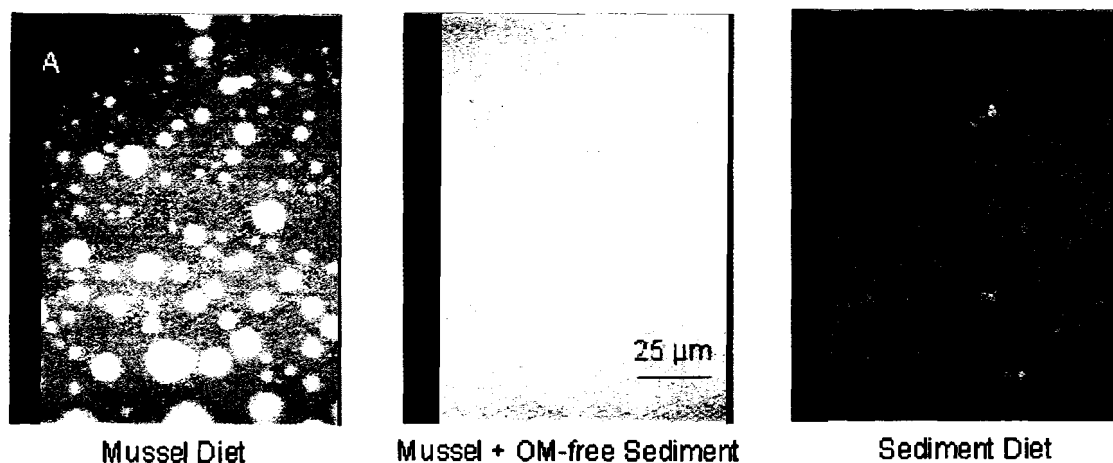
Figure 2.4: Nile Red fluorescence images from *N. virens* gut fluids from individuals with different diets.

All fluids showed no autofluorescence without the addition of Nile Red. For worms on a mussel diet, emulsion droplets are distinct within the gut fluid (A). Worms ingesting sediment have few or no visible droplets. However, all fluids have a fluorescent "haze" in the background indicating solubilization of Nile Red into surfactant micelles.

Table 2.2: Emulsion droplet diameter (μm)

Sample	Min	Max	Mean ± SD
A. marina gut fluid + SELCO	0.6	21.7	4.8 ± 4.2
G. dibranchiata gut fluid	0.5	23.3	4.4 ± 4.1
N. virens gut fluid (Mussel diet)	1.0	25.1	7.7 ± 6.2
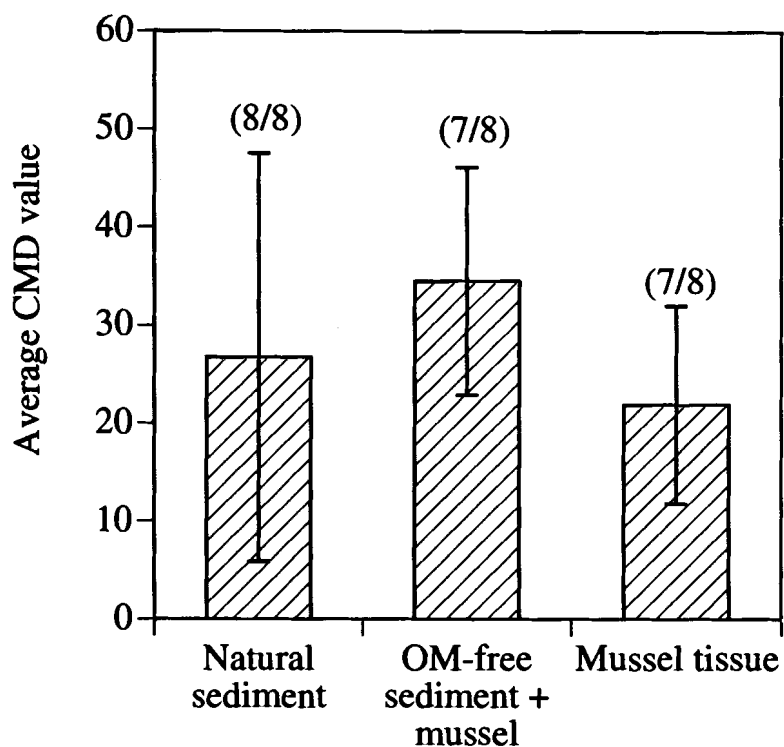
Figure 2.5: Average CMD values for *N .virens* individuals with different diets. There are no differences between CMD values for any of the treatments. Numbers in parentheses indicate the number of individuals (out of 8) showing evidence of micelles in the gut.

Table 2.3: Total organic material associated with *N. virens* feeding treatments

Sample	Organic carbon (mg g⁻¹)	Nitrogen (mg g⁻¹)	C:N ratio
Natural sediment	3.42	0.37	9.24
OM-free sediment	0.37	0.03	12.33
OM-free sediment + mussel	3.85	0.74	5.20
Mussel tissue	401.58	82.79	4.85

Data on emulsion droplet sizes and frequencies are in Table 2.2 and Fig. 2.6. The

three fluids containing emulsion droplets (unadulterated fluid from the carnivorous *G.*

dibranchiata, gut fluid from *N. virens* fed mussel meat, and *A. marina* gut fluid amended

with SELCO) had similar maximum droplet sizes. Minimum values, though similar, are

a function of the analytical window – colloids smaller than ~1 μm fall out, as contact

angle titrations and red haze after incubation with Nile Red confirm that smaller colloids,

e.g., micelles, were present. *Glycera* and modified *Arenicola* gut fluids have similar

dispersions of emulsion droplets, while *N. virens* gut fluids tend to have more

intermediate and larger (> 4 μm) droplets.

2.4.3. Controls of emulsification

Mussel flesh readily formed an emulsion when mixed with deposit-feeder gut

fluid above a solid:fluid ratio of ~80 g L^{-1} (Fig. 2.7). This experiment supports the

concept of a threshold in the relative concentrations of surfactant and solubilizates before

emulsification occurs. Paucity of lipids in the gut may, therefore, explain the lack of

emulsions in deposit-feeder guts.

2.5. Discussion

We find that filtration by sediments may be an important impediment to lipid

assimilation in marine deposit feeders. Under the *in vitro* conditions used, filtration

decreased delivery of lipids by 40%. Though we cannot say that this same level of effect

would occur during delivery of lipids to enterocytes lining the digestive tract *in vivo*,

transport mechanics clearly puts an ecological pressure to limit the size of lipoidal
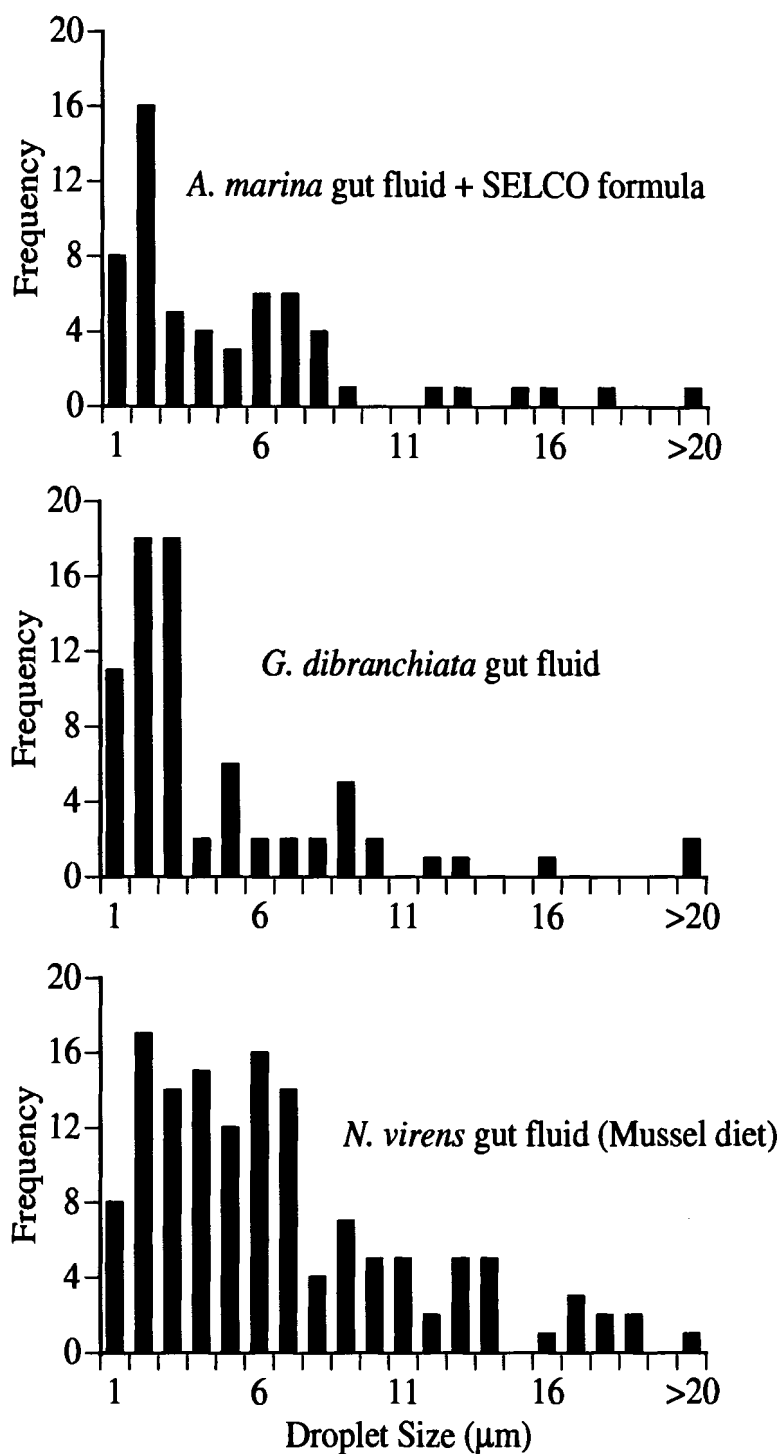
Figure 2.6: Droplet size frequency for three gut fluids containing emulsions. Abscissas are the bins for droplets of specific sizes. For example, a value of 2 on the x-axis represents droplets of size = $1 < x \leq 2$. Ordinates are the numbers of droplets of a particular size.

colloids used to transport dietary lipids in the guts of deposit feeders. Further evidence of this pressure is that all of the deposit feeders investigated and the *N. virens* individuals with sediment in the diet had only micelles in their guts.

Filtration likely affects only one feeding guild, the deposit feeders. This specificity is due to two dietary prerequisites: (i) a large fraction of the diet must be indigestible matter and (ii) enough emulsifying lipid must be ingested to form an emulsion in the gut. These conditions can be thought of as opposite endpoints along the spectrum of diets available to marine invertebrates. For example, carnivores eating soft-bodied prey ingest a diet high in emulsifying lipids that can be almost completely assimilated. Thus, there is no "filter" of indigestible material. Therefore, in the guts of carnivores, large emulsion droplets can be used to maximize the mobilization of nutritional lipids for delivery to the gut wall without consequence, as was found in the guts of *Glycera* (Fig. 2.3).

At the other end of the food quality spectrum, deposit feeders are commonly thought to eat a diet of low quality. There may not be enough lipid material in ordinary diets to emulsify when mixed with endogenous lipids in the gut; thus under normal dietary conditions, filtration may not be an issue. However, deposit feeders must also take advantage of episodic events that deliver a windfall of nutritious material. For example, deep-sea sipunculids cache labile food within their burrows (Jumars et al., 1990). When processing these windfalls, deposit feeders may favor the use of small colloids that are less susceptible to filtration. They can do so by limiting the amount and frequency of emulsifying lipid in the digestive tract, e.g., by taking smaller, less frequent "bites" or by diluting ingested lipid with sediment.
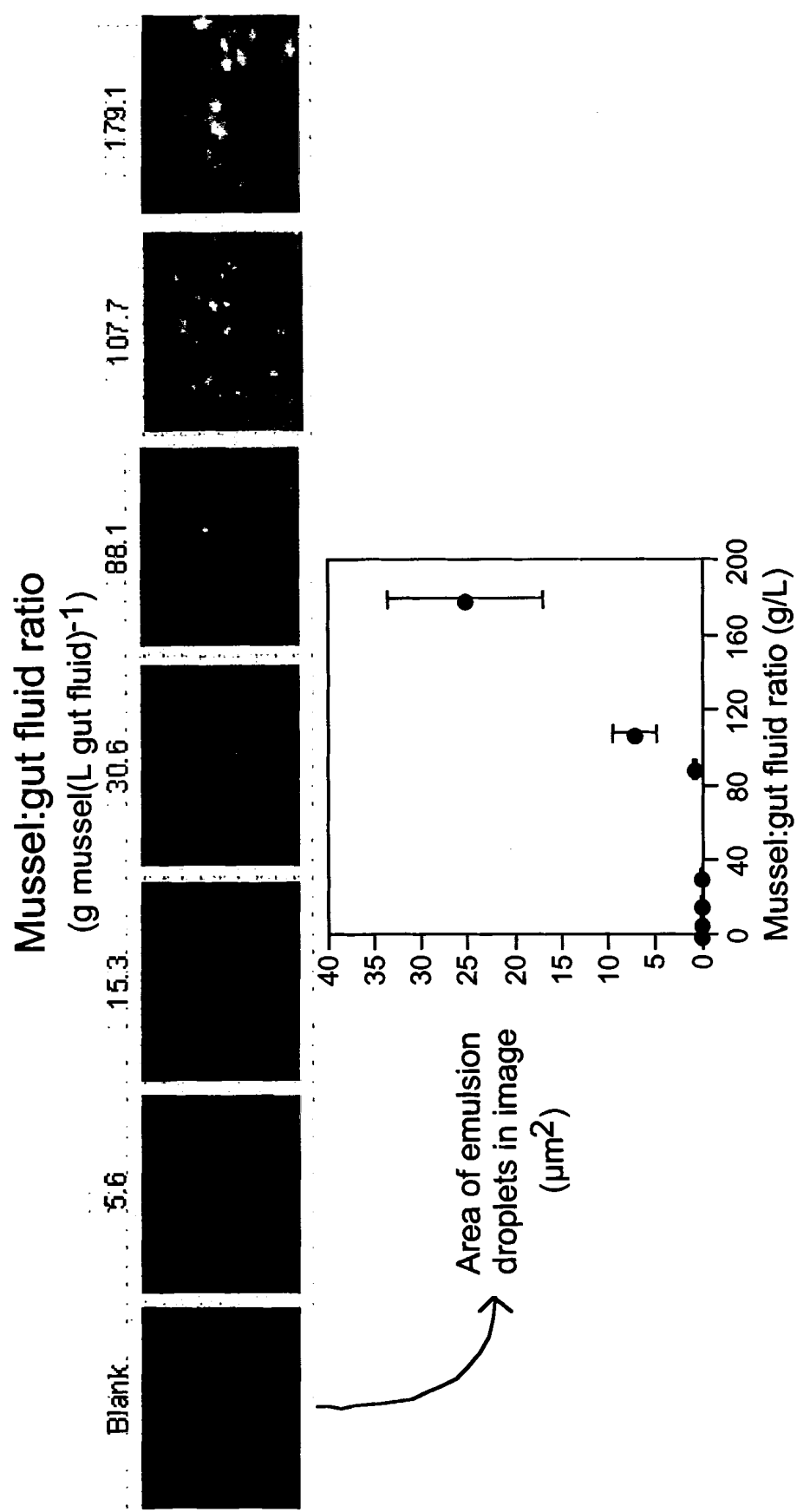
Figure 2.7: Emulsion droplets form when mussel meat is mixed with *A. marina* gut fluid above a solid:fluid ratio of ~80 g L^{-1}. For the graph, error bars are ± 1 SD.

2.5.1. Mechanics of filtration

The factors governing emulsion filtration have been well studied because of the importance to many industrial processes. Thinking of emulsion droplets as particles (albeit deformable ones) in suspension allows significant intuition into the controls of filtration. Passage through a porous medium can capture emulsion droplets as well as decrease the bulk fluid's permeability as pores become clogged and fluid is forced to flow through a reduced number of pores or via more circuitous routes (Soo & Radke, 1984). Capture increases dramatically as the ratio of droplet size approaches pore size, though droplets larger than a specific size can sometimes be forced through by application of additional force to the fluid phase (McAuliffe, 1973; Briscoe et al., 2000). Permeability to colloids is positively related to solid-particle size, smoothness of grain surfaces, and the porosity of the medium (Yan et al., 1991, Vidrine et al., 2000). Electrostatic interactions between the charges on the surface of the colloid and on the surface of the solid phase can also affect filtration (Hofman & Stein, 1991). For example, opposite charges can immobilize droplets to the solid surface, followed by aggregation and formation of larger emulsion particles (Vidrine et al., 2000).

2.5.2. Potential biological responses to filtration

Emulsion formation requires concentrations of surfactants and solubilizates that surpass some threshold value. We used *A. marina* gut fluid mixed with mussel flesh to investigate this threshold concept under realistic, digestive conditions and found that 80 mg of mussel flesh, when mixed with 1 mL of gut fluid, forms an emulsion (Fig. 2.7). We did not identify and quantify the particular components of the gut fluid (the

surfactants) and the mussel flesh (the emulsifying lipids) responsible for emulsification. The proportion of lipids present in mussel flesh likely varies with season, and the enzyme activities of gut fluid show tremendous plasticity (Bock & Mayer, 1999; Mayer et al., 1997). For example, lipase activity on triglycerides creates monoglycerides, which readily form emulsions (Hofmann & Borgstrom, 1962).

Identification of the chemical constituents in emulsions would allow creation of phase diagrams that could be more generally applied. Phase diagrams, indicating the type of aggregate formed at different relative concentrations of constituents, are widespread for different combinations of surfactants and solubilizates important during vertebrate lipid digestion (e.g., Fig. 7 in Carey et al., 1983; Fig. 8 in Staggers et al., 1990). These are well-described systems, in which the identities and concentrations of the important constituents, i.e., the concentrations of particular bile salts present in the gut and the concentrations of emulsifying lipids, are already known. Identification of the specific surfactants and emulsifying lipids present would be required to create phase diagrams for the conditions in deposit-feeder guts. The digestive surfactants of *A. marina* have been identified (Smoots et al., in prep.), but the suites of lipids present in deposit feeders' guts have yet to be identified.

Deposit feeders could adjust their dietary solubilizate:surfactant ratio by modifying either the amount of solubilizate (ingested lipid) delivered to the gut or the amount of surfactant secreted into the gut during digestion. For example, filtration could force a behavioral response whereby animals with lipid-rich diets ingest smaller amounts of food less frequently in order to decrease the amount of solubilizate in the gut. I am unaware of feeding studies with deposit feeders that have explicitly measured the lipid

content of the diet concurrently with rates of gut throughput. However, Mayer et al.

(1993) found that a deposit feeder's (*Streblospio benedicti*) ingestion rate slowed when

feeding on greater concentrations of low-density material from sediment. Though the

authors focused on the (increased) protein content, the low-density material was likely

also enriched in lipid compared to natural sediment. Changes in the feeding rate may not

necessarily have to do with the chemical composition of the particles, but rather increased

sorting and selecting of material for ingestion from a complex mixture of particles

(Jumars et al., 1982; Taghon 1982).

When eating lipid-rich food, animals could also increase surfactant concentration

in the gut to "break" emulsions into smaller colloids, as is used when oil recovery is

stymied by emulsification. Though our results indicated no change in the gut surfactancy

of *N. virens* individuals ingesting diets with varying lipid content (Fig. 2.5), Bock &

Mayer (1999) had different results – no indication of micelles in *N. virens* individuals

feeding on mussel meat. Only individuals ingesting sediment in the diet had micelles in

the gut. These disparate results are perplexing; there were no apparent differences in

animal handling and food preparation between my study and theirs. I consulted with M.

Bock prior to initiating the feeding experiment in order to match the conditions of his

study. Perhaps some uncharacterized aspect of the food was different. Concentrations of

emulsifying lipids in mussel meat may have differed; high amounts in Bock & Mayer's

(1999) mussel treatment may have obviated the need for surfactants to mobilize lipids

within the gut. This difference in emulsifying lipids is conjecture and, as such, a rather

unsatisfying explanation of the differences between these studies.

Deposit feeders may also control an as yet undetermined quality of the surfactants secreted into the gut during the digestion of an especially lipid-rich meal. Our measure of CMD offers only a general picture of the surfactancy of the gut fluids. More information as to the concentration and composition of surfactants secreted to the gut would be useful. For example, *A. marina* has three structurally distinct surfactants in its gut fluids (Smoot et al., in prep.) that seem to vary in relative concentration among individuals (Voparil, unpub. data). Animals may adjust the surfactants' relative concentrations in response to the lipid or other contents of the diet or to their own dietary histories and current nutritional conditions.

2.5.3. Lipid transport mechanisms in guts

Marine invertebrates utilize a number of different strategies with which to overcome the basic difficulty of transporting lipids (hydrophobic compounds) through the aqueous phase that exists in the digestive tract. Many animals use some combination of micelles and emulsion droplets to enhance lipid mobilization in the gut. But what about *Molpadia* and *Cucumaria*, the species that solubilize lipids to the same extent as seawater? How do they mobilize ingested lipids? Instead of using extracellular digestion, these species may rely on the intracellular digestion of dietary solids. Echinoderms, in general, are thought to be capable of intracellular digestion (Rosati, 1968; Filimonova & Tonkin, 1980; Lawrence 1982), which circumvents the dissolved-phase transport step entirely by advecting particles through the gut lumen, then engulfing them into the cells lining the digestive tract (phagocytosis). Other examples of animals

that use intracellular digestion include platyhelminths, nemerteans, some annelids, arthropods, and deuterostomes (Brusca & Brusca, 1990).

I suggest three mechanisms used to mobilize ingested lipids (Fig. 2.8) and propose that dietary constraints guide the choice of mechanism for a particular animal. Potential mechanisms for lipid transport include: (i) the direct uptake of solids via phagocytosis; (ii) mobilization in colloids as large as emulsion droplets; and (iii) mobilization in surfactant micelles.

Animals that ingest food of very high quality, free of indigestible components, have all of the pathways of lipid transport available to them. For those animals using extracellular digestion, lipid mobilization can be maximized via the formation of emulsions containing large droplets. Emulsions provide two advantages relative to micelles: 1) they mobilize more lipid in the aqueous phase than micelles as emulsion droplets are much larger, and 2) they require less investment in solubilizing "aids" such as surfactants (often in combination with proteins) than micelles per amount of solubilizate mobilized. Assuming that colloids are spherical, that surfactants are located at the lipid-water interface, and that solubilizates tend to partition to a colloid's interior, then the ratio of solubilizates to surfactants (the molar solubilization ratio described in the introduction, MSR) should be proportional to the volume-to-surface area ratio of a sphere:

$$(4/3\pi r^3)/(4\pi r^2) = r/3$$

MSR values, therefore, increase with the radius of the colloid (r), indicating that the chemical investment in surfactants per solubilizate molecule is minimized when size is maximized, e.g., emulsion droplets.

For animals whose diets contain greater fractions of indigestible material, extracellular digestion allows enzymes and surfactants to penetrate ingested material in order to access labile compounds. The fraction of indigestible matter and the specific composition of the food create conditions favorable to the use of either micelles or emulsions. When the bulk of material is refractory, as is the case for deposit feeders, colloids are required to navigate the tortuous paths between sediment grains to the gut wall for assimilation.

In the gut of a deposit feeder (*A. marina*), diffusion alone is not rapid enough to deliver micelles or emulsion droplets for assimilation. The Stokes-Einstein equation indicates that the diffusion coefficient decreases with increased hydrodynamic radius of the particle undergoing diffusion:

$$D = \frac{KT}{6\pi\eta R_H}$$

where D = diffusion coefficient ($cm^2\ s^{-1}$)

K = Boltzmann's constant (1.3807×10^{-16}) ($g\ cm^2\ s^{-2}\ K^{-1}$)
T = absolute temperature (°K)
η = solvent dynamic viscosity ($g\ cm^2\ s^{-1}$)
R_H = hydrodynamic radius of particle (cm)

Inserting realistic values for temperature (292 K) and seawater viscosity ($1.1 \times 10^{-2}\ g\ cm^2$ s^{-1}; Denny, 1993), the diffusion coefficient for a micelle (radius = 2×10^{-7} cm) is $1.0 \times 10^{-6}\ cm^2\ s^{-1}$; that for a small emulsion droplet (radius = $0.5\ \mu m$) is $4.0 \times 10^{-9}\ cm^2\ s^{-1}$ (250 times slower). Therefore the diffusive delivery of micelles is much faster than that of emulsion droplets and would allow, for example, much shorter gut residence times (GRT) for ingested lipid material if diffusion alone were responsible for transporting material to the absorptive cells lining the gut wall.
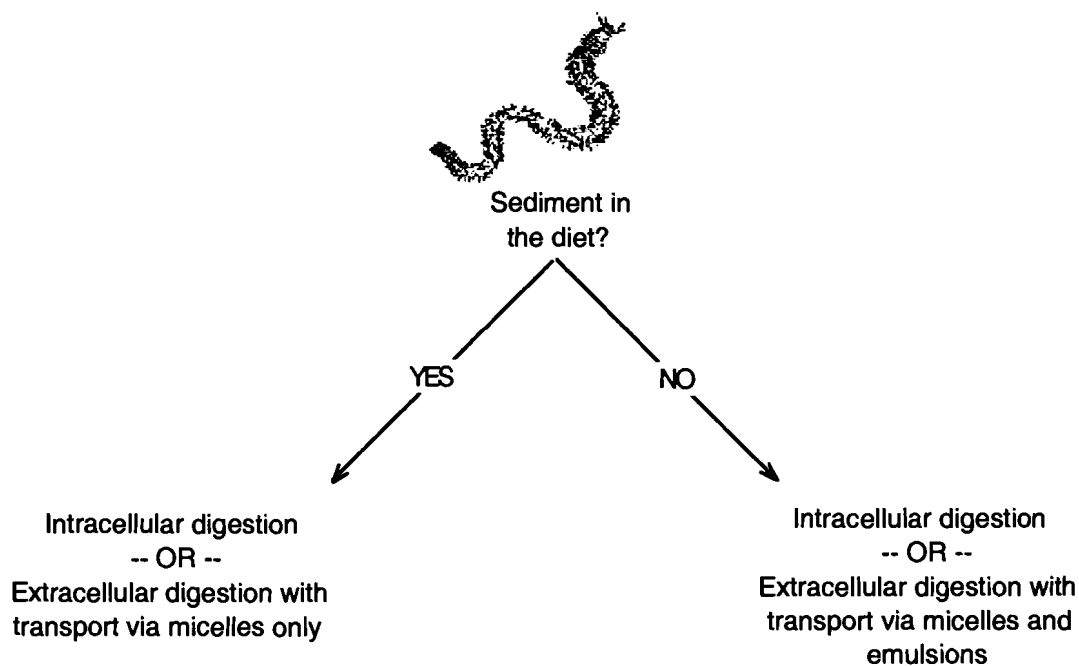
Figure 2.8: General pathways for lipid mobilization in the guts of marine invertebrates. The presence of sediment in the diet forces animals to modify the mechanisms used for lipid mobilization. Those animals using intracellular digestion must select and sort ingested material thoroughly to ensure that only nutritious material undergoes phagocytosis. Animals using extracellular digestion in the presence of sediment can only transport lipids in micelle-sized colloids, rather than larger, otherwise more efficient emulsion droplets.

However, the gut diameter of *A. marina* suggests that the gut is too big for micelles to reach enterocytes during the amount of time that food resides in the gut (Fig. 2.9). Traversing a distance equal to the internal radius of *Arenicola's* gut within the period defined by the GRT (r = 0.25 cm and 5400 s, respectively; Plante & Mayer, 1996) would require a diffusion coefficient of 1.2×10^{-5} cm^2 s^{-1} for a micelle. Only much smaller compounds, e.g., O_2, have diffusion coefficients in this range. Therefore, *A. marina* must use advection to radially deliver lipoidal colloids to the absorptive cells. Differences in the gut size (r = 0.1 cm; pers. obs.) and GRT of food (72000 s; Vahl, 1976) for *Glycera dibranchiata* make delivery of micelles via diffusion possible for this animal. However, emulsion droplets are too large to be delivered to the gut wall without advection.

Arenicola marina

Gut Residence Time

r = gut radius

r = 0.25 cm (Plante & Mayer, 1996)

GRT = 5400 s (Plante & Mayer, 1996)

$$D_{real,\,Arenicola} = \frac{r^2}{GRT} = 1.16 \times 10^{-5}\ cm^2\ s^{-1}$$

Glycera dibranchiata

Gut Residence Time

r = 0.10 cm (pers. obs.)

GRT = 72000 s (Vahl, 1976)

$$D_{real,\,Glycera} = \frac{r^2}{GRT} = 1.39 \times 10^{-7}\ cm^2\ s^{-1}$$
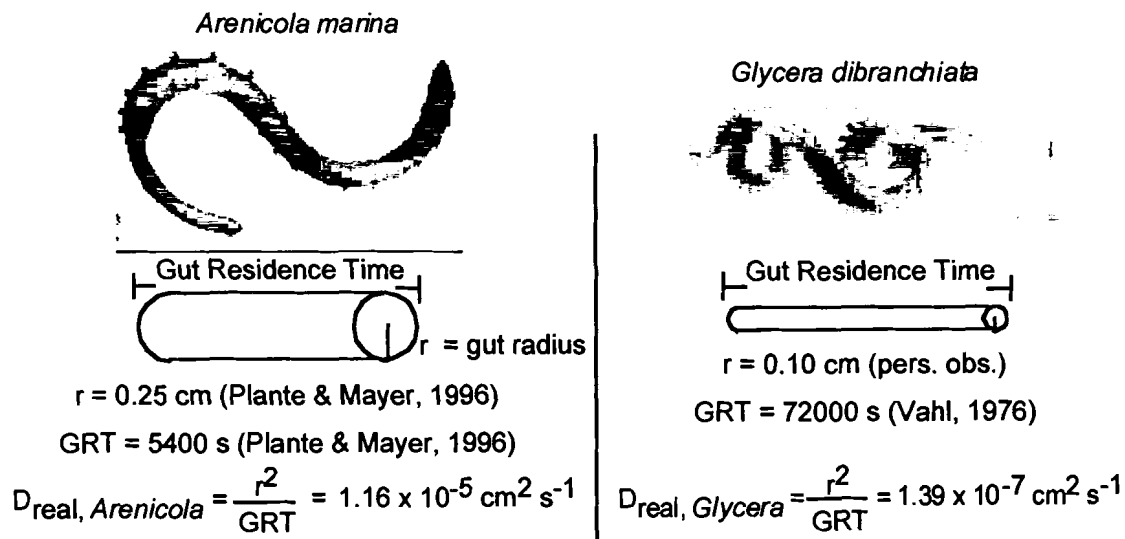
Figure 2.9: The internal gut radius and gut residence time of ingested material sets a diffusion requirement ($D_{required}$) for delivery.

The Stokes-Einstein equation allows calculation of diffusion coefficients for colloids the size of micelles and emulsion droplets (see text). *In vivo* conditions in *A. marina* guts make it clear that diffusion of micelles and emulsion droplets is too slow; these animals must advect gut contents for their delivery to enterocytes. In *G. dibranchiata* guts, conditions may allow diffusing micelles to reach the gut wall, but emulsion droplets are too slow.

Chapter 3 - INTERACTIONS AMONG CONTAMINANTS AND NUTRITIONAL LIPIDS DURING MOBILIZATION BY DIGESTIVE FLUIDS FROM MARINE INVERTEBRATES

3.1. Abstract

Coastal sediments contain complex mixtures of hydrophobic compounds, including organic contaminants such as polycyclic aromatic hydrocarbons and biogenic compounds such as cholesterol and phospholipids. Within the guts of benthic invertebrates these mixtures are subjected to digestive, chemical conditions that can be rich in surfactants and proteins. Using *in vitro* incubations as proxy for digestive exposure, we studied the solubilization of binary mixtures of nutritional and contaminant lipids into artificial seawater and six marine invertebrate gut fluids (*Molpadia intermedia*, *Cucumaria frondosa*, *Arenicola marina*, *Arenicola brasiliensis*, *Parastichopus californicus*, and *Nereis virens*). For animals with surfactant micelles or high protein concentrations, solubilization interactions were frequent. For example, in *A. marina* gut fluid benzo(a)pyrene enhanced the solubilization of hexadecane (491% of the compound alone) and palmitic acid (130%), but hindered cholesterol (83%). Benzo(a)pyrene concentrations increased in gut fluids in the presence of cholesterol (137% of BaP alone), phenanthrene (154%), lecithin (140%), and hexadecanol (232%). In *A. marina* gut fluid, dilution with seawater indicated that these enhancements occur only when micelles are present. Sediment-water partitioning models, used to predict the bioavailability of hydrophobic organic chemicals, do not account for such interactions between

solubilizates (compounds solubilized in micelles). However, for animals exposed via a

digestive tract containing micelles or high protein concentrations, digestive

bioavailability and perhaps bioaccumulation are likely influenced by these interactions.

3.2. Introduction

Coastal sediments are often contaminated with complex mixtures of hydrophobic

organic compounds, e.g., polycyclic aromatic hydrocarbons (PAH), that can be toxic and

procarcinogenic (LaFlamme & Hites, 1978). Current mechanistic models of sediment

toxicity and bioaccumulation suggest that the biological effects of a mixture of PAH can

be predicted by the sum of all individual components' toxicity (the ΣPAH model; Swartz

et al., 1995). Inherent in this model is the assumption that exposure to a mixture of

hydrophobic chemicals should also be additive, resulting from equilibrium partitioning

between the aqueous phase and the animal. When very hydrophobic compounds are

freely dissolved in water, molecules are generally thought to be too dilute to influence

one another (Schwarzenbach et al., 1993). However, water is not the only pathway of

exposure, nor even the primary means of exposure, for some types of benthic animals.

Deposit feeders are often exposed to these contaminants primarily via the

digestive tract (Landrum, 1989; Weston, 1990; Leppanen, 1995; Weston et al., 2000;

Ahrens et al., 2001). The gut is a complex reaction zone of hydrolytic enzymes,

dissolved organic matter and surfactants that solubilize organic compounds at many times

their seawater, and equilibrium partitioning-predicted, solubilities (Voparil & Mayer,

2000). In the gut fluids of the deposit-feeding polychaete *Arenicola marina*, surfactant

micelles solubilize benzo(a)pyrene (BaP) at concentrations 1000 times greater than the

concentration of freely-dissolved molecules in seawater (Voparil & Mayer, 2000). The compounds incorporated into digestive micelles in gut fluid appear to be bioavailable (Ahrens et al., 2001), unlike humic-type micelles that bind hydrocarbons in an unavailable form (Boehm & Quinn, 1973). The situation is analogous to vertebrate lipid digestion, in which bile salt micelles shuttle lipids through the bulk aqueous solution to the digestive epithelium. Not only are lipids in bile salt micelles bioavailable, but solubilization into micelles is required for efficient digestive assimilation (for review, see Shaiu 1987).

Solubilizates (those compounds within the micelle, Carey & Small, 1970) are concentrated in micelles and can interact therein to influence each other's apparent solubilities. For example, bile salt micelles alone have a poor capacity for cholesterol, carrying on average less than 1 molecule per micelle (Spanner & Bauman, 1937). Solubilization of lecithin causes bile salt micelles to expand, increasing their capacity to 125 cholesterol molecules per micelle (Small et al., 1966). By doing so, lecithin increases the *in vivo* digestive bioavailability of cholesterol dramatically (Simmonds et al., 1967). Within the guts of deposit feeders, exogenous lipids from sediment such as phospholipids, pigments, hydrocarbons, and potential contaminants (Volkman et al., 1986; Harvey & Johnston, 1995; Parrish, 1988; Pinturier-Geiss et al., 2002) mix with endogenous lipids such as cholesterol (Voparil, unpublished). With the multitude of compounds present in marine invertebrate guts, interactions among solubilizates are likely to influence digestion and absorption, and hence influence the assimilation of nutritious lipids as well as the bioaccumulation of sedimentary contaminants.

This research tests whether solubilization into micelles leads to interactions between pairs of lipids in digestive fluids from marine polychaetes and holothuroids. We used *in vitro* incubations of gut fluids with pure substrates because they offer a simplified system for study. *In vitro* incubations compare well to *in vivo* deposit-feeder assimilation of very hydrophobic compounds (Ahrens et al., 2001; Weston & Mayer, 1998). Specific pairs of lipids were used to emphasize the impact of a common environmental contaminant (benzo(a)pyrene) on the bioavailability of both nutritional lipids and other nonpolar contaminants. In this paper, the term "lipid" is used to refer to compounds that are more soluble in organic solvents than in water, following others' functional definitions (Folch et al., 1957; Bligh & Dyer, 1959; Parrish, 1988). By doing so, we group a wide variety of compounds together, e.g., fatty acids, sterols, PAH, aliphatic hydrocarbons, in a way that may be unusual for some readers.

3.3. Materials & Methods

The solubilities of various lipid substrates were measured alone and in combination with another substrate (a binary mixture). During binary incubations, a radiolabeled substrate was paired with an unlabeled substrate; thus only one component of the mixture was quantified. Two separate incubations were therefore necessary when measuring both substrates' concentrations in the mixture. This approach avoids problems of "crosstalk" between counting windows when attempting to measure two different isotopes, e.g., ^3H and ^{14}C, in a single sample.

Selected solvents including artificial seawater (ASW) and gut fluids were incubated with excess substrate (about one hundred-fold excess over the amount

solubilized, confirmed visually at the end of the incubation) in 300-μL glass tubes.

Substrates were introduced in a carrier solvent (toluene or ethanol) which was then

evaporated with N_2 gas. Most substrates were solids at the experimental temperature

(22°C), except hexadecane, which was liquid. Substrates and fluid were incubated on an

inverting table for 1 h, which approximates gut residence time in some deposit feeders

(Plante & Mayer, 1994). Fluid was removed via glass pipette, passed through PFTE

(Teflon) 0.45-μm filters, dispensed into 10 mL of ScintiVerse BD cocktail (Fisher

Scientific), and counted on a LKB Wallac 1217 RackBeta liquid scintillation counter

with the detection window set at either 8-110 for ^{3}H or 50-165 for ^{14}C quantification.

Data were quench-corrected by comparison to quench curves generated by adding

different concentrations of ^{14}C or ^{3}H toluene scintillation standard to each of the gut

fluids. As the PFTE filters were housed in plastic, some absorptive loss of lipids may

have occurred during filtration; iterative filtrations indicate no more than 20% loss due to

absorption. We did not correct for these possible losses. As a result, solubilization data

reported may be underestimates. All incubations were performed in triplicate.

3.3.1. Solvents

Artificial seawater was made following the recipe of Parsons et al. (1984). For animals,

six species of benthic marine invertebrates were collected (Table 3.1) that were expected

to represent a range of protein and surfactant concentrations based upon previous work

(Mayer et al., 1997). Polychaetes were collected from intertidal regions while

holothuroids were from subtidal environments. Animals were collected with care to

avoid injury and dissected immediately for digestive (gut) fluids from the midgut –

digestive agents attain maximal concentrations in this section – and frozen at –80°C until use. Each gut fluid was characterized for surfactancy, protein content, and pH. Surfactant activity and micelle presence were assessed using contact angle titrations with artificial seawater (Mayer et al., 1997). Total protein in gut fluids was measured using a Pierce Micro BCA Protein Assay Reagent Kit (www.piercenet.com) and compared to standard solutions of bovine serum albumin (BSA); pH was measured using an Orion Research probe (www.thermo.com).

3.3.2. Substrates

We chose representative compounds from different classes of lipids expected in marine invertebrates' guts, either from dietary or endogenous sources. Hexadecane is a member of the aliphatic hydrocarbons which are anthropogenic contaminants (Pinturier-Geiss et al., 2002) and markers for terrestrial organic matter and plants (Parrish, 1988). Hexadecanol is a free aliphatic alcohol and is a co-surfactant in some deposit feeders' guts (Findlay, pers. comm.), a minor constituent of sediment lipids (Pinturier-Geiss et al., 2002), and a product of wax ester digestion (Place, 1992). Cholesterol belongs to the sterols which are found in eukaryotic cell membranes (Brock & Madigan, 1988) and at high concentrations in deposit-feeder guts (unpublished data) likely due to sloughing of digestive cells. Palmitic acid represents the free fatty acids, which are major constituents of settling particles (Parrish, 1988) and a major product of lipase and esterase activities on more complex dietary lipids. Lecithin (phosphatidlycholine) is a phospholipid, a major component of cell membranes and often the major class of lipids in deposit-feeder

Table 3.1: Gut fluids investigated in this study

Species (location)	Taxon	Feeding mode	CMD [A]	Protein (g/L)	pH
Molpadia intermedia (WA)	Holothuroid	Deposit feeder	ND	0.23	6.88
Cucumaria frondosa (ME)	Holothuroid	Suspension feeder	ND	23.92	6.35
Parastichopus californicus (WA)	Holothuroid	Deposit feeder	40%	0.89	6.33
Nereis virens (ME)	Polychaete	Omnivore	29%	36.24	6.7
Arenicola brasiliensis (CA)	Polychaete	Deposit feeder	22%	40.25	6.67
Arenicola marina (ME)	Polychaete	Deposit feeder	15%	39.28	6.38
" for CMD experiment	"	"	40%	NM	6.43

[A] CMD = critical micelle dilution. This is the dilution below which surfactant micelles are converted to monomers. ND indicates that micelles were not detected. For *A. marina*, two gut fluids were used; fluid with a CMD of 40% was used only for BaP incubations with hexadecanol at gut fluid dilutions. NM indicates "not measured".

guts (unpublished data). Benzo(a)pyrene (BaP) is a PAH and a common contaminant of coastal sediments (LaFlamme & Hites, 1978).

Radiolabeled lipids were purchased from either American Radiolabeled Chemicals (palmitic acid [1-^{14}C], benzo(a)pyrene [^{3}H(G)], and cholesterol [4-^{14}C]; www.arc-inc.com) or Amersham (hexadecane [1-^{14}C], hexadecanol [1-^{14}C]; www.apbiotech.com). All non-radiolabeled or "cold" lipids were purchased from Sigma (www.sigma-aldrich.com). All had a radiopurity of at least 98%. Incubations of the substrates with DI water were in good agreement with previously reported values of aqueous solubility (Howard & Meylan, 1997).

3.3.3. Substrate combinations tested

Various binary-substrate combinations were tested (Table 3.2). To determine whether interactions occur only when micelles are present, ^{3}H-BaP and cold hexadecanol

were incubated with serial dilutions of *A. marina* gut fluids and ASW. Above that gut

fluid's critical micelle dilution (CMD; 40% gut fluid, 60% ASW), micelles are present.

When diluted below 40%, micelles disaggregate and surfactants exist as monomers in

solution. Artificial seawater and full-strength *A. marina* gut fluid were also incubated

with ^{3}H-BaP alone and with each of cholesterol, phenanthrene, palmitic acid, and lecithin

to determine whether the presence of micelles leads to solubilization interactions between

lipids that do not occur when ASW is the solvent. To corroborate results with *A. marina*

in other species, artificial seawater and all animals' gut fluids were incubated with binary

mixtures of unlabeled BaP and each of radiolabeled hexadecane, hexadecanol,

cholesterol, and palmitic acid. These animals vary widely in their contents of digestive

agents and presence of micelles, so that this comparison served as an interphyletic test of

the importance of micelles in facilitating solubilization interactions.

3.4. Results

3.4.1. Gut fluid characteristics

Gut fluid characteristics presented here (Table 3.1) agree in general with previous

work (Mayer et al., 1997; Mayer et al., 2001). Of the holothuroids, *M. intermedia* gut

fluid was most like seawater, being low in protein and devoid of surfactant micelles.

Though *C. frondosa* fluid did not have micelles, protein concentrations were similar to

the polychaetes' fluids. *Parastichopus* fluid was the converse of *Cucumaria*, having

micelles, but little protein. All polychaetes' gut fluids had both micelles and high protein

contents. Differences in CMD for the two collections of *Arenicola marina* gut fluids

suggests that digestive physiology is somewhat pliable and may respond to

environmental and animal conditions that have not been accounted for in this study.

Table 3.2: Experimental solubilizate-solvent combinations

Solubilizate A	Solubilizate B	Solvent
hexadecane*	BaP	ASW, all animal gut fluids
hexadecanol*	BaP	"
cholesterol*	BaP	"
palmitic acid*	BaP	"
BaP*	palmitic acid	ASW and *A. marina* gut fluid
BaP*	cholesterol	"
BaP*	phenanthrene	"
BaP*	lecithin	"
BaP*	hexadecanol	"
BaP*	hexadecanol	*A. marina* diluted with ASW

* indicates the measured solute. ASW = artificial seawater.

3.4.2. Mixture solubilization

The solubilization of BaP in *A. marina* gut fluid diluted to various extent with

ASW showed a biphasic concentration plot, which implies solubilization into micelles

(Fig. 3.1). The dramatic increase in BaP concentrations with increasing concentrations of

gut fluid above the CMD (40% gut fluid in seawater) reflects the onset of micelles and

their enhanced ability to mobilize this compound (Voparil & Mayer, 2000). By fitting a

linear regression to BaP concentration data below the CMD and extrapolating to 100%

gut fluid, we calculate that nonmicellar constituents of gut fluid are responsible for 8% of

the BaP solubilization – micelles are responsible for 92% of the BaP in full-strength gut

fluid, in general agreement with previous work (Voparil & Mayer, 2000).

Hexadecanol has no effect on BaP solubilization without micelles in the gut

fluids. With micelles, i.e., above the CMD, addition of hexadecanol enhanced BaP
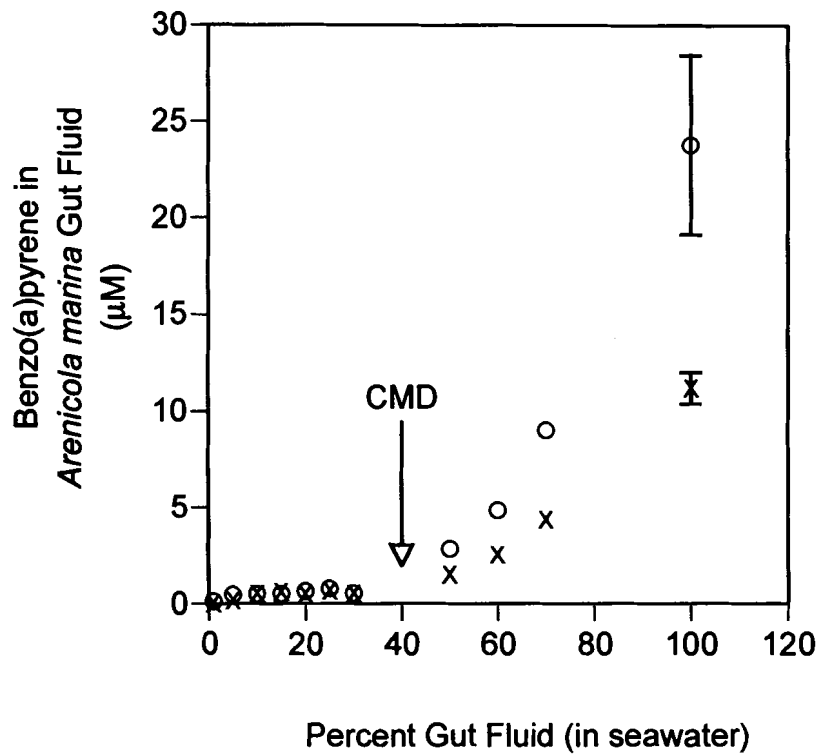
Figure 3.1: Benzo(a)pyrene solubilization with (x) and without (o) hexadecanol in *A. marina* gut fluids titrated with clean seawater.

Abscissa represents the dilutions of the original gut fluid. Ordinate is the concentration of BaP solubilized. Error bars are ± 1 SD.
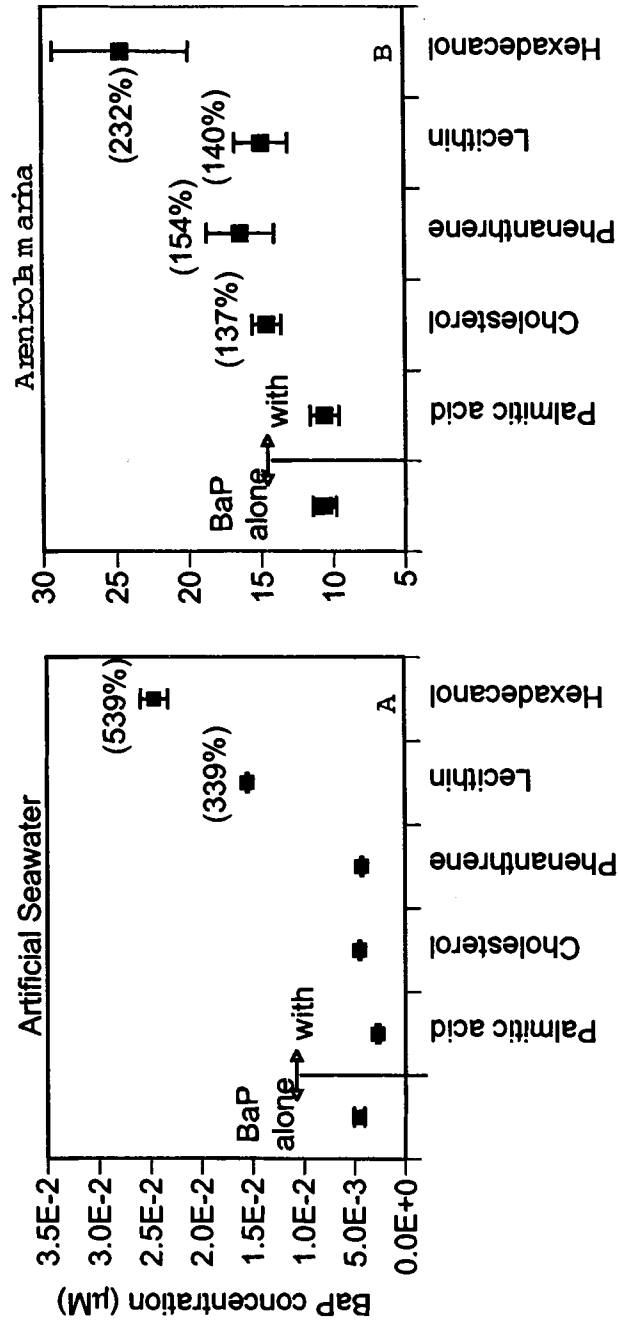
Figure 3.2: The effects of co-solubilizates on benzo(a)pyrene dissolution in artificial seawater (A) and *A. marina* gut fluids (B).

Abscissas represent the specific co-solubilizates tested: palmitic acid, cholesterol, phenanthrene, lecithin, and hexadecanol. Ordinates are the concentration of BaP – notice the change of scale between A and B. Significant differences (unpaired t-test, $P \le 0.05$) between lipid with and without BaP are indicated in parentheses as the percent change of the lipid's average solubility with BaP compared to the solubilizate alone. Error bars are $\pm$ 1 SD.

solubilization, allowing full-strength gut fluid to solubilize $23.8 \pm 4.4 \ \mu M$ BaP – more

than double the concentration without hexadecanol ($11.2 \pm 0.8 \ \mu M$). Some lipids

enhanced BaP solubilization in full-strength *A. marina* gut fluid, but had no effect in

ASW (Fig. 3.2). In the micellar gut fluid, cholesterol and phenanthrene enhance BaP's

apparent solubility approximately the same amount as lecithin (140%).

In ASW, neither of these compounds had an effect. In both gut fluid and ASW, lecithin

and hexadecanol increased BaP solubilization; hexadecanol has the greatest effect,

increasing relative BaP concentrations to 539% and 232% in ASW and gut fluid

respectively. Increases in actual concentrations were orders of magnitude higher in the

gut fluid than in ASW. In *Arenicola* gut fluid, BaP solubilization increased by 14 μM

with hexadecanol present. In ASW, the absolute increase of BaP due to hexadecanol was

0.02 μM. Palmitic acid had no effect on BaP solubility in either solvent.

3.4.3. Interphyletic corroboration

Gut fluids generally solubilized more of each individual lipid than ASW, except

for *Molpadia's* and *Cucumaria's* dissolution of hexadecane (Figs. 3.3, 3.4). Hexadecanol

was especially enriched in all gut fluids; even *Molpadia*, with little protein and no

micelles, solubilized at least two orders of magnitude more

hexadecanol than did seawater. Gut fluids with surfactant micelles were able to

solubilize much greater concentrations of all of the lipids than fluids without micelles;

note the change of ordinates between Fig. 3.3 and Fig. 3.4.

In fluids without micelles or proteins (ASW and *Molpadia* gut fluids), BaP had no

observed effect on any of the other lipids' solubilities (Fig. 3.3). However, solubilization

interactions were seen between certain pairs of lipids in all other gut fluids, even *Cucumaria*. In fluids with either micelles or protein (i.e., all species except *Molpadia*), cholesterol solubilization was inhibited by the presence of BaP in the mix. The greatest relative effect was with *Cucumaria* fluid, which lost 78% of its cholesterol solubilization when BaP was present (Fig. 3.3C); *A. marina* solubilization of cholesterol decreased least – by only 17% (Fig. 3.4A).

The effects of BaP on the other lipids varied among species. For example, hexadecane solubility increased to 491-844% in *A. marina, A. brasiliensis*, and *Cucumaria* fluids to concentrations approaching those found in *Parastichopus* and *Nereis*, which both showed no effect of BaP. Opposite effects of BaP on palmitic acid solubilization were found in different micellar gut fluids; in both arenicolids BaP caused a slight increased capacity for palmitic acid (both 130%), but in *P. californicus* and *N. virens* fluids decreased dissolution of palmitic acid (to 55 and 41% respectively).

3.5. Discussion

In marine invertebrate digestive fluids, a mechanism that concentrates solubilizates, such as the formation of surfactant micelles or solubilization by proteins, is necessary for interactions among lipid substrates to occur. In fluids lacking these agents, interactions are unlikely, unless a substrate is amphiphilic and can aggregate on its own. When concentrated in a micelle or the hydrophobic domain of a protein, the distance between solubilizates is much less than when freely dissolved in water making solubilization interactions more likely.
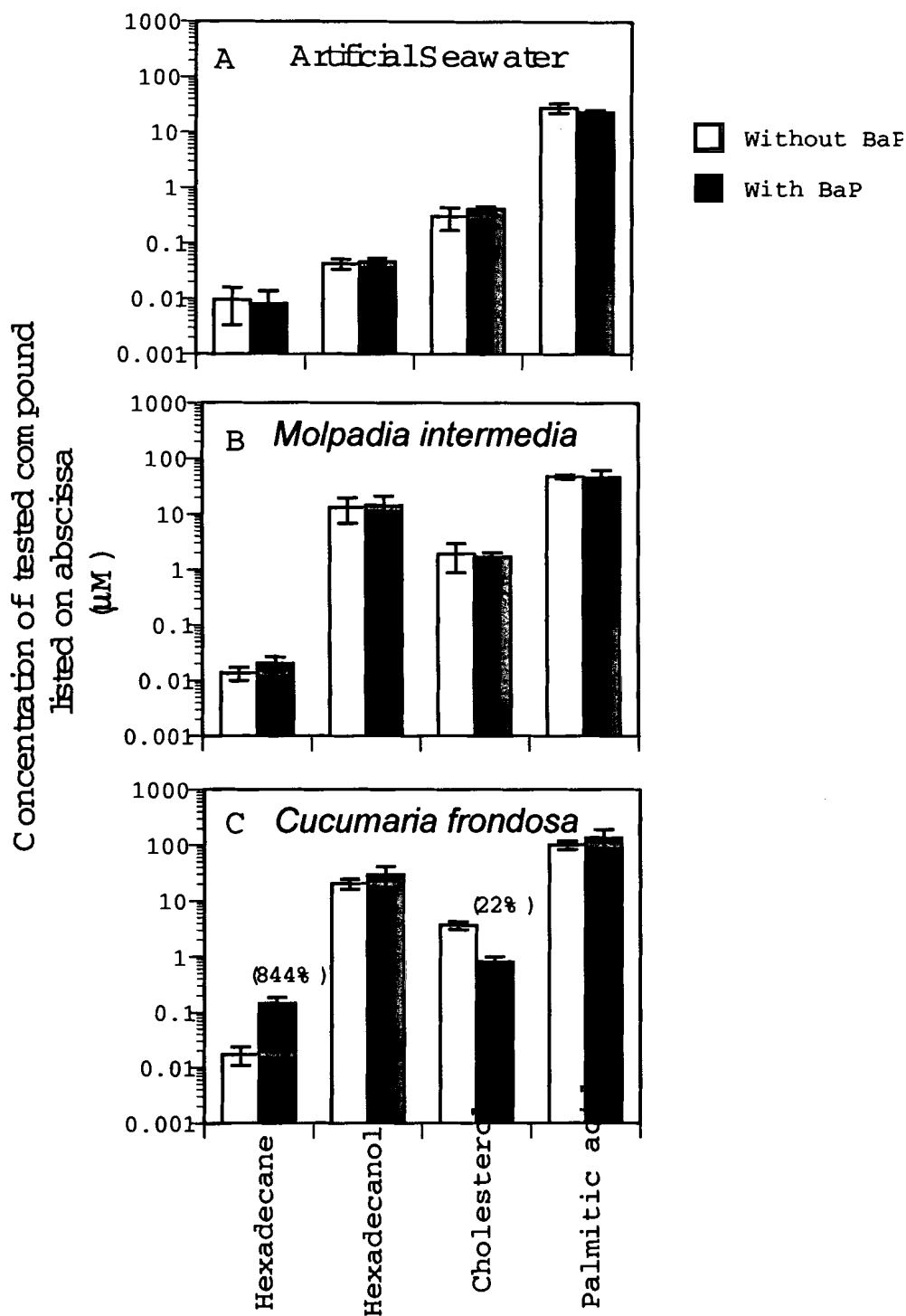
Figure 3.3: Solubilization of substrates, without (□) and with (■) added BaP, in gut fluids without surfactant micelles.

Abscissa is the particular substrate tested. Ordinate is the substrate's concentration. Each figure is a different fluid. Significant differences (unpaired t-test, $P \leq 0.05$) between concentrations with and without BaP are indicated in parentheses as the percent change of the substrate's solubility with BaP compared to the substrate alone. Error bars are ± 1 SD.
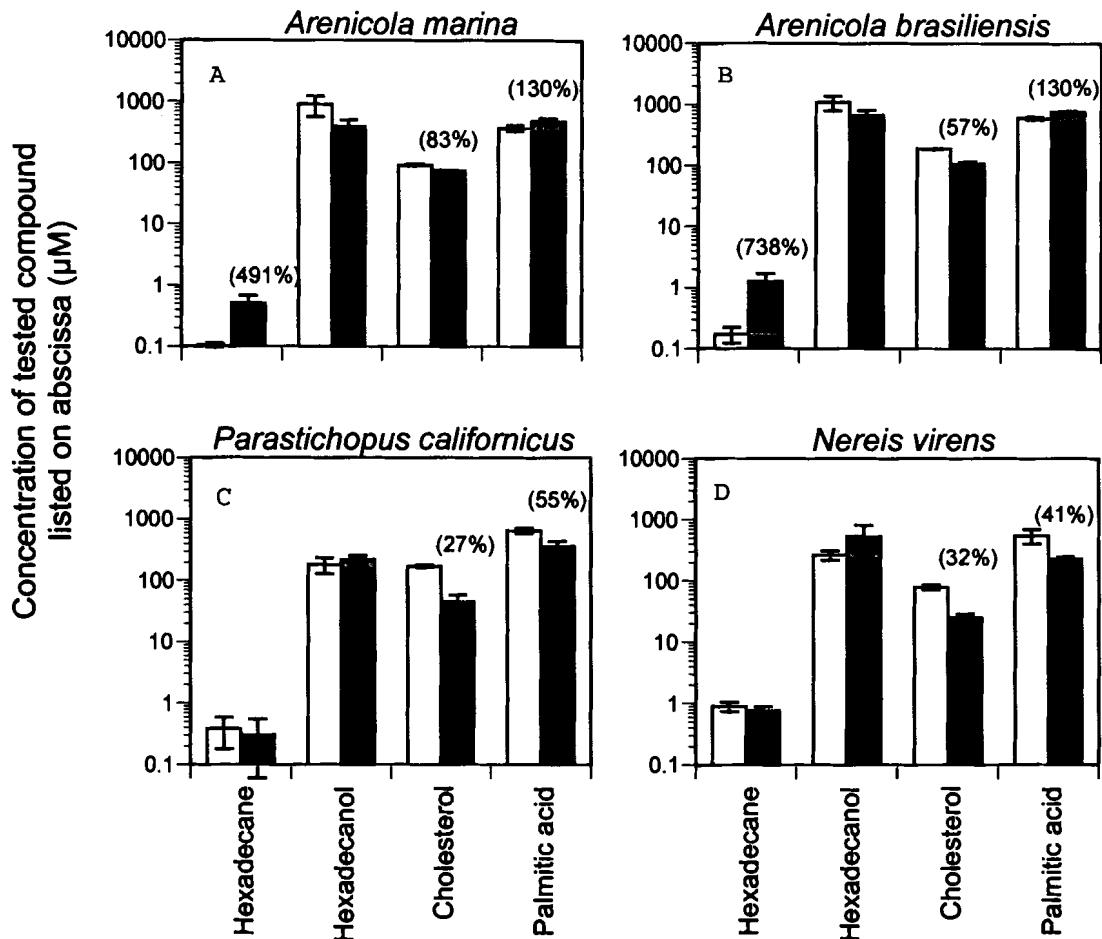
Figure 3.4: Solubilization of various lipids in gut fluids with surfactant micelles, without (□) and with (■) added BaP.

Abscissa is the particular substrate tested. Ordinate is the substrate's concentration. Each figure is a different gut fluid: A) *Arenicola marina*, B) *Arenicola brasiliensis*, C) *Parastichopus californicus*, and D) *Nereis virens*. Significant differences (unpaired t-test, $P \leq 0.05$) between concentrations with and without BaP are indicated in parentheses as the percent change of the substrate's solubility with BaP compared to the substrate alone. Error bars are ±1 SD.

These invertebrate gut fluids are complex blends of ingested materials in various states of digestion combined with different proportions of secreted digestive agents. The weakest gut fluid (*Molpadia*) shows no solubilization interactions, just like ASW (except for hexadecanol and lecithin). In these solvents, the intermolecular distance between any two freely-dissolved lipids makes their interaction unlikely. For example, hexadecane's aqueous solubility (0.0095 μM) indicates that ~ 6 x 10^9 water molecules surround each hexadecane molecule, assuming dispersion throughout the solution. Indeed, no interactions among binary mixtures of chlorinated organics and hydrocarbons in water have been found at aqueous solubility (Munz & Roberts, 1986; Banerjee, 1984) or below (Burris & MacIntyre, 1986).

However, both lecithin and hexadecanol increased BaP dissolution in ASW (Fig. 3.2A), even though hexadecanol is only sparingly soluble [we have no data for lecithin; reported aqueous solubility is 4.6 x 10^{-4} μM (Smith & Tanford, 1972)]. Because of their amphiphilic structures, these compounds can form polymolecular aggregations in water that would not have been removed by our 0.45-μm filtration (Hoffman & Anacker, 1967; Carey & Small, 1970). For example, lecithin aggregates in a number of different polymeric forms such as vesicles and liposomes that contain distinct hydrophobic regions that allow the apparent solubilities of more hydrophobic compounds, like BaP, to increase (Small, 1986).

In gut fluids with greater amounts of digestive agents, surfactant micelles and proteins were already present in solution; thus no hexadecanol or lecithin were required to form hydrophobic phases. Lipids are not only dispersed throughout these fluids as monomers, but also concentrated in micelles and in the hydrophobic domains of proteins.

This concentration increases the likelihood of interactions between solutes in two ways: (i) by allowing more substrate to be solubilized within the bulk aqueous phase and (ii) by concentrating hydrophobic molecules in specific regions within the fluid – the micelles or proteins. For example, hexadecanol enhanced BaP solubility in *A. marina* gut fluids only when micelles were present (at dilutions > 40%; Fig. 3.1). Within micelles, BaP and other solubilizates are in closer proximity than when freely dissolved in seawater. For example, with 98% of the BaP in micelles of gut fluids with hexadecanol (Fig. 3.1) and a surfactant concentration of 5 mM (Smoot et al., in prep), the BaP:surfactant molecule ratio is 4.5×10^{-3}. At aqueous solubility, the BaP:water molecule ratio is 1×10^{-14}. This ratio of solubilizate to solvent, called the molar solubilization ratio (MSR), is commonly used to compare the relative effectiveness of surfactants in mobilizing compounds of interest (Edwards et al., 1991).

Hydrophobic domains of proteins may also serve as loci for lipid solubilization (Voparil & Mayer, 2000; Backus & Gschwend, 1990) and interactions. Compared to micelles, these regions may not be as easily modified by the presence of other lipids because of the many different levels of organization involved in protein structure. BaP appeared to displace cholesterol for the limited space within *C. frondosa* proteins (Fig. 3.3). As a result, the amount of cholesterol in solution dropped towards aqueous solubility – the concentration of free monomers in solution.

3.5.1. Role of solubilizates

Interactions among substrates in arenicolids' gut fluids are qualitatively consistent with previous work using vertebrate bile salts and commercial surfactants. Solubilizates influence the size and capacity of micelles according to their interactions with water and

their location within the micelle. For example, nonpolar compounds compete with one another for space in a micelle's interior. More-hydrophobic compounds are enhanced by, as well as inhibit, the solubilization of less-hydrophobic solubilizates (Nagarajan et al., 1984; Guha et al., 1998; Chun et al., 2002). We found this same effect when BaP enhanced solubilization of the more hydrophobic hexadecane (Fig. 3.3C, 3.4A, 3.4B) and was enhanced by the less hydrophobic phenanthrene (Fig. 3.2B). Although cholesterol is not a nonpolar lipid, it partitions like a PAH in micellar systems (Carey & Small, 1972). Being slightly less hydrophobic, cholesterol enhances BaP solubilization (Fig. 3.2) at its own expense (Fig. 3.4).

Polar lipids, like lecithin and hexadecanol, increase the solubility of nonpolar compounds (Carey & Small, 1972; Rosen, 1989). Polar lipids are solubilized by micelles at the micelle-water interface and act to decrease the interfacial tension between the hydrophobic core of the micelles and the surrounding aqueous phase (Nagarajan et al., 1984), thereby allowing the cores to swell and decreasing the surfactant concentration at which micelles form (the critical micelle concentration). These interactions within micelles suggest, rather counter-intuitively, that polar nutritional lipids, like phospholipids or their partially digested lyso-forms, may increase the digestive solubilization of nonpolar contaminants like BaP (Fig. 3.3B). These powerful co-surfactant effects of amphiphilic lipids during vertebrate digestion have been widely reviewed for nutritional compounds like fatty acids, cholesterol, and the hydrophobic vitamins (Carey & Small, 1972; Shiau, 1987) and contaminants like PAH (VanVeld, 1990; Vetter et al., 1985) and PCB (Laher & Barrowman, 1983). We believe our study is the first to suggest this co-surfactant effect in marine invertebrate guts.

Characterization of the surfactants and other lipids in guts of these marine invertebrates would allow clearer interpretation of the interactions found herein. Differences in the surfactants secreted by the arenicolids from those of *Parastichopus californicus* and *Nereis virens* gut fluids might explain their opposing effects on palmitic acid solubilization with BaP. For example, decanol enhances aliphatic hydrocarbon solubilization by polyoxyethylene surfactants (Shinoda et al., 1963), but has no effect with bile salt surfactants (Ekwall et al., 1956). However, the surfactants in the guts of marine invertebrates have received little attention, with only crustacean (Lester et al., 1975) and *A. marina* (Smoot et al., in prep.) surfactants currently identified.

In ternary and quaternary systems, due to additional solubilizate interactions, substrates' solubilities often deviate from values obtained in binary systems (Eganhouse & Calder, 1976). A rough accounting suggests that the individual substrates solubilized in these experiments were a small fraction of the total lipid present in these gut fluids. In *A. brasiliensis*, the amount of palmitic acid solubilized ($600 \ \mu M = \sim 0.15$ g/L) is only 5% of the lipid already present in the fluid (2.86 g/L according to unpublished thin-layer chromatography-flame ionization detection of another sample of *A. brasiliensis* fluid). Even in *Molpadia* fluid, the radiolabeled palmitic acid ($48 \ \mu M = 0.0123$ g/L) was only 21% of the total lipid (~ 0.06 g/L according to data from another *Molpadia* fluid). Clearly, the spiked lipids are greatly outweighed by uncharacterized lipids in these fluids.

3.5.2. Relevance for *in vivo* contaminant bioavailability

In this paper, we report measurements of the solubilities of pure, lipid substrates in digestive fluids extracted from benthic invertebrates. These *in vitro* incubations have

been found to directly relate to digestive bioavailability (Weston & Mayer, 1998a; Mayer et al., 2002; Mayer et al., 1996), though this is a somewhat contentious idea. In support of the link between gut fluid release and bioavailability, deposit-feeder assimilation efficiencies for PAH (Weston & Mayer, 1998a) and polychlorinated hydrocarbons (Ahrens et al., 2001) have been predicted by *in vitro* incubations with contaminated sediments. After transport to the mucosa, lipid uptake into the epithelial cells lining the digestive tract is thought to be a passive process limited by the concentration of lipid solubilized by digestive fluids. Therefore, we suspect that interactions occurring during *in vitro* gut fluid incubations reflect actual bioaccumulation *in vivo*; synergistic interactions between solubilizates would result in enhanced bioaccumulation.

Our approach of incubating gut fluids with excess substrate probably serves to maximize interactions between two compounds, because substrates are at relatively high concentrations – their respective solubility limits. At lower concentrations, which are more representative of field conditions, solubilization interactions may be reduced. For example, naphthalene at ~ 27% of aqueous solubility in a solution of TX100 micelles enhances phenanthrene solubility, but only 61% of the enhancement that occurs when an excess of naphthalene is available (Guha et al., 1998). However, the multitude of lipids potentially present in contaminated sediments may have compounded effects on a particular substrate of interest.

Solubilization interactions in the gastrointestinal tract may help explain the current inaccuracy in predicting organic contaminant bioaccumulation using equilibrium partitioning theory (about one order of magnitude; EPA, 1998). Interactions of BaP on hexadecane (Figs. 3.3, 3.4) and hexadecanol on BaP (Fig. 3.2) resulted in hundreds of

percent increases in gut fluid concentrations and do not bode well for expectations of increased accuracy from the current iterations of EqP-based bioaccumulation models applied to deposit-feeders. We suggest that *in vitro* gut fluid extractions may serve to fill this void in sediment assessment techniques (Weston et al., 2002). Gut fluid extractions are able to provide empirical data on solubilization interactions that cannot be predicted using current theoretical models. We also developed a cocktail of commercially available chemicals that mimic *A. marina* gut fluid's solubilization of organic contaminants from sediments in hopes of allowing more widespread use of these *in vitro* methods and the possibility of more through characterization of solubilization (Chapter 5).

Chapter 4 - DIGESTIVE BIOAVAILABILITY TO A DEPOSIT FEEDER (*Arenicola marina*) OF PAH ASSOCIATED WITH ANTHROPOGENIC PARTICLES

4.1. Abstract

Marine sediments around urban areas serve as catch basins for anthropogenic particles containing polycyclic aromatic hydrocarbons (PAH). These particles can have detrimental effects on marine animals and their predators, depending on the bioavailability of the PAH. Using incubations of particles with gut fluids extracted from *Arenicola marina*, a deposit-feeding polychaete, we determined the digestive bioavailability of PAH from fly ashes, coal dusts, diesel soots, tire tread materials, and urban particulates (SRM 1649). We found that gut fluids dissolve significant concentrations of PAH from two tire treads, two diesel soots, and the urban particulate matter. However, PAH in fly ashes and coal dusts were not available to the digestive agents in gut fluid. Potential digestive exposure to PAH is much greater than that predicted to be available from these materials using equilibrium partitioning theory (EqP). With a contaminated sediment, amendment with fly ash decreased phenanthrene solubilization by gut fluid. In contrast, tire tread additions caused increased concentrations of four PAH in gut fluid. The addition of anthropogenic particles to sediments may therefore result in net additions to bioavailable PAH rather than net losses as predicted by soot-amended EqP. Difficulty in predicting the amount of change due to amendment may be due to interactions occurring among the mixture of compounds solubilized by gut fluid.

4.2. Introduction

Marine sediments around urban areas serve as repositories for anthropogenic particles (AP) including aerosols from the combustion or pyrolysis of organic materials (e.g., soot carbon), and particles derived from asphalt, brake-linings, tire treads, and material from construction sites. Delivery of these particles via atmospheric deposition and surface-water runoff leads to their elevated concentrations in sediments: these particles constitute the majority of non-mineral matter in sediments around cities. For example, up to 30% of total organic carbon in coastal sediments is from soot (Bucheli & Gustafsson, 2000). Furthermore, tire tread debris approaches 15% of the total sediment (by mass, including minerals) in areas surrounded by heavy automobile traffic (Reddy et al., in prep.; Spies et al., 1987; Kumata et al., 1997).

Organic solvent extractions of many types of AP (e.g., soot, coal, and tire treads) release polycyclic aromatic hydrocarbons (PAH) – a class of hydrophobic contaminants that can have toxic and carcinogenic effects on marine animals (for review see Meador et al., 1995). Sediments close to urban areas show characteristic PAH enrichments, and presumably much of the PAH is associated with AP, although petrological sources of PAH are also prevalent in these areas (Lake et al., 1979). Though clearly a global trend and problem (LaFlamme & Hites, 1978), PAH contamination of sediments surrounding the USA is particularly well documented and pervasive (Johnson et al., 1985; NOAA, 1989). In Boston Harbor for example, sedimentary concentrations of individual PAH range as high as 120 μg g^{-1} (McLeese et al., 1985; Voparil & Mayer, 2000).

Though available to organic solvents, much of the PAH associated with AP are sequestered in forms that are not thought to be bioavailable (i.e., not available to

animals). Bioavailability is a function of both the geochemical characteristics of the AP as well as the specific physiological pathways that expose a particular organism to contaminants. For example, pyrogenic materials like soot carbon and fly ashes sorb PAH very strongly and, when present in sediments, can depress interstitial water concentrations of PAH (McGroddy & Farrington, 1995; McGroddy et al., 1996). For animals exposed to PAH only via interstitial water, the PAH associated with pyrogenic AP ought to be less bioavailable than petrogenic PAH. Empirical studies of soot carbon (Knutzen, 1995; Maruya et al., 1997; Naes et al., 1999) and coal-associated PAH (Bender et al., 1987; Chapman et al., 1996; Paine et al., 1996) in sediments have generally conformed; the biological effects of PAH are suppressed.

Deposit feeders are conspicuously rare in areas with high concentrations of PAH, even when associated with pyrogenic AP (Oug et al., 1998), suggesting an additional route of exposure besides interstitial water for these animals. Recent results suggest that deposit feeders receive the majority of their exposure to sedimentary organic contaminants via digestion (Leppanen, 1995; Mayer et al., 1996; Weston & Mayer, 1998a, Mayer et al., 2001). Surfactant micelles are responsible for the bulk of sedimentary PAH solubilization in a deposit-feeder's gut fluids (Voparil & Mayer, 2000) and form a nonpolar pseudophase for the partitioning of hydrophobic compounds. As a result of micelle formation, digestive fluids may be better able than interstitial water, both kinetically and thermodynamically, to solubilize PAH from AP.

We studied extractability of 12 PAH from nine types of anthropogenic particles by digestive fluids of a deposit-feeding polychaete, *Arenicola marina*. Then, we determined whether patterns of PAH release from AP alone hold under more realistic

conditions in which AP are only a fraction of a contaminated sediment (by weight) by

using a sediment amended with either a tire tread or a fly ash. We considered only

solubilization (exposure) of PAH with our *in vitro* incubations, not their uptake across the

digestive tract (assimilation), or metabolic transformations once inside the animal

(biotransformation). The importance of the digestive pathway of exposure is made clear

by comparison of the amount of PAH solubilized by digestive fluids to the amount

predicted to be freely dissolved in interstitial water by equilibrium partitioning theory.

4.3. Materials & Methods

4.3.1. Collection of *Arenicola marina* gut fluid

Arenicola marina (lugworms) were dissected to remove mid-gut digestive fluids

as described previously (Voparil & Mayer, 2000), except that gut fluids were extracted

on the day of collection and were clarified by filtration (0.45-μm PFTE membrane)

instead of centrifugation. *A. marina* has high surfactant activity in its gut (Mayer et al.,

1997; Smoot et al., in prep.) – enough to form surfactant micelles, which are responsible

for the bulk of PAH solubilization, though other compounds such as proteins are

probably also involved (Voparil & Mayer, 2000). The gut fluid used in this study was

surfactant-rich, having a critical micelle dilution of ~ 20% using the contact angle

dilution method (Mayer et al., 1997). In other words, gut fluids could be diluted 80%

with artificial seawater before surfactant micelles would disaggregate into individual

surfactant monomers.

Table 4.1: Anthropogenic particles' descriptions

Sample	Source	Description
GF80A tire tread	Rouse Rubber, Vicksburg, MS, USA	Extremely fine, black powder
GR16 tire tread	Baker Rubber, South Bend, IN, USA	Coarse, black powder
Diesel soot	Interstate Diesel Equipment Service, Inc., North Kingstown, RI, USA	Fine, black, powder scrapped from exhaust systems of diesel vehicles
SRM 1650 Diesel Soot	National Institute of Standards and Technology, Gaithersburg, MD, USA	Fine, black, powder collected from a heat exchanger fed by four diesel engines
SRM 1649 Urban Particulates	National Institute of Standards and Technology, Gaithersburg, MD, USA	Very fine, atmospheric particulate material collected with large diameter filters
Valley Power Plant coal dust	Wisconsin Electric, Milwaukee, WI, USA	Unburned, fine, black, bituminous coal powder from Pennsylvania
Dal-Tex coal dust	New England Power Company, Somerset, MA, USA	Unburned, coarse, black, bituminous coal powder from Pennsylvania/West Virginia
Class "F" fly ash	Wisconsin Electric, Milwaukee, WI, USA	Burned, very fine, gray-black, bituminous coal powder from Colorado
High carbon fly ash	U.S. Generating Company, Somerset, MA, USA	Burned, very fine, high carbon, powder prepared in proprietary process used to separate bituminous Venezuelan coal fly ash into high and low carbon fractions

4.3.2. Anthropogenic particle characterization

A variety of AP were acquired (Table 4.1). Most were collected from their sources, without environmental mixing or weathering. However, SRM 1649 was collected from urban atmospheres with a large-diameter filter and is presumably weathered. SRM 1650 was collected from heat exchangers after 200 h of diesel engine operation. All samples were donated by the company named in the "Source" column of Table 4.1, except for SRM 1650 Diesel Soot and SRM 1649 Urban Particulates, which were purchased from the U.S. National Institute of Standards and Technology (www.nist.org). All samples were used without additional modification.

All particles were analyzed for Total Organic Carbon (TOC) using either a Perkin-Elmer Series II CHNO/S 2400 elemental analyzer (Wilton, CT) or Carlo Erba NA 1500 elemental analyzer (Fisons Instruments, Beverly, MA). Samples were treated by direct acidification with 1 M HCl to remove calcium carbonate. Soot carbon for all samples was determined using the thermal fractionation method (375°C for 24 h in excess oxygen) of Gustafsson et al. (1997). Surface area was measured by N_2 adsorption and multipoint B.E.T. analysis of freeze-dried samples, except for the tire tread samples. Both tire tread materials liquefied when subjected to the high temperatures (150°C) required for complete outgassing of N_2; thus measured values would not represent the samples' true environmental state.

To measure total PAH concentrations in these particles, dry samples were extracted with acetone/dichloromethane in a wrist-action shaker (Burrell Corporation, Pittsburgh, PA) for 48 h at a medium mixing rate (level '5'). After extraction, the solvent was passed through a glass fiber filter (GF/C, Whatman International Ltd., Maidstone,

England), dried with anhydrous sodium sulfate, and the contents transferred to a turbo-evaporation tube (Zymark Corporation, Hopkinton, MA) for volume reduction and exchanged into hexane. Final extracts were stored at 4°C in the dark until analyzed by gas chromatography / mass spectrometry (GC/MS) using a Hewlett-Packard 5890 Series II gas chromatograph equipped with a 7673A autosampler, electronic pressure control, and 5971A mass selective detector monitoring ions at 188, 240, 264 (internal standards phenanthrene-10d, benzo(a)anthracene-12d and perylene-12d, respectively) and target PAH mass units. PAH concentrations in the samples were quantified by comparison to peak areas from calibration standards of authentic compounds (Supelco, Belafonte, PA). A multi-point (4 - 5 level) internal standard calibration curve was used to determine calibration response factors.

4.3.3. Collection of sediment

During a low tide, sediment was collected with a shovel from Little Mystic Channel, Boston Harbor, MA, USA (LMC sediment). Within a day of collection, sediment was twice washed to remove salt with artificial seawater/ DI water (5/95 by volume), centrifuged at 8000 g for 15 min, freeze-dried, and stored in the dark at 5°C. Sediment from this location is contaminated with PAH and has organic carbon:specific surface area ratios that suggest an already heavy loading with anthropogenically-derived organic material (Voparil & Mayer, 2000). While a more pristine sediment may have provided a clearer test of how release of PAH from AP occurs in sediment, our choice of LMC sediment was an effort to maintain environmental realism with regards to the types of sediment that are actually impacted by AP.

4.3.4. Gut fluid incubations

A. marina gut fluid was incubated in triplicate for 4 h in the dark with each pure

AP type at a solid-fluid ratio of ~ 0.05 g mL^{-1}. This solid-fluid ratio was lower than the

sediment-fluid ratio normally found in a deposit-feeder's gut (Plante & Mayer, 1994), but

ensured that enough fluid would be available for analysis after incubation with these

materials. Gut fluids were clarified by filtration (0.45 μm) and then liquid/liquid

extracted with nanopure water and dichloromethane (DCM), partitioning PAH into the

DCM phase. Deuterated PAH (phenanthrene-D10, benzo(a)anthracene-D12, &

benzo(a)pyrene-D12) were added to the DCM extracts to serve as internal standards.

Typical recoveries were 56 ± 11%, 103 ± 14% and 111 ± 28% respectively. DCM

extracts were purified in the dark by passage through sodium sulfate and ENVI-Florisil

columns (Supelco. #5-7058) to remove polar and sulfur compounds, and dried under

nitrogen gas at 38°C. Dried samples were reconstituted in 1:1 acetonitrile:water (v/v),

passed through a 0.45-μm syringe filter and injected into a Hitachi D-7000 high pressure

liquid chromatograph (HPLC). A Vydac 201 TP, 5 μm, 250 x 4.6 mm column was used

under the following operational conditions: flow rate = 1.0 mL min^{-1}; temperature = 29

°C; injection volume = 250 μL; mobile phase = 1:1 acetonitrile:water (v/v) for 5 min,

ramping to 100:0 in 15 min, and holding for 8 min. PAH were identified using retention

time and absorbance spectrum when concentrations permitted and quantified using

fluorescence detection. Fluorescence detection limits were approximately 0.1 μg (L gut

fluid)$^{-1}$ for individual PAH.

4.3.5. Amended sediment incubations

To prepare amended sediments, freeze-dried sediment was mixed with GF80A

tire tread at 3.25% and High carbon fly ash at 3.48% (by weight) – reasonable for

sediments close to the source of contamination, but high for more distant sediments.

Gustafsson et al. (1997) measured ~ 0.6% soot in outer Boston Harbor sediments. Gut

fluids were incubated with LMC sediment (control) and sediment amended with either

GF80A tire tread or High carbon fly ash (treatments) at a solid-fluid ratio of ~ 0.25 g

$(mL)^{-1}$. Incubations and subsequent PAH measurements were performed as described

above.

4.3.6. Statistics

All values reported are means and standard deviations of triplicate samples. The

effects of added AP on gut fluids' release of PAH from sediment were determined by

analysis of variance (ANOVA) of contrasts between the unadulterated sediment (control)

and each of the amended sediment treatments using Systat 9 (SPSS, Inc.) statistical

software on a PC computer. Risk of Type I error was controlled at 0.05.

4.4. Results

4.4.1. Anthropogenic particle characteristics

Organic solvents extracted PAH from all of the samples except for the fly ashes

(Table 4.2). In the other AP, most individual PAH concentrations were on the order of

several $\mu g\ g^{-1}$ (dry weight). The coal dusts were relatively enriched in phenanthrene, the

Table 4.2: Geochemical characteristics of the anthropogenic particles

Measurement	Matrix									
	GF80A tire tread	GR16 tire tread	Diesel Soot	SRM 1650 Diesel Soot	SRM 1649[c] Urban Particulates	PPPP coal dust	Daltex coal dust	Class "F" fly ash	High carbon fly ash	LMC Sediment
Total Organic Carbon (including Soot) (%)	98.0	81.9	61.2 ±3.5	60.7	18.2	60.5 ±16.3	73.7	30.0 ±7.5	19.2 ±0.2	71.7
Soot Carbon (%)	23.1	10.3	28.9 ±6.1	53.1	5.2	42.4 ±0.1	15.3	26.4 ±6.4	20.9 ±0.1	NM
Surface Area ($m^2 g^{-1}$)			17.18	48.00	3.55	1.43	0.98	7.16	9.33	
Total PAH ($\mu g\ g^{-1}$)										
Phenanthrene	3.81 ±0.07	12.80 ±0.25	29.90 ±5.87	49.9 ±0.10	4.14 ±0.37	16.50 ±0.78	18.30 ±3.65	ND	ND	120.0
Anthracene	0.32 ±0.05	1.25 ±0.05	0.71 ±0.23	0.86 ±0.16	0.43 ±0.08	13.50 ±0.61	0.39 ±0.08	ND	ND	18.2
Fluoranthene	8.70 ±0.08	6.11 ±0.43	8.81 ±1.34	37.7 ±2.03	6.45 ±0.18	1.86 ±0.11	2.53 ±0.33	ND	ND	146.0
Pyrene	42.80 ±0.76	35.90 ±2.50	23.20 ±8.16	33.4 ±1.99	5.29 ±0.25	3.70 ±0.21	3.35 ±0.49	ND	ND	74.2
Benzo[a]anthracene	1.04 ±0.08	0.80 ±0.07	1.17 ±0.41	9.25 ±0.72	2.21 ±0.07	2.45 ±0.11	2.12 ±0.45	ND	ND	37.6
Chrysene	6.73 ±0.07	3.80 ±0.17	5.03 ±1.57	39.2 ±1.36	3.05 ±0.06	3.10 ±0.15	3.69 ±0.71	ND	ND	50.9
Benzo[b]fluoranthene	6.60 ±0.36	NM[b]	3.28 ±0.36	NM	6.45 ±0.64	NM	3.55 ±0.64	NM	NM	36.2
Benzo[k]fluoranthene	ND[a]	NM	3.37 ±0.42	2.1[c]	1.91 ±0.03	NM	3.36 ±0.60	NM	NM	16.8
Benzo[a]pyrene	2.49 ±0.19	0.77 ±0.08	0.32 ±0.05	1.12 ±0.25	2.51 ±0.09	1.96 ±0.12	1.78 ±0.44	ND	ND	43.0
Dibenzo[a,h]anthracene	0.62 ±1.08	0.09	ND	0.85 ±0.57	0.29 ±0.02	0.12 ±0.02	ND	ND	ND	2.5
Benzo[ghi]perylene	18.10 ±1.83	2.85 ±0.18	0.45 ±0.09	2.53 ±0.69	4.01 ±0.91	0.52 ±0.04	2.08 ±0.62	ND	ND	24.4
Indeno(123-cd)pyrene	1.11 ±1.08	0.30 ±0.01	0.32 ±0.14	1.50 ±0.54	3.18 ±0.72	0.18 ±0.01	0.42 ±0.39	ND	ND	18.2

[a] ND = Not Detected

[b] NM = Not Measured

[c] Concentrations supplied by the National Institute of Standards and Technology, USA, but not measured in this study. Reported values are the mean of analytical duplicates, unless followed by "±", which indicates that values are the mean of triplicates ± SD.

uniform distribution of PAH concentrations. Tire tread materials, diesel soots, and coal

dusts all contained more than 50% organic carbon, with significant fractions as soot

carbon.

4.4.2. Gut fluid release of PAH from AP

PAH release to gut fluids varied widely among the nine particles (Table 4.3). No

PAH was detected from fly ash after incubation with gut fluid – to be expected, as

extraction with organic solvents indicated that there were no PAH in these samples

(Table 4.2). No PAH were released from either of the coal dust samples, except for

traces of phenanthrene and pyrene at levels close to analytical detection limits.

Gut fluids released PAH from GF80A and GR16 tire treads, Diesel Soot, SRM

1649, and SRM 1650, but the amounts released did not correlate strongly with the total

PAH present in any of the samples, except for GR16 tire tread (i.e., Spearman

nonparametric rank coefficients were all non-significant with $p > 0.05$, except GR16 tire

tread 0.90 with $n = 5$). The greatest concentration of PAH released was BaP from

GF80A tire tread (0.0506 μg mL^{-1}). Gut fluid PAH concentrations were greater than

seawater solubility only for PAH with molecular weight ≥ 252 (Table 4.3).

Significant fractions of some of the PAH associated with these particles are

extractable in gut fluid. When calculated as the fraction of the total PAH in the samples,

40 and 33% of the BaP and dibenzo(a,h)anthracene, respectively, in GF80A tire tread,

24% of BaP in Diesel Soot #1, and 44 and 28% of the anthracene in SRM 1650 and SRM

1649, respectively, were released to gut fluid (Table 4.4). Releases of these PAH were

exceptional, less than a few percent of most other PAH were released from these samples.

Table 4.3: *Arenicola marina* gut fluid concentrations of PAH after incubation

PAH (µg L⁻¹)	Molecular Weight (g)	Seawater [a] Solubility	GF-80A Tire Tread	GR16 Baker Tire Tread	Diesel Soot	SRM 1650 Diesel Soot	SRM 1649 Urban Particulates	PPPP [c] Coal Dust	Daltex [c] Coal Dust	Class "F" Fly Ash	High carbon Fly Ash
Phenanthrene	178.2	725.0	6.9±0.5	4.5±0.8	14.9±1.2	ND	ND	0.1	0.1	ND	ND
Anthracene	178.2	29.0	0.5±0.1	0.7±0.2	0.3±0.1	5.7±1.7	3.5±1.3	ND	ND	ND	ND
Fluoranthene	202.3	152.0	2.0±0.1	1.1±0.1	1.0±0.6	5.5±0.6	2.0±0.3	ND	ND	ND	ND
Pyrene	202.3	83.0	11.7±1.7	17.7±1.6	2.0±0.4	19.3±1.8	15.2±5.3	ND	ND	ND	ND
Benzo[a]anthracene	228.3	6.7	2.4±0.1	ND	3.3±0.4	ND	3.1±0.7	0.3	ND	ND	ND
Chrysene	228.3	1.2	0.4±0.1	1.9±0.6	1.1±0.1	4.4±1.1	2.6±0.3	ND	ND	ND	ND
Benzo[b]fluoranthene	252.3	0.9	17.6±7.5	ND	8.0±2.0	ND	ND	ND	ND	ND	ND
Benzo[k]fluoranthene	252.3	0.5	12.5±1.1	2.3±1.2	4.0±2.5	ND	5.0±0.9	ND	ND	ND	ND
Benzo[a]pyrene	252.3	2.3	50.6±27.77	ND	3.9±2.5	2.4±2.1	5.3±0.6	ND	ND	ND	ND
Dibenzo[a,h]anthracene	278.4	0.3	10.2±1.1	ND	2.4±0.9	ND	ND	ND	ND	ND	ND
Benzo[ghi]perylene	276.3	0.2	3.2±0.4	ND	0.4±0.1	ND	ND	ND	ND	ND	ND
Indeno(123-cd)pyrene	276.3	0.7	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Xie et al., 1997
[b] ND = Not Detected
[c] Single measurements, not replicated

Table 4.4: Percentage of total PAH in the sample released to *A. marina* gut fluids.

PAH (%)	GF-80A Tire Rubber	GR16 Tire Rubber	Diesel Soot	SRM 1650 Diesel Soot	SRM 1649 Urban Particulates
Phenanthrene	3.64	0.70	0.99	0.00	0.00
Anthracene	2.86	1.20	0.84	43.80	28.32
Fluoranthene	0.47	0.35	0.24	0.98	1.14
Pyrene	0.55	0.98	0.18	3.85	10.86
Benzo(a)anthracene	4.55	0.00	5.70	0.00	3.47
Chrysene	0.12	1.00	0.45	0.74	1.22
Benzo(b)fluoranthene	5.35		4.89		0.00
Benzo(k)fluoranthene			2.35	0.00	5.21
Benzo(a)pyrene	40.64	0.00	24.44	14.18	5.14
Dibenzo(a,h)anthracene	33.02	0.00		0.00	0.00
Benzo(ghi)perylene	0.35	0.00	1.85	0.00	0.00
Indeno(123-cd)pyrene	0.00	0.00	0.00	0.00	0.00

Values are calculated sing the average concentration of each PAH released
by gut fluid. [A] Blank cells result when a specific PAH was measured in gut fluids,
but the samples had not been analyzed for that compound.

4.4.3. Release of PAH from amended sediment

A. marina gut fluids solubilized PAH from Little Mystic Channel sediment at a

range of concentrations spanning 2.9×10^{-4} μg mL^{-1} (anthracene) to 3.5×10^{-2} μg mL^{-1}

(BaP; Fig. 4.1). Adding High carbon fly ash decreased the amount of phenanthrene

released to gut fluids by 31%, but had no significant effect on other PAH. Addition of

GF80A tire tread significantly ($P < 0.05$) increased gut fluid solubilization of

fluoranthene, benzo(a)anthracene, benzo(k)fluoranthene, and dibenz(a,h)anthracene by

1.6×10^{-2}, 1.6×10^{-3}, 5.4×10^{-2}, and 6.2×10^{-4} μg mL^{-1}, respectively, compared to

sediment alone.

4.5. Discussion

4.5.1. Biological aspects of bioavailability

The bioavailability of sedimentary organic chemicals depends upon both the geochemical characteristics of the sediment as well as the biochemical pathways through which animals are exposed. Benthic organisms can accumulate contaminants through interstitial water and dietary exposures. Ingestion of contaminated sediment is a major route of exposure to hydrophobic organic contaminants for deposit feeders (Weston, 1990; Boese et al., 1990; Lepannen, 1995; Mayer et al., 2001). Dissolved organic material in guts is composed of food hydrolysates, digestive enzymes, and surfactants (Mayer et al., 1997) that render xenobiotic compounds available for absorption. *In vitro* gut fluid incubations offer a simple way of predicting deposit-feeder bioaccumulation of hydrophobic sedimentary contaminants (Weston & Mayer, 1998b; Ahrens et al., 2001). Though our results are directly applicable only to one animal, *Arenicola marina*, the common use of surfactant micelles to solubilize lipids in deposit-feeder gut fluids (Mayer et al., 1997) suggests that this work is also applicable to other deposit feeders.

We can compare exposure to PAH via digestion (as measured by gut fluid incubations) and via interstitial water (as predicted by equilibrium partitioning theory – EqP; DiToro et al., 1991). Normal sediment-water partitioning (K_d; L kg^{-1}) behaviors of organic contaminants such as PAH are described as:

$$K_d = C_s / C_d = f_{oc} K_{oc}$$

which has been extended by Gustafsson et al. (1997) to account for sedimentary soot partitioning as:

$$K_d = C_s / C_d = f_{oc} K_{oc} + f_{sc} K_{sc}$$
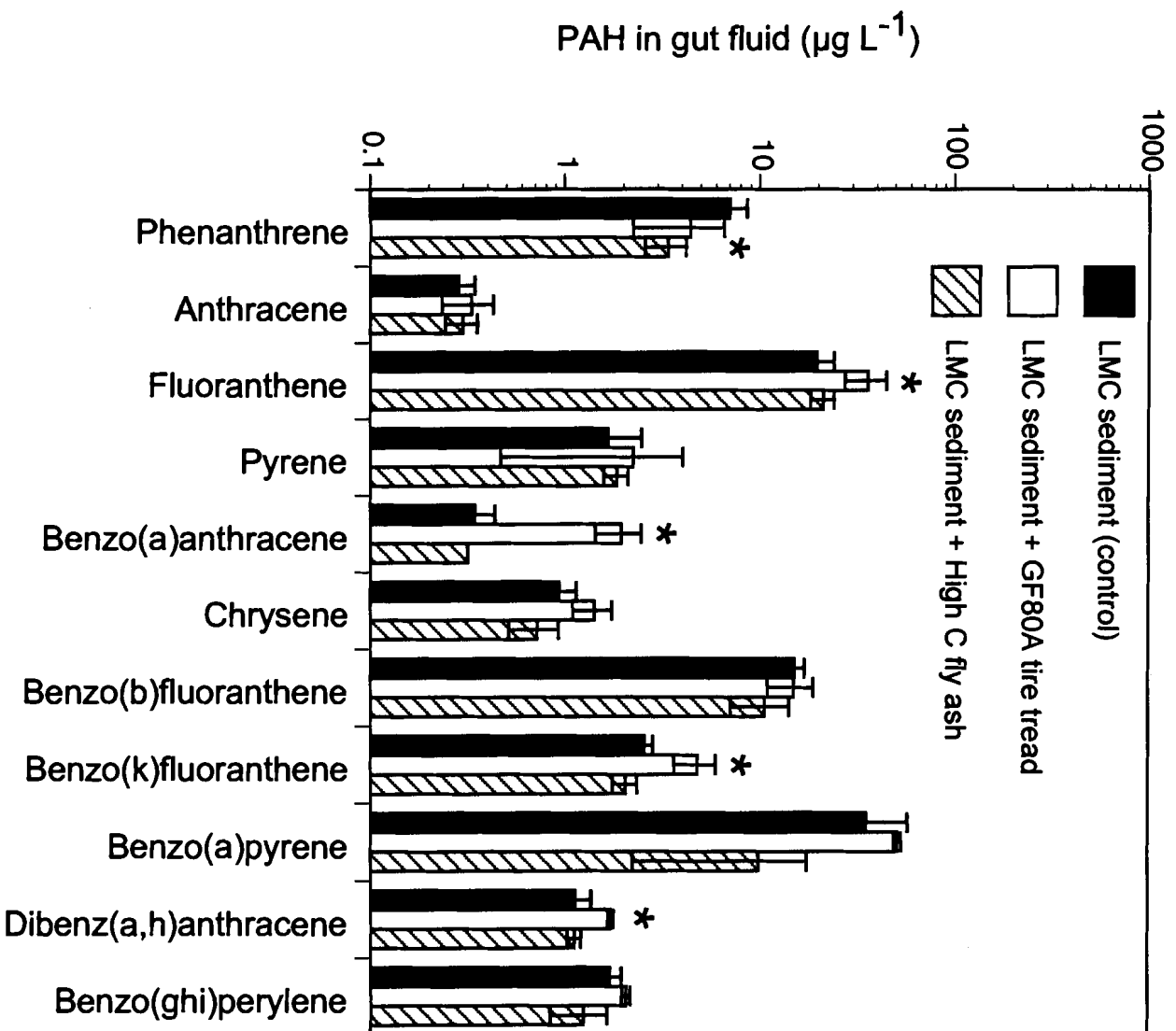
Figure 4.1: Amending Little Mystic Channel sediment with GF80A tire tread increased the amount of PAH solubilized by *A. marina* gut fluids. There was a significant increase in fluoranthene, benzo(a)anthracene, benzo(k)fluoranthene, and dibenz(ah)anthracene concentrations. The addition of High carbon fly ash decreased the amount of phenanthrene released to gut fluids. No other PAH was affected. Asterisk indicates a difference from control at $P < 0.05$.

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where C_s is the PAH sediment concentration (μg kg^{-1}), C_d is the PAH dissolved

concentration (μg L^{-1}), f_{oc} is the weight fraction of organic carbon in the sediment (g g^{-1}),

K_{oc} is the partition coefficient between sediment organic carbon and interstitial water (L

kg^{-1}; often predicted by the octanol-water partitioning coefficient K_{ow}), f_{sc} is the fraction

of soot carbon (g g^{-1}), and K_{sc} is the soot-carbon normalized partition coefficient (L kg^{-1};

predicted by activated carbon-water partitioning coefficients (Walters & Luthy, 1984;

Luehrs et al., 1996)). The equilibrium dissolved concentration of PAH and other

nonionic organic compounds is:

$$C_d = C_s / (f_{oc} K_{oc} + f_{sc} K_{sc})$$

Digestive fluids released considerably more PAH than these calculations predict

to be dissolved from AP, and this trend increases with molecular weight of the PAH (Fig.

4.2). For example, gut fluid solubilizes 20 times more pyrene and almost 1000 times

more BaP from SRM 1650 than EqP predicts to be freely-dissolved from this material.

Because hydrophobic compounds adsorb even more strongly to soot than to normal

organic material in sediments (as reflected in soot partitioning coefficients that are greater

than organic carbon partitioning coefficients), soot-corrections caused EqP to

underestimate the amount of PAH released to gut fluid by an additional two orders of

magnitude.

Why is digestion so important, especially for the more hydrophobic PAH? Gut

fluids do not solubilize very hydrophobic compounds particularly strongly. In fact,

Arenicola marina digestive fluids solubilize a wide size range of PAH to similar extent

(Table 4.3 and Chapter 5 herein; Voparil & Mayer, 2000). In contrast, EqP theory

assumes that compounds available for bioaccumulation come from a freely dissolved
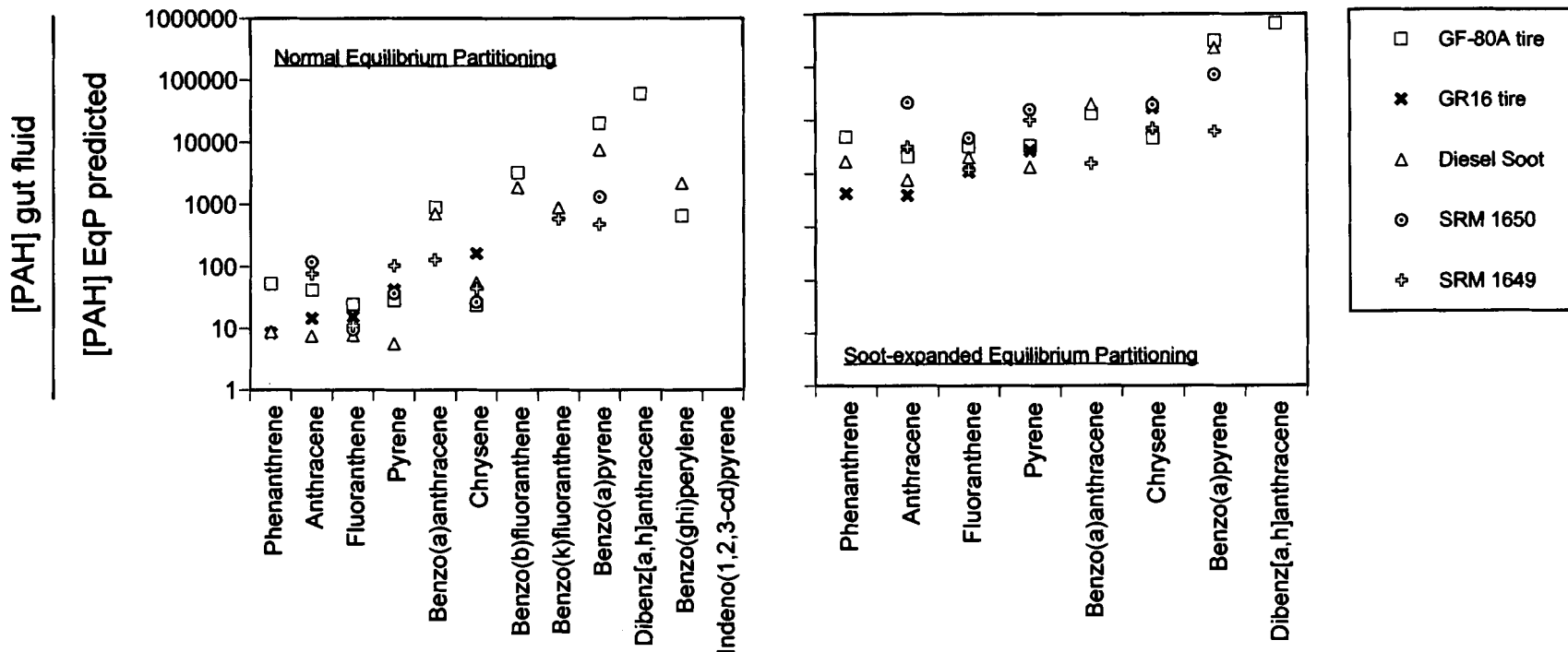
Figure 4.2: Gut fluids solubilize more PAH from anthropogenic particles than would be predicted by equilibrium partitioning theory. The Y-axis is the amount PAH measured in gut fluids divided by that predicted to be dissolved by EqP. Values greater than 1 on the Y-axis represent more digestively available PAH than EqP would have predicted. Soot-partitioning coefficients were not available for all PAH, therefore fewer PAH are in the graph on the right. Data are not shown for fly ash and coal dust samples due to low PAH concentration.

state and therefore uses partition coefficients between some phase and water (e.g. the

organic carbon-water partition coefficient (K_{oc})), to determine how much is available.

Because water is an increasingly poor solvent for PAH of increasing molecular weight

(Whitehouse, 1984; Afgan & Chau, 1989), EqP theory predicts that less of the very

hydrophobic compounds are available.

4.5.2. Geochemical aspects of bioavailability

Our use of isolated AP detected differences in PAH bioavailability due to the type

of material, such as the apparent lack of bioavailable PAH in coal dusts and fly ashes.

For example, the diesel soots, tire tread materials, and SRM 1649 released

PAH to *A. marina* gut fluids.

4.5.2.1. Fly ashes

There were likely no PAH in our fly ash samples, even though they comprised ~

20% soot carbon, as neither organic solvents (Table 4.2) nor gut fluids (Table 4.3) were

able to mobilize PAH from these particles. Fly ashes may contain PAH due to low

temperature and excessive fuel-to-air ratios during combustion (Liskowitz et al., 1978;

Mott & Weber, 1992; Liu et al., 2000).

Because of their capacity for sorbing low molecular weight organic contaminants

in aqueous solutions, fly ashes have been proposed as barriers to contaminant transport in

groundwater systems (Mott & Weber, 1992; Banerjee et al., 1995). Our results suggest

limited utility as an adsorbent in sediment systems in which concern is for deposit-feeder

exposure to PAH. The addition of 3% of High carbon fly ash did decrease gut fluid's

release of phenanthrene from Boston sediment by 31%. However, this reduction in

phenanthrene is not likely to have toxicological significance and, more importantly, none

of the other PAH were affected by the addition of the fly ash. We cannot speak to the

utility of the addition of fly ash at greater concentrations in sediment.

4.5.2.2. Coal

Unburned coal can be a significant source of total PAH in marine coastal

sediments (Tripp et al., 1981; Barrick et al., 1984; Dickhut et al., 2000). Our coal dusts

contained PAH, as measurements of total PAH indicate (Table 4.2), but they are

apparently sequestered in ways that make them unavailable to the digestive agents in gut

fluid. PAH sorbed to coal particles have high desorption activation energies (Talley et

al., 2002) that inhibit dissolution into water and digestive fluids. The weak digestive

fluids of suspension feeders like *Crassostrea virginica* and *Mytilus edulis* and even the

stronger fluids of the deposit feeders *A. marina, Rhepoxynius abronis*, and *Neanthes*

arenaceodentata seemingly cannot desorb PAH from coal particles (Bender et al., 1987;

Chapman et al., 1996; this study). Therefore, while coal may have some detrimental

biological effects on benthic animals by diluting sediment and decreasing its volumetric

nutrient value, PAH associated with this bituminous coal do not appear to be

bioavailable.

4.5.2.3. Diesel soot

Our results with the diesel soot samples – finding significant fractions of some

PAH to be released to gut fluid – are at odds with other research on the interstitial water

solubility (and assumed bioavailability) of diesel soot-PAH. Soot-associated PAH are

generally thought to be less bioavailable than PAH associated with sedimentary organic matter (e.g., Knutzen, 1995; Paine et al., 1996; Maruya et al., 1997). Diesel soot is a much stronger PAH adsorbent than natural organic matter (Bucheli & Gustafsson, 2000) and causes enhanced partitioning of sedimentary PAH to the particulate phase rather than interstitial water (McGroddy et al., 1996). However, our deposit feeder's digestive fluids solubilized PAH from diesel soots at concentrations greater than seawater solubility (Table 4.3) and much greater than equilibrium partitioning would suggest (Figure 4.4). These results suggest that digestive micelles of deposit feeders may access PAH in diesel soot that is not available to other animals.

Two factors may have influenced our results. First, approximately 50% of the carbonaceous material in Diesel Soot and 12% of that in SRM 1650 is removed by oxidation at 375°C and thus does not fit the analytical definition of soot-like material proposed by Gustafsson et al. (1997). This other fraction of organic carbon in these "soots" may be responsible for part or all of the bioavailable PAH.

Second, our clarification of gut fluid (passage through a 0.45 μm filter) may not have removed all soot particulates from the fluid-phase after incubation; for example, soot particles as small as 180 nm are discerned when suspended in ethanol (Gustafsson et al, 1997). If such particles were present in gut fluid after clarification, then PAH measurements would include PAH associated with colloidal particles, rather than only solubilized PAH, and thus overestimate bioavailability. It is not clear whether particles this small would be present in an aqueous system like gut fluid, as soot cannot be suspended in water (Gustafsson pers. com.). Calculations indicate that 2.14 mg of SRM 1650 Diesel soot would have to pass through a 0.45 μm filter in order to deliver enough

solid-phase to match the amount of BaP we measured in gut fluids – equivalent to 5% of the amount in the incubation, which would likely be visible. However, gut fluid filtrates were clear to the naked eye. In fact, the gut fluid solubilization of PAH we noted from soots was not remarkable, actually quite reasonable, when compared to previous work with contaminated sediments. After incubation with these soots, the concentrations of PAH and percentages of total PAH released to gut fluids are similar to previous work with A. *marina* gut fluids and contaminated sediments (Voparil & Mayer, 2000). Therefore, though we cannot discount solid-phase contamination of the gut fluids, we suspect little bias due to the clarification step.

4.5.2.4. Tire tread materials

We believe this paper to be the first report on the bioavailability of PAH associated with tire tread materials. Gut fluids incubated with tire treads alone released many PAH from these matrices. From GF80A tire tread, all PAH with molecular weight greater than or equal to 252, except for indeno(1,2,3-cd)pyrene, were solubilized at concentrations above aqueous solubility. From GR16 Baker, chrysene and benzo(k)fluoranthene were solubilized at the same concentration as aqueous solubility (Table 4.3). As both types of tire tread contained PAH (Table 4.2), the differences in PAH solubilization are likely due to compositional differences of the tires. However, we have no other data to characterize these samples. In general, tires contain a melange of organic compounds from synthetic polymers and pitches, oils, tars, rubber, and carbon black that have been mixed together to modify the life and workability of the tire. PAH

may make up over 200 μg g^{-1} of the final product (Jungclaus et al., 1976; Rogge et al., 1993).

The number of cars and trucks in urban areas suggests that tire tread material may be a significant source of bioavailable PAH contamination to surrounding sediments. Reddy & Quinn (1997) estimated that roughly 1.3 x 10^9 kg of tire is released each year into the environment around the United States. From the concentrations of benzothiazoles, the estimated amounts of tire tread material in sediments can approach 15% by mass (Reddy et al., in prep) in areas surrounding heavy automobile traffic such as the San Francisco-Oakland Bay Bridge (Spies et al., 1987) and metropolitan Tokyo (Kumata et al., 1997). As benzothiazoles are water soluble and photolytic (Reddy & Quinn, 1997), they may underestimate actual amounts of tire particles in sediments. The relative bioavailability of PAH from different tire tread formulations may therefore be an important area for future research, though in the field they will be averaged out.

4.5.2.5. Urban particulates

Although PAH associated with urban dusts have long been known to have biological effects on terrestrial mammals, we believe that this is the first test of their digestive bioavailability to a marine deposit feeder. Though PAH contamination in sediments around urban areas is often thought to derive from urban dusts, only recently has a molecular fingerprint been used to confirm this hypothesis; Marvin et al. (2000) noted that bottom and suspended sediments from Hamilton Harbor, Ontario, Canada, had thia:arene ratios characteristic of SRM 1649. We found that SRM 1649 contained seven

digestively bioavailable PAH (Table 4.3) which suggests a potential biological impact of these particles on benthic communities.

4.5.3. AP in sediments

Our amended sediment experiments were meant to address the implications of AP additions to real world systems. A number of conceptual models could be used to interpret our results (e.g., equilibrium partitioning theory (EqP)). According to EqP, our addition of either GF80A tire tread or High carbon fly ash to LMC sediment ought to reduce the interstitial water concentration of all PAH – the additional sorptive power of the added organic carbon phase has greater influence than the added PAH – and would presumably decrease bioavailability. However, these predicted changes in PAH solubility are small and probably not detectable as they are less than the standard deviations of our PAH measurements. For the fly ash treatment, in which there were no changes of PAH concentrations except for phenanthrene, we therefore cannot rule out the validity of the soot-amended EqP model.

However, when tire tread material is added, measured changes in some PAH' digestive bioavailabilities are opposite EqP predictions; that is, fluoranthene, BaA, BkF, and DB(ah)A) concentrations increased. We tried to predict the effects of tire tread amendment with an additive model in which concentrations of PAH solubilized from LMC sediment were increased by the bioavailable percentage of each PAH in GF80A tire tread (Table 4.4) multiplied by the weight of tire tread added to these incubations. The additive model underpredicted actual changes in fluoranthene and benzo(a)anthracene solubilization, but overpredicted dibenz(a,h)anthracene concentrations (data not shown).

We are thus unable to explain these levels of additional PAH release, but note that experiments with pairs of lipids have shown both inhibitions and enhancements during gut fluid solubilization (Chapter 3). Surfactant micelles, which solubilize lipids in *A. marina* gut fluid (Voparil & Mayer, 2000), dynamically adjust sizes and shapes depending on their solubilized constituents (Carey & Small, 1970). For example, fatty alcohols serve as co-surfactants, increasing micelle size and capacities for more nonpolar compounds like cholesterol (Carey & Small, 1970). Therefore, the differences between the actual and predicted changes in gut fluid solubilization from amended sediments may result from the presence of an unmeasured constituent in GF80A tire tread, or from the many-component interactions of compounds solubilized in gut fluid.

4.6. Conclusions

This paper demonstrates the potential for harmful biological impacts of anthropogenic particles to benthic communities. Organic contaminants associated with these particles tend to be strongly bound, resulting in increased solid-water partitioning coefficients relative to natural organic matter in sediments, and decreased predictions of bioavailability using EqP. However, the gut fluids of a deposit feeder (*Arenicola marina*) solubilize much greater concentrations of PAH from some anthropogenic particles than are available to water. This enhanced exposure is likely due to surfactants in gut fluids, which are common in deposit feeders.

Chapter 5 - COMMERCIALLY-AVAILABLE CHEMICALS THAT MIMIC A DEPOSIT FEEDER'S (*Arenicola marina*) DIGESTIVE SOLUBILIZATION OF LIPIDS

(*submitted to Environmental Science and Technology*)

5.1. Abstract

In vitro incubations of sediments with digestive fluid extracted from marine invertebrates have been shown previously to mimic digestive exposure to sediment-associated hydrophobic contaminants. The *in vitro* approach has a number of advantages over bioaccumulation studies, including applicability to widely disparate sediments, decreased cost, and rapid results. However, collection of digestive fluids is time-consuming and usually fatal for target animals. To overcome these problems, we developed a mixture of commercially available chemicals that mimics a deposit feeder's (*Arenicola marina*) gut fluid solubilization of lipids. Commercially available surfactants (Triton X-100, Brij 35, sodium dodecylsulfate, and sodium taurocholate) and proteins (chicken egg albumin, bovine hemoglobin, casein, and bovine serum albumin (BSA)) were compared to *A. marina*'s luminal fluids in their ability to solubilize polycyclic aromatic hydrocarbons (PAH). Sodium taurocholate was the most accurate mimic tested, and was incubated with additional contaminant and nutritional lipids alone and in various combinations. Of all the proteins tested, bovine serum albumin was the most efficient in the solubilization of PAH. Finally, we compared a cocktail of sodium taurocholate and BSA to *A. marina* gut fluid for efficacy in solubilization of 12 PAH from four different contaminated sediments (from Boston, Charleston, Jacksonville, and San Diego harbors).

The two solutions released most PAH to similar extents; 40 of 48 PAH-sediment combinations were released in ratios of 0.5 to 2 in cocktail and gut fluid solutions.

5.2. Introduction

Deposit feeders transport large volumes of sediment through their guts each day in search of nutritious material (Cammen, 1980; Rice, 1986). Ingestion of contaminated sediment can therefore become a major route of exposure to hydrophobic organic compounds, and result in increased bioaccumulation by deposit feeders relative to animals that do not normally ingest sediment, such as suspension feeders (Lynch & Johnson, 1982; Foster et al., 1987; Boese et al., 1990; Lake et al., 1990; Meador et al., 1997). Therefore, deposit feeders can serve as sensitive ecological indicators of anthropogenic, sediment-associated contaminants (Oug et al., 1998).

In vitro incubations of the digestive fluids of deposit feeders with sediments can be used to quantify those contaminants that become digestively available to an animal during gut passage (Mayer et al., 1996). After mixing sediment and digestive fluids under physiologically reasonable conditions, one quantifies the amount of contaminant desorbed into the fluid, based on the presumption that contaminants must first be solubilized by digestive fluids in order to be bioavailable. For lipids in general, solubilization in the gut is thought to be the limiting step during assimilation, as uptake into the cells lining the gut is passive and a function of concentration in the digestive fluid (reviewed in Shiau 1986). For very hydrophobic organic compounds like polycyclic aromatic hydrocarbons (PAH; Weston & Mayer, 1998) and chlorinated

hydrocarbons (Ahrens et al., 2001), gut fluid solubilization has thus far been shown to mimic bioaccumulation.

Gut fluid extraction has a number of advantages over conventional bioaccumulation studies using live animals for sediment risk assessment (Weston et al., 2002). First, *in vitro* incubations can be consistently applied to sediments with a wider range of abiotic parameters (e.g., salinity, sediment grain size, total organic carbon) than could be tolerated by any single animal species for bioaccumulation studies. Second, bioaccumulation experiments often run for 28 days while *in vitro* incubations last only a few hours, with associated cost savings and faster results. Third, extent of gut fluid solubilization is a metric for an animal's exposure to contaminants that is not confounded by metabolic transformations once inside the animal.

A disadvantage of *in vitro* incubations is the limited quantity of gut fluid in most animals. Studies on gut fluid chemistry have been limited to macroinvertebrates with tubular guts that allow gut fluid extraction without contamination by other body fluids (Mayer et al., 1997, 2001). Even in large polychaetes like *Arenicola marina*, usually only 1 mL of fluid is available per individual (Mayer et al., 2001). The small volume of gut fluid available from each animal becomes problematic for replication, as well as a threat to communities of animals compelled to support this endeavor. Moreover, the compositional variation of gut fluids among individuals, physiological states and developmental stages.

The development of a "cocktail" of commercially available substances that mimic the digestive agents in gut fluids would allow easier and more widespread adoption of the *in vitro* approach, offering the advantages of gut fluid incubations without the difficulties

of animal collection and dissection. For the bioavailability of hydrophobic chemicals, important constituents of the cocktail are surfactants and proteins (Mayer et al., 2001). For the weakly selective, deposit-feeding polychaete *Arenicola marina* (lugworm), surfactant micelles are responsible for 80-90% of the solubilization of the PAH benzo(a)pyrene by gut fluids, with the rest likely due to globular proteins (Voparil & Mayer, 2000; Chapter 3). Ahrens et al. (2001) recently used a solution of sodium dodecyl sulfate (SDS) to mimic bioaccumulation of two organic contaminants (hexachlorobenzene and tetrachlorobiphenyl) by two deposit feeders (*Nereis succinea* and *Pectinaria gouldii*).

In this paper, we develop and test a cocktail of commercially available proteins and surfactants to closely match *Arenicola marina's* digestive solubilization of lipids. The comparison started with testing of solubilization of single lipids, then advanced to the release of multiple PAH from contaminated sediments collected from the field.

5.3. Materials & Methods

5.3.1. *Arenicola marina* gut fluids

Arenicola marina (Linnaeus, 1758) (lugworm) individuals were collected from sandflats near Lubec, Maine, USA, in July, 1999. Animals were stored in seawater for up to 4 h and gut fluids were removed by carefully cutting open the body wall and inserting a pipette tip directly into the stomach. Fluids from the stomach have maximal enzyme activity and surfactant concentration (Mayer et al., 1997). Individuals' fluids were pooled, passed through a 0.45-μm PTFE membrane filter, decanted into plastic containers, and stored at -80°C until use.

To quantify the surfactancy of the collected *A. marina* fluids, we measured the contact angle of gut fluid titrated with artificial seawater (ASW, using the recipe of Parsons et al., 1984) on Parafilm following the methods of Mayer et al. (1997). The critical micelle dilution (CMD), the dilution at which micelles form, was 15% for this fluid. As surfactant concentration at the critical micelle concentration is 2.0 mM (Smoot et al., in prep.), these gut fluids have a surfactant concentration of approximately 13.3 mM as found in the animals.

A. marina gut fluid proteins were isolated by cold ethanol precipitation. A sample of the gut fluid was mixed with ice-cold 100% ethanol (1:9 by vol.), vortexed for 30 s, and centrifuged for 15 min at 4°C at 10000 g. The protein precipitate was washed twice more with ethanol, dried under N_2 gas, and reconstituted in ASW to the same concentration as the original gut fluid (42.0 g L^{-1}).

5.3.2. Commercial surfactants and proteins

Sodium dodecyl sulfate (SDS; PanVera Corp., Madison, WI, USA), sodium taurocholate (a vertebrate bile salt; U.S. Biochemical Corp., Cleveland, OH, USA), Triton X-100 (TX-100), and Brij 35 (both from Sigma, www.sigma-aldrich.com) were mixed with ASW to create solutions with the same CMD as *A. marina* gut fluids (15%). A surfactant's critical micelle concentration (CMC) depends on the ionic strength of the medium, so it was necessary to determine the CMC of these surfactants in ASW, instead of relying on reported values measured in distilled water. However, Ca^{2+} caused significant precipitation of SDS, TX-100, and Brij 35, making determinations of CMD

tenuous. As a result, these three surfactants were dissolved in new ASW without Ca^{2+}

and retested for CMD.

Four commercially available proteins were tested to mimic PAH solubilization by

nonmicellar components of gut fluid. Chicken egg albumin (Fraction V), bovine

hemoglobin, casein, and bovine serum albumin (Fraction V) (all from Sigma) were

dissolved in ASW at the same concentration as the material isolated from *A. marina* gut

fluid by ethanol precipitation. Bovine hemoglobin and casein were difficult to dissolve at

the concentration required, and the actual amount of these proteins in solution is below

the nominal concentrations reported; their solubility behavior in ASW excluded them

from further use in cocktail development.

5.3.3. Pure lipid solubilization experiments

The four commercially available surfactants were tested for their abilities to

solubilize seven PAH (naphthalene, fluorene, phenanthrene, pyrene, chrysene,

benzo(a)pyrene, and dibenzanthracene (all from Sigma)). Purified gut fluid proteins and

the four commercial protein solutions were incubated with each of phenanthrene, pyrene,

and benzo(a)pyrene. Seawater solutions with different concentrations of BSA were

incubated with benzo(a)pyrene. Sodium taurocholate (the best gut fluid mimic) and

Arenicola surfactant solutions were further scrutinized with an expanded set of lipids

(cholesterol, palmitic acid, myristic acid, stearic acid, oleic acid, linoleic acid,

arachidonic acid, tripalmitin, hexachlorobenzene, and hexachlorobiphenyl –all ^{14}C-

labeled compounds). Because different surfactants can solubilize multiple lipids in

different patterns (Carey & Small, 1971), binary pairs of each of palmitic acid,

tripalmitin, hexadecanol, cholesterol, and lecithin with ^{3}H-benzo(a)pyrene were tested

with both solutions. All radioisotopes were purchased from American Radiolabeled

Chemicals (www.arc-inc.com) except for hexachlorobenzene (Amersham,

www.apbiotech.com) and hexachlorobiphenyl (Sigma) – gifts from D. Weston (UCal-

Berkeley).

All experiments with pure lipids were done in triplicate. Lipid dissolved in carrier

solvent (chloroform for PAH, toluene for all others) was added to clean glass test tubes

and dried under N_2 gas for 15 min. Fluids of interest (gut fluids or solutions of the

commercial compounds) were added and incubated on a rotary shaker (120 rpm) in the

dark (PAH being photosensitive (Payne & Phillips, 1985)) for 4 h. The amount of solid

lipid dried on the bottom of the tube was at least 1000 times more than that ultimately

dissolved by the test solutions. After incubation, solutions were clarified by filtration

(0.45-μm PFTE membrane) and analyzed.

5.3.4. PAH analysis

PAH were extracted from the solution phase by chloroform (1:10 by volume) in

triplicate and quantified using a Hitachi F-4500 fluorescence spectrophotometer at the

following (excitation/emission) wavelengths: naphthalene (275/325 nm), fluorene

(265/305 nm), phenanthrene (280/360 nm), pyrene (335/380 nm), chrysene (270/380

nm), benzo(a)pyrene (370/430 nm), and dibenzanthracene (300/388 nm). We corrected

for background autofluorescence of these fluids - always less than 10% of the measured

PAH concentrations. Samples were quantitatively diluted until measurements at three

different dilutions were linear and proportional, as fluorescence of PAH in chloroform

can be quenched above certain concentrations resulting in a nonlinear relationship between fluorescence and concentration. Concentrations were calculated by comparison to external standards of individual PAH in chloroform.

5.3.5 Radioisotope analysis

After filtration as described above, fluids were dispensed directly into 10 mL of ScintiVerse BD cocktail (Fisher Scientific), and counted on a LKB Wallac 1217 RackBeta liquid scintillation counter. Data were quench-corrected by comparison to quench curves generated by adding different concentrations of ^{14}C or ^{3}H toluene scintillation standard to each of the gut fluid and the sodium taurocholate solutions.

5.3.6. Contaminated sediment experiments

Work with pure compounds allowed selection of two surfactants and one protein that best mimicked *A. marina* gut fluid solubilization of PAH; cocktail 1 was sodium taurocholate and BSA in ASW, cocktail 2 was SDS and BSA in ASW without Ca^{2+}. Cocktails were tested for their abilities to match *A. marina* gut fluid's release of 12 PAH from four contaminated marine sediments from around the United States. Boston, MA, (referred to as Little Mystic Channel sediment in Voparil & Mayer, 2000) and Charleston Harbor, SC, sediments were intertidal and collected by hand. San Diego, CA, (referred to as Pier 8 sediment in Voparil & Mayer, 2000) and Jacksonville Harbor, FL, sediments were subtidal and collected by grab from a ship. The original Jacksonville Harbor sediment was diluted 1:10 with clean sediment (for another project) and the characteristics reported here are for the diluted sediment; PAH concentrations in pure

Jacksonville Harbor sediment are therefore 10 times those reported and used here. Sediments were subsampled to measure water content; all incubations were with wet sediments. The total PAH concentration in San Diego, Boston, and Charleston sediments was measured by HPLC according to Voparil & Mayer (2000). Total PAH concentrations in the Jacksonville sediment were measured by the Army Corps of Engineers, ERDS, using EPA method SW 846, clean-up by silica gel (3630C), and analysis by GCMS (8270).

Sediments and either gut fluids or cocktails were incubated as described above for pure PAH. Incubations were fixed at a solid-fluid ratio of 0.25 g dry weight sediment (mL solution)$^{-1}$, as solid-fluid ratio can influence the amount of PAH released (Voparil & Mayer, 2000). Although sediments with very high water contents might dilute the surfactants in gut fluid and the cocktails to below their respective CMC, this dilution would require sediment with > 98% water content. None of our sediments were so dilute, so we continued to use wet sediments, as freeze-drying might affect the bioavailability of contaminants (Weston et al., 2001). Although incubations did not dilute the solutions below the CMD, we felt it critical to ensure that the cocktails had the same CMD as the gut fluids, so that potential dilution with sediments' interstitial fluids would affect both equally. Following incubation, the sediment-solution slurry was centrifuged (1200 g for 12 min) and the fluid phase filtered (0.45-μm PFTE filter).

A modified liquid-liquid separation was used to extract PAH from gut fluids and cocktails (Bligh-Dyer, 1959), except dichloromethane (DCM) was substituted for chloroform because DCM is less toxic (Chen et al., 1981). The fluid sample was mixed with DCM and ice-cold methanol, vortexed for 15 s, DI-water was added, and vortexed

again for 1 min. Deuterated internal standards (phenanthrene-d_{10}, benzo(a)anthracene-d_{12}, & benzo(a)pyrene-d_{12}) were added. PAH partitioned to the organic phase (DCM) were concentrated under a gentle stream of N_2.

PAH in DCM extracts were further isolated using EPA Method 3630C Silica Gel Cleanup (EPA, 1996). DCM extracts were exchanged into cyclohexane and trapped in silica gel columns, with a top layer of sodium sulfate. Interfering compounds were eluted with pentane, the compounds of interest (PAH) were eluted in DCM/pentane (2:3 by vol.), and then exchanged with acetonitrile for quantification of PAH via high pressure liquid chromatography (HPLC).

The HPLC (Hitachi D-7000 system) used a Supelcosil LC-PAH column (Sigma part no. 58229) under the following operational conditions: flow rate = 1.0 mL min^{-1}; temperature = 29 °C; injection volume = 50 μL; mobile phase = 40:60 acetonitrile:water (v/v) for 5 min, ramping to 100:0 at 30 min, and holding for 16 min. PAH were identified by retention time and absorption spectrum when possible. Using UV absorption (at 254 nm), detection limits were approximately 0.002 μg (mL initial solution)$^{-1}$ for individual PAH. However, fluorescence detection could also be used with limits of detection more than an order of magnitude less than with absorption by using a complex set of sequential fluorometer excitation/emission wavelengths: 250/390 nm until min 16, 252/378 nm until min 27.5, 265/385 nm until min 33.5, 287/415 nm until min 39, 290/420 nm until min 41, and finally 290/405 nm. Because of these switches, fluoranthene, benzo[ghi]perylene, and indeno[1,2,3-cd]pyrene could not be quantified with fluorescence.

5.3.7. Statistics

All statistics were calculated in Systat 9 (www.systat.com). We compared pairs

of samples using a paired t-test with unequal variances and $P \leq 0.05$. To determine which

commercial surfactant solubilized PAH most like A. *marina* gut fluid, molar

solubilization ratio (MSR, see below) data were log-transformed (to normalize error

variance for data that span more than 3 orders of magnitude) and a simple linear

regression was fitted to scatter plots for the gut fluid against each commercial surfactant.

Gut fluid MSR was treated as the dependent variable. While a model II regression would

be appropriate for our data (as both variables are subject to error), we followed the

suggestions of Sokal & Rohlf (1997) for conditions when there is a causal relation

between variables and used a simple linear model. A regression of $y = x$ with $R^2 = 1$

would indicate that the solution was a perfect match of gut fluid. This approach was also

used to compare *Arenicola* gut fluids and sodium taurocholate solubilization of the

expanded set of lipids, as well as the comparison of gut fluid and cocktail release of PAH

from contaminated sediments.

5.4. Results

5.4.1. Commercial surfactant solubilization of pure lipids

To compare the relative effectiveness of a particular surfactant in solubilizing a

given solute, data are expressed as a molar solubilization ratio (MSR). The MSR is

defined as the number of moles of organic compound solubilized per mole of surfactant

added to solution (Edwards et al., 1991) and normalizes for differences in the molecular

weight of each surfactant and PAH. Samples with most of their surfactant in micelle

form (i.e., a low CMD) ought to have higher MSR values than in fluids having only monomers of surfactants in solution, because micelles are particularly able to solubilize lipids.

Ranges among PAH MSRs in any particular surfactant solution (~ 10^2) were less than the solubility range in water (~ 10^5) (Fig. 5.1). For an individual PAH, MSRs in different surfactants usually fell within one order of magnitude, except for phenanthrene and naphthalene, which spanned two orders of magnitude. Triton X-100 was the most efficient solubilizer of all PAH except naphthalene and phenanthrene. The rank order of solubilization of different PAH by A. marina gut fluid and sodium taurocholate solutions followed aqueous solubility (Spearman rank correlation coefficients p < 0.001).

Sodium taurocholate best mimicked A. marina gut surfactants' solubilization of pure PAH. The calculated regression was:

Log(Arenicola MSR) = 0.64 x Log(taurocholate MSR) - 0.65, R^2 = 0.76,

which had a slope closer to unity, a smaller y-intercept, and greater R^2 value than the second best surfactant, SDS.

Our choice of surfactant for the cocktail is based on MSR values of PAH solubilization. A few of the PAH-surfactant combinations tested can be corroborated by previous work. Measured MSRs for Brij 35 and TX-100 solubilization of phenanthrene, and pyrene are within a factor of two of reported values (Edwards et al., 1991, Grimberg et al., 1995; Guha et al., 1998; Prak & Pritchard, 2002) as is BaP solubilization into sodium taurocholate (Laher & Barrowman, 1983). However, our TX-100 solubilization of naphthalene was one order of magnitude lower than found by Edwards et al. (1991) and Guha et al. (1998). The differences between our measurements and those previously

Molar Solubilization Ratio (moles PAH (mole surfactant)$^{-1}$)

Legend:
- Arenicola marina gut fluid
- Triton X-100 (no Ca2+)
- Sodium Dodecyl Sulfate (no Ca2+)
- Brij 35 (no Ca2+)
- Sodium taurocholate
- Arenicola marina (Voparil & Mayer, 2000)

Abscissa categories: Naphthalene, Fluorene, Phenanthrene, Pyrene, Chrysene, Benzo(a)pyrene, Dibenz[a,h]anthracene

	log (Arenicola MSR) vs log (Surfactant MSR) regression	R^2
Triton X-100	v = 0.57x - 1.26	0.70
Sodium dodecyl sulfate	y = 0.60x - 1.03	0.75
Brij 35	v = 0.30x - 1.66	0.34
Sodium taurocholate	y = 0.64x - 0.65	0.76

Figure 5.1: Solubilization of individual PAH by commercial surfactants and gut fluid.
Abscissa is the specific PAH arranged according to decreasing aqueous solubility in water. Ordinate is the molar solubilization ratio; note the log scale. In order to determine which surfactant best mimicked A. *marina* gut fluid, PAH solubilization data for each surfactant were regressed against those for A. *marina* (see text). SDS and sodium taurocholate behaved most like gut fluids for all of the PAH tested. Error bars are ±1 SD.

reported may be due to our use of ASW as the solvent, rather than distilled water. Salinity affects the CMCs of surfactants (Rosen 1989) as well as the aqueous solubility of PAH (Whitehouse, 1984).

We found that MSRs tend to normalize variation caused by plasticity in an animal's digestive physiology (i.e., different surfactant concentrations) as long as micelles are the dominant form of surfactant in the fluid. In Fig. 5.1, phenanthrene, pyrene, and benzo(a)pyrene solubilities in another *A. marina* gut fluid are included (marked as *Arenicola marina* (Voparil & Mayer, 2000)). This fluid was collected during a different season the previous year and had different surfactancy (CMD of 30 vs. 15%), yet the MSR for benzo(a)pyrene overlaps the value for the gut fluid studied herein (3.74 x 10^{-3} ± 0.10 x 10^{-3} vs. 3.48 x 10^{-3} ± 0.18 x 10^{-3}). Only the MSR for phenanthrene was significantly different, increasing from 3.69 x 10^{-3} to 6.38 x 10^{-3} in the fluid with lower CMD. For lipids with aqueous solubility greater than that of phenanthrene, the MSRs may have been affected by a significant fraction of the solute being solubilized by non-micellar components of the gut fluid. If so, a fluid with a high CMD (low surfactancy) would display lower MSRs than a surfactant-rich solution. For more hydrophobic compounds, in which solubilization is almost completely micellar, this effect on MSR is insignificant. For example, three additional *A. marina* gut fluids were collected and tested for BaP solubilization. These fluids had CMDs of 12, 20, and 70% (this last sample was unusually weak) and one standard deviation of the MSRs for BaP solubilization was ± 24% with these three fluids. However, with gut fluid diluted with artificial seawater below the CMD (from Fig. 2D of Voparil & Mayer, 2000), the MSR was 5.25 x 10^{-4} – almost an order of magnitude lower than with micelles present.

For the expanded set of test lipids, sodium taurocholate matched *Arenicola*

surfactant solubilization even better than when tested with only PAH –with the regression

(Fig. 5.2):

Log(*Arenicola* MSR) = 0.91 x Log(taurocholate MSR) – 0.01, R^2 = 0.91.

The intercept indicates that gut fluid tended to solubilize slightly more of a particular

compound, on average, than did sodium taurocholate. Both *A. marina* gut fluid and

sodium taurocholate preferentially solubilized free fatty acids such as myristic, palmitic,

and stearic acid over naphthalene and had a strong preference for BaP; thus solubilization

by surfactants does not strictly follow aqueous solubility. However, the rank orders of

lipid solubilization into each surfactant solution were strongly related to that of their

aqueous solubility (Spearman rank correlation coefficients p < 0.0001).

A concentration factor of lipid in micelles can be expressed as the MSR in

surfactant micelles divided by the MSR relative to water (water is 55.5 mol L^{-1}). Using

this ratio, BaP is ~ 3.2 x 10^7 times more soluble in *A. marina* gut fluid micelles than in

water, and 2.7 x 10^7 times more soluble in sodium taurocholate micelles than in water.

These results suggest that both surfactants' micelles are 3x to 4x more effective solvents

of BaP than octanol, which solubilizes BaP ~ 8.2 x 10^6 times more than water (using an

log octanol-water partitioning constant of 5.97 (Howard & Meylan 1997), and

normalizing for the number of octanol and water molecules in 1 L of each).

Solubilization interactions between pairs of lipids are qualitatively similar for

both gut fluid and sodium taurocholate (Fig. 5.3). Hexadecanol, cholesterol, and

lecithin all increased the concentration of BaP in both solutions, while palmitic acid and

tripalmitin had no effect. However, sodium taurocholate and *A. marina* gut fluid differed
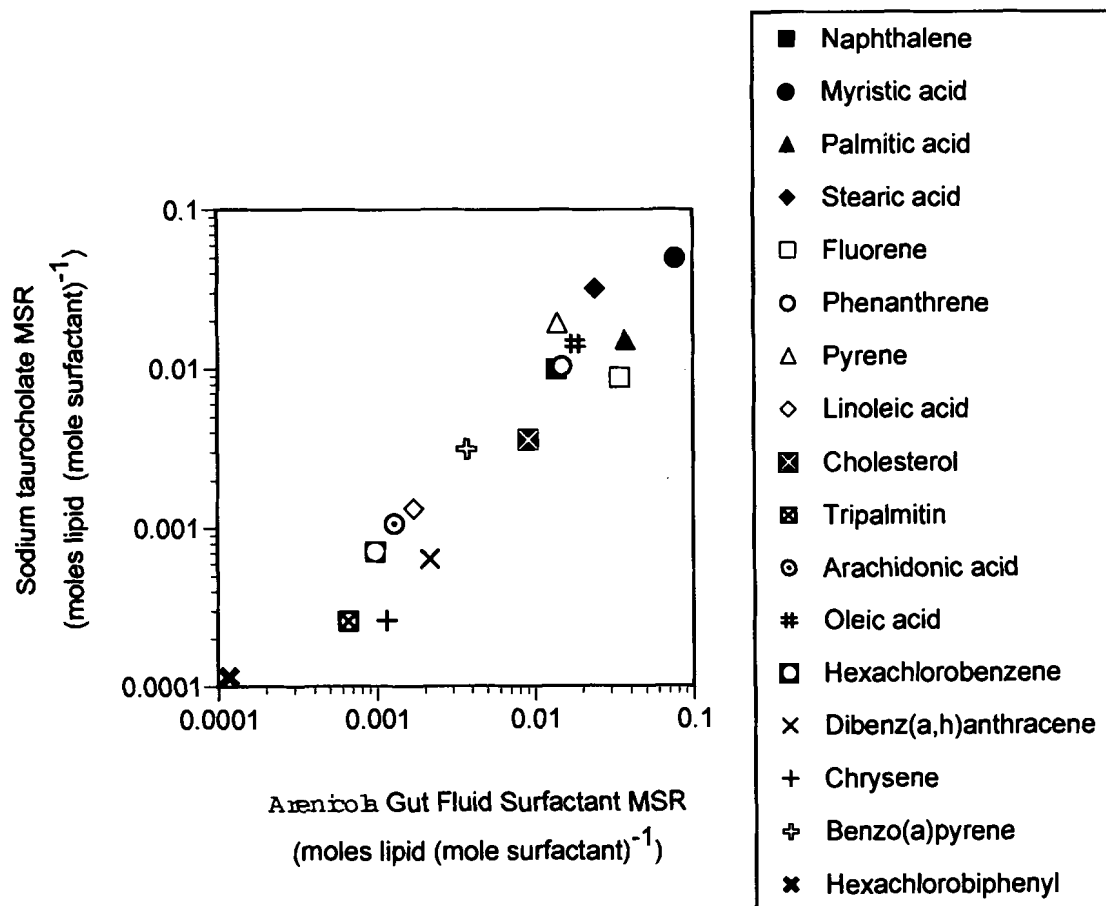
Figure 5.2: Comparison of *A. marina* gut fluid and sodium taurocholate solubilization of lipids.

Abscissa is the solubilizate's MSR in *Arenicola* fluid and ordinate is the MSR in sodium taurocholate solution. Solubilizates are arranged in the legend according to aqueous solubility with less-soluble compounds at the bottom. Error bars are ±1 SD.
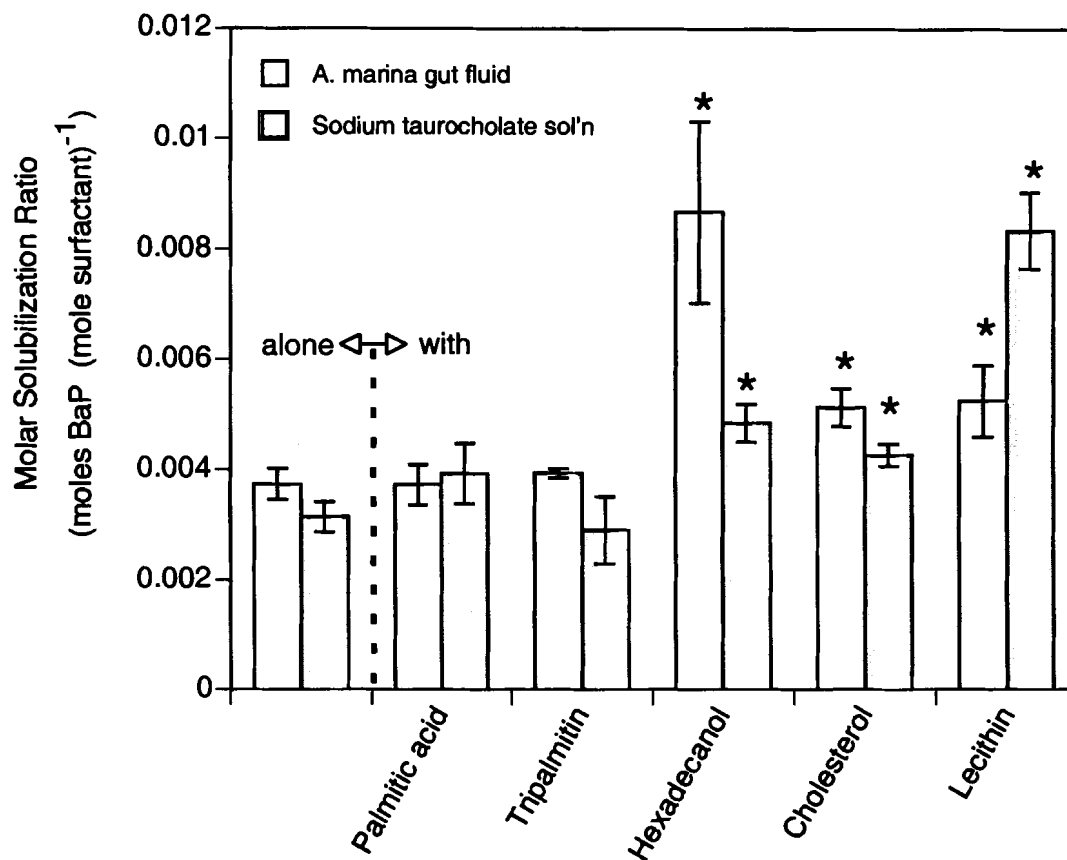
Figure 5.3: *A. marina* gut fluids and sodium taurocholate solution show similar abilities to solubilize benzo(a)pyrene (BaP) when exposed to binary mixtures of lipids. Abscissa is the lipid incubated with BaP, except the first column pair, which is the solubility of BaP alone in gut fluid (white) or sodium taurocholate solution (gray). Ordinate is the MSR of BaP. Asterisks above a column indicate a significant difference from MSR of BaP alone in the particular fluid (t-test with $P \leq 0.05$).

in the solute that most enhanced BaP solubilization. In *A. marina* gut fluid, hexadecanol

was the most effective co-surfactant of BaP, increasing BaP solubility to 232% of the

amount of BaP alone. In sodium taurocholate, lecithin was the most effective co-

surfactant; BaP concentrations were 265% greater with lecithin present.

5.4.2. Commercial protein solubilization of pure PAH

Bovine serum albumin (BSA) was the most efficient protein for solubilizing PAH,

followed by bovine hemoglobin (Fig. 5.4). Though proteins variably enhanced

the solubilization of phenanthrene and benzo(a)pyrene, the complexation of pyrene by

most proteins was remarkably consistent at ~ 1 μmol g^{-1}, except for BSA. Lacking

molecular characterization of the proteins in gut fluid that would allow normalization per

mole of protein, we normalize the concentration of PAH to the weight of protein in

solution (i.e. μmol PAH (g-protein)$^{-1}$). Although bovine hemoglobin (BH) and chicken

egg albumin (CEA) most closely matched *A. marina* gut fluid proteins, BSA was chosen

for use in further cocktail development based upon BSA's greater efficiency in

solubilizing PAH and its ready dissolution in seawater. BaP solubilization was linearly

related to BSA concentration over a wide range of BSA concentrations from 0-30 g L^{-1} in

seawater (Fig. 5.5). The linear relationship between BSA and BaP indicates that there is

no aggregation behavior of BSA that creates hydrophobic regions for BaP solubilization,

in the range of concentrations investigated. As a result, BSA at a lower concentration can

be used to mimic *A. marina* gut fluid proteins' solubilization of PAH. A BSA solution at

5.0 g L^{-1} matched the BaP dissolution by non-micellar constituents of the specific *A.

marina* gut fluid used in this study.
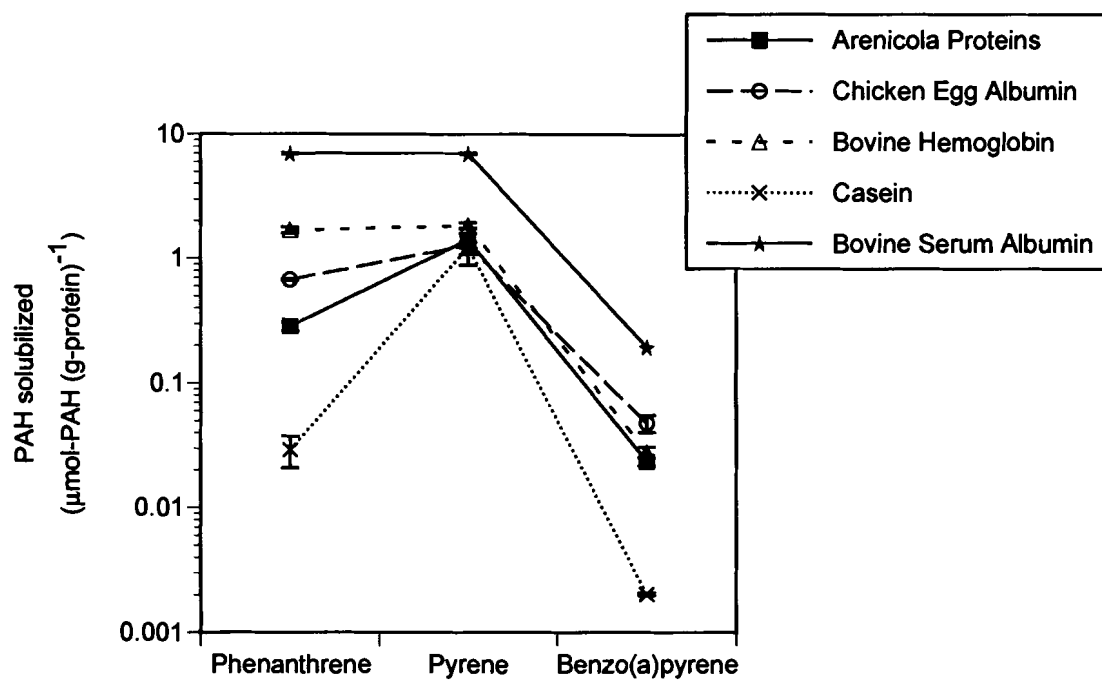
Figure 5.4: Enhanced solubilization of individual PAH by proteins.
Abscissa is the particular PAH. Ordinate is the amount of PAH dissolved by protein solution, normalized by weight. Bovine serum albumin is the most efficient solubilizer of PAH. Among the PAH, proteins solubilized benzo(a)pyrene the least. Error bars are ±1 SD.
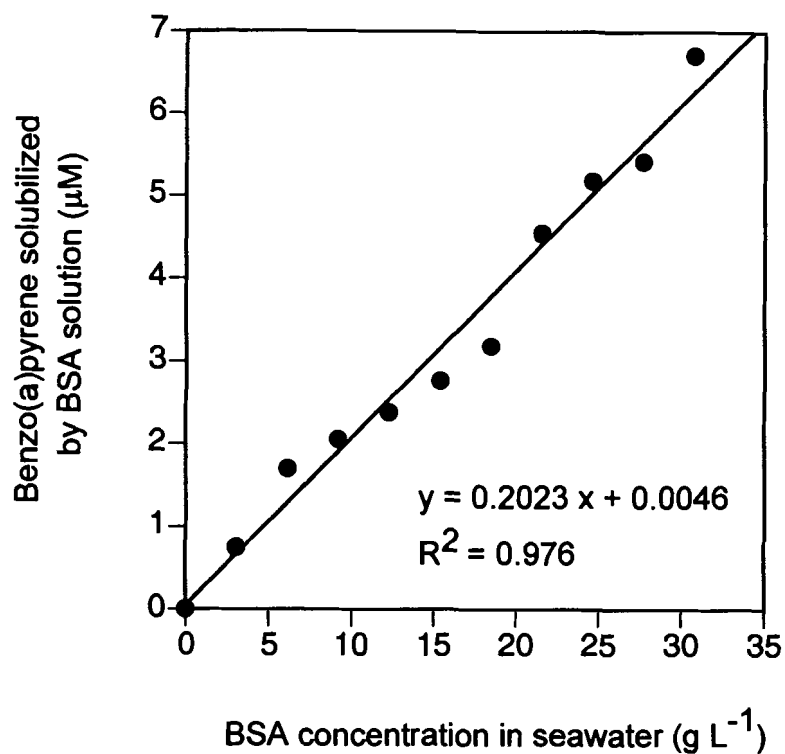
Figure 5.5: Various concentrations of bovine serum albumin (BSA) in seawater
solubilize BaP in linear relation to the concentration of the protein.
Abcissa is the BSA concentration in seawater. Ordinate is the concentration of BaP
solubilized.

5.4.3. Work with contaminated sediments

Sediment concentrations of individual PAH span more than an order of magnitude (Fig. 5.6). These harbors are all highly contaminated with PAH, compared to sediments from other harbors in the United States (NOAA, 1989). Other organic contaminants such as aliphatic hydrocarbons are likely present, but were not measured.

In general, the amount of PAH solubilized by gut fluids, expressed as an MSR, correlates positively with the sedimentary concentrations of PAH (Fig. 5.6, 5.7). Gut fluid solubilized pyrene at the greatest concentration from all sediments, except for Jacksonville Harbor. Pyrene was the dominant PAH in Charleston and San Diego sediments, but only the third most concentrated PAH in Boston sediment. From Jacksonville sediment, phenanthrene was the most concentrated PAH in gut fluid and in sediment.

PAH bioavailability differs among sediments (Fig. 5.7). For example, Boston and San Diego Harbor sediments have similar levels of PAH contamination, yet the amounts of PAH, expressed as MSRs, are an order of magnitude lower for Boston sediment. For differences among sediments, we suspect that bulk sedimentary organic carbon modifies PAH solubilization by gut fluid, as has been noted for interstitial water partitioning (DiToro et al., 1991). Specific types of organic matrices, e.g., soot, likely also modify PAH solubilization by gut fluids (Chapter 4).

Two cocktails were formulated to match *A. marina* gut fluid's release of PAH from these sediments. Cocktail 1 consisted of 13.0 mM sodium taurocholate and 5.0 g L^{-1} BSA in ASW. Cocktail 2 was 11.5 mM SDS and 5.0 g L^{-1} BSA in ASW without Ca^{2+}. Preliminary use of cocktail 2 with sediment spiked with a single PAH showed no release
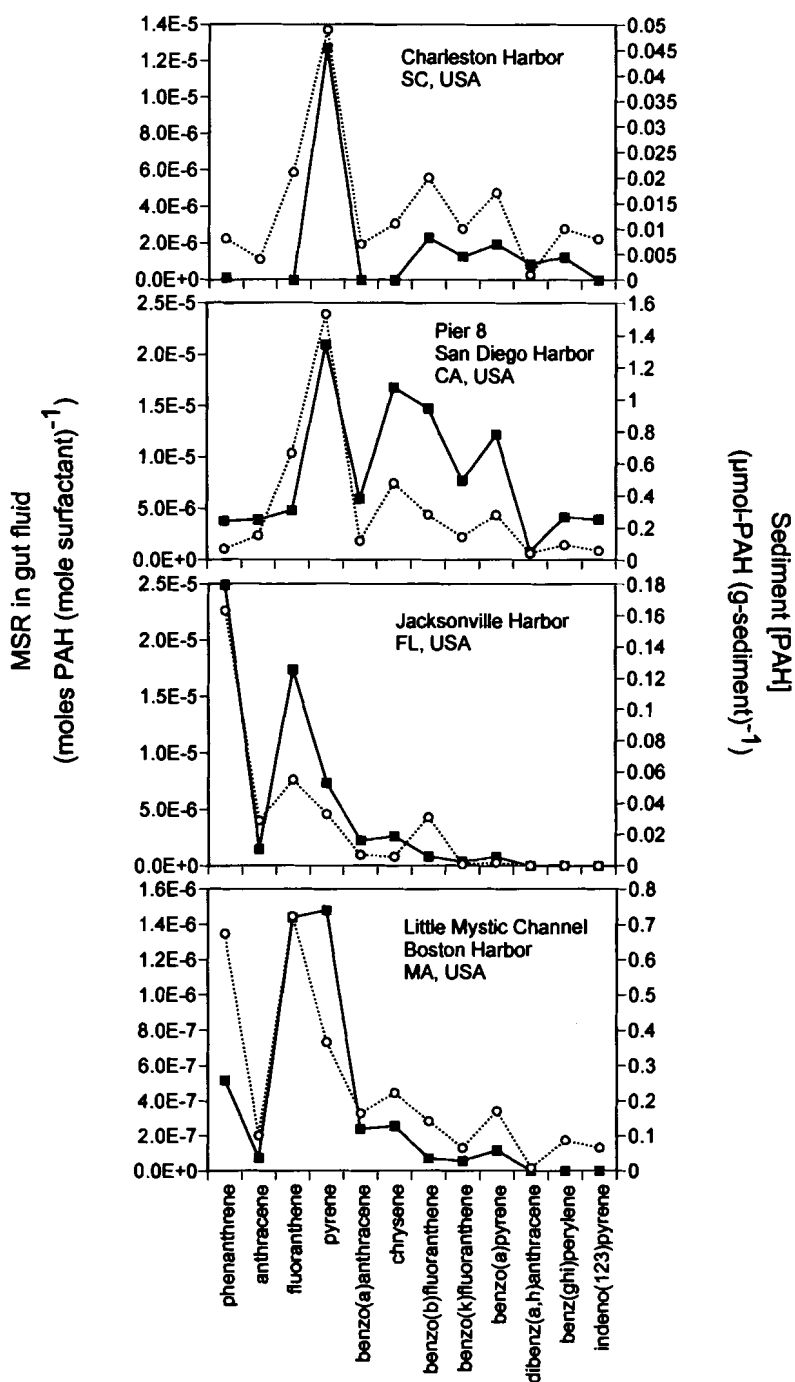
Figure 5.6: Total PAH concentration in sediment and MSR of that released to *A. marina* gut fluid.

Abscissas are the 12 PAH quantified and are the same for each graph. The left ordinate is the molar solubilization ratio of PAH released to gut fluid and changes scale for each sediment's graph (■). Right ordinate is the total amount of each PAH in sediment via organic solvent extraction (O). Average MSR values are plotted.
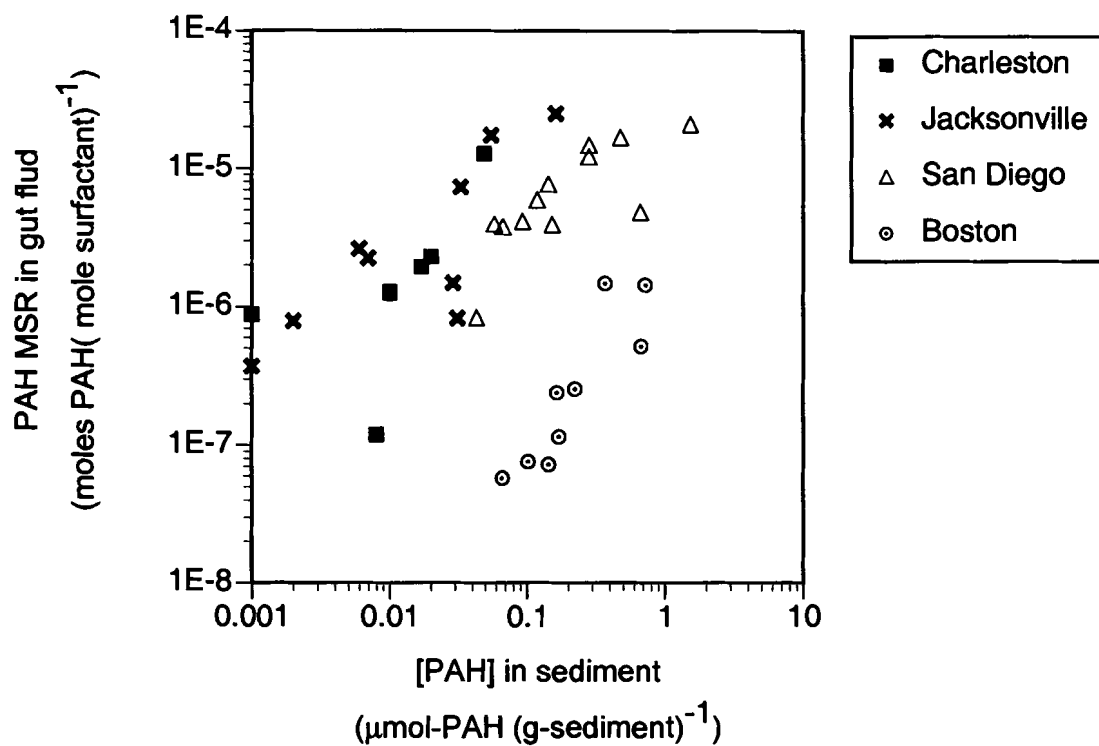
Figure 5.7: Ratio of amount of PAH solubilized by *A. marina* gut fluid to total PAH
    tends to cluster according to sediment.
Abscissa is the concentration of PAH in sediments. Ordinate is the amount released by
gut fluid as MSR.

of PAH (data not shown). Cocktail 2 also failed to release PAH from San Diego

sediment – the CMD shifted from 20% before incubation to 70% after incubation,

signifying loss of SDS micelles (Fig. 5.8). We suspect SDS adsorption onto sediment

organic matter or SDS precipitation due to divalent cations (e.g. Ca^{2+}) present in sediment

interstitial water, similar to that noted when initially trying to determine the critical

micelle concentration of SDS in ASW. In comparison, contact angles and CMD for the

sodium taurocholate cocktail remained constant after incubation with sediment. We

confirmed the constancy of micelle concentration in gut fluid and cocktail 1 before and

after incubation with six other contaminated sediments by comparison of the fluids'

abilities to solubilize BaP (Voparil, unpub.). There was no difference in BaP solubility

between any of the post-incubation treatments and the pre-incubation controls. As a

result, cocktail 2 (with SDS) was not used further and only cocktail 1 (with sodium

taurocholate) was tested with the other contaminated sediments.

The patterns of PAH release by *A. marina* gut fluid are complex for the different

sediments, yet cocktail 1 reproduces them reasonably well (Fig. 5.9). Cocktail 1 and gut

fluid PAH concentrations for 40 of 48 PAH-sediment combinations are within a factor of

two. Release of PAH from Boston sediment was the most difficult to mimic; cocktail 1

underestimated fluoranthene and pyrene solubilization by ~ 20%, but overestimated by

several fold benzo(b)fluoranthene, benzo(k)fluoranthene, and benzo(a)pyrene.

Regression analysis of Cocktail 1 solubilization versus gut fluid solubilization for

all sediment-PAH combinations indicates that the cocktail is accurate in reproducing gut

fluid PAH concentrations (Fig. 5.10). With all sediment-PAH combinations grouped

together, the fitted linear regression is:

$$\text{Log}(Arenicola \text{ MSR}) = 1.06 \text{ x Log(Cocktail1 MSR)} + 0.30, R^2 = 0.84$$

The 1:1 line is contained in the 95% confidence interval of the regression's slope and

intercept (-0.60 < intercept < 1.20; 0.90 < slope < 1.22); thus we cannot detect a

difference between the cocktails' MSR regression and $A.$ $marina$ gut fluids' MSR

regression.
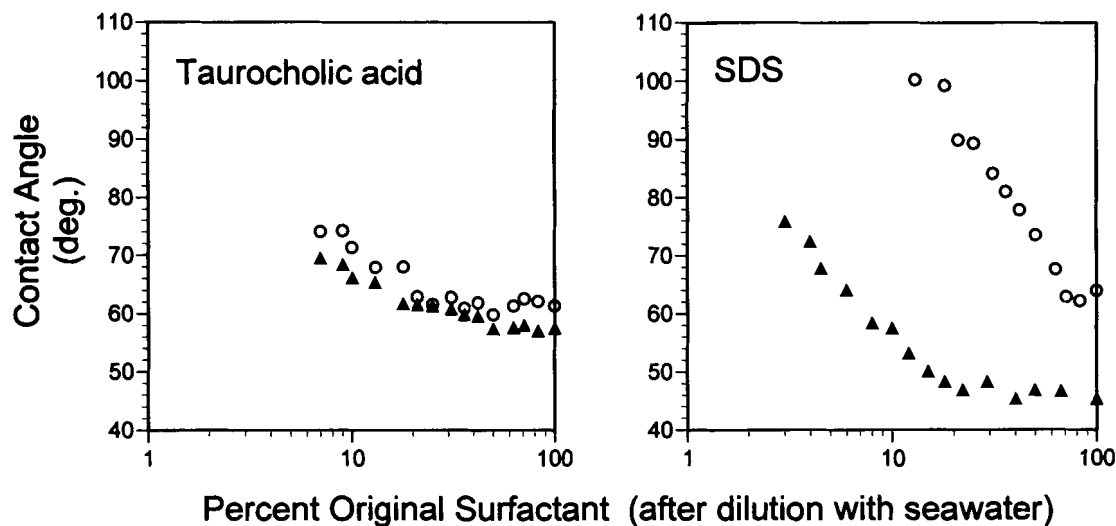
Figure 5.8: The effects of incubation with San Diego sediment on the surfactancy of the sodium taurocholate and SDS solutions.

Abscissas for both graphs are the percent dilution of solution with ASW. Ordinates are the contact angle. Plots of contact angles of surfactant solutions before (π) and after (O) incubation with sediment are relatively unchanged for taurocholic acid. However, with SDS, the CMD is shifted from 20% before incubation to 70% after incubation, signifying loss of SDS micelles.
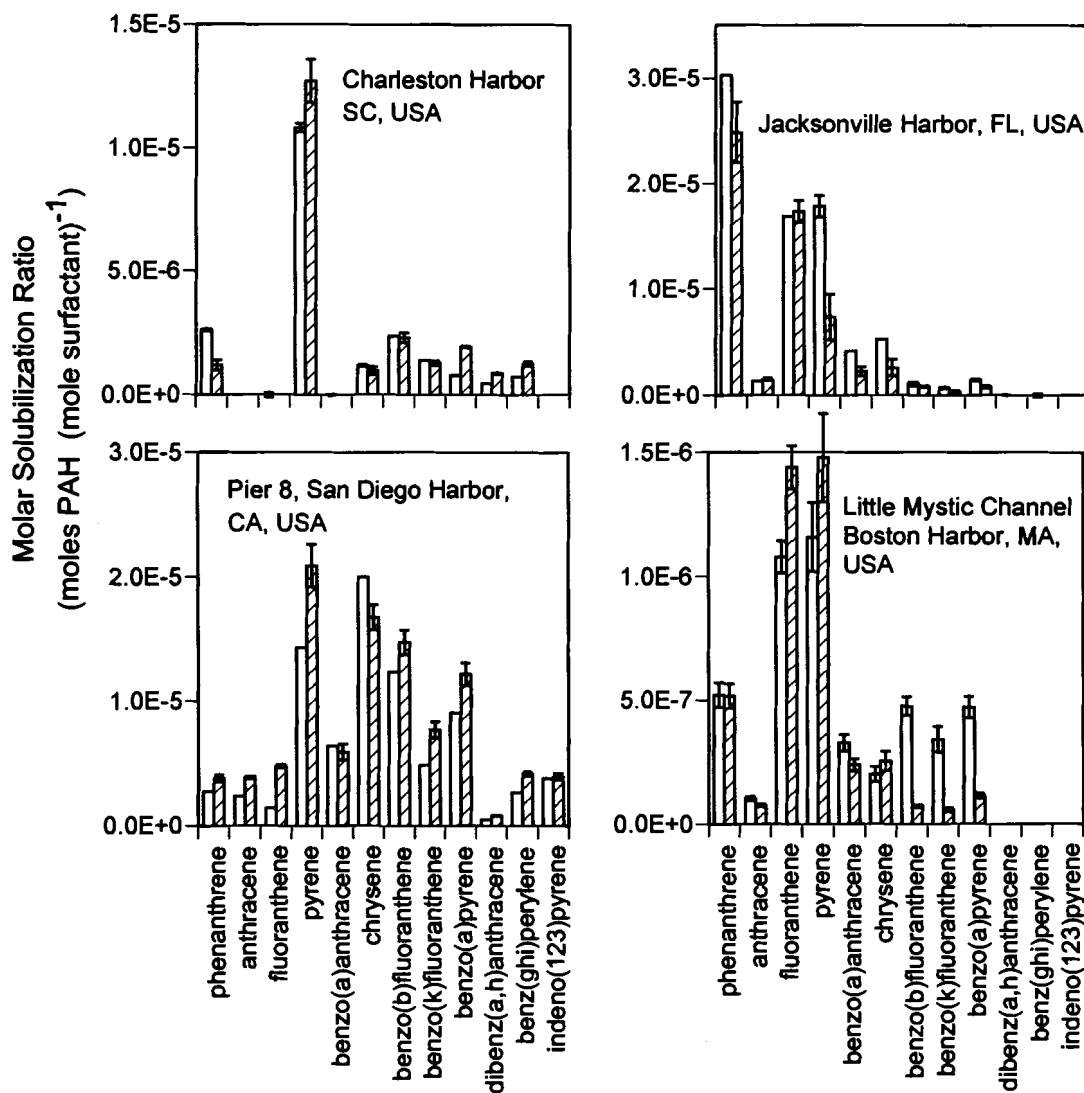
Figure 5.9: Comparison of Cocktail 1 and *A. marina* gut fluid release of PAH from 4 sediments.

Each graph is for a different sediment; white bars are for Cocktail 1, hatched bars are for *A. marina* gut fluid. The abscissa for all graphs is the same, and as appears in the bottom graphs. The ordinate for all graphs is the MSR of each individual PAH. For most sediments, the cocktail mimics both the absolute amount of individual PAH released by gut fluid and the relative trends among PAH. Error bars are ±1 SD.

Figure 5.10: Regression analysis shows that Cocktail1 is both precise and accurate in predicting *A. marina* gut fluid release of PAH from these sediments.

MSR data was log-transformed to normalize error variance. The 1:1 line is contained in the 95% confidence interval.
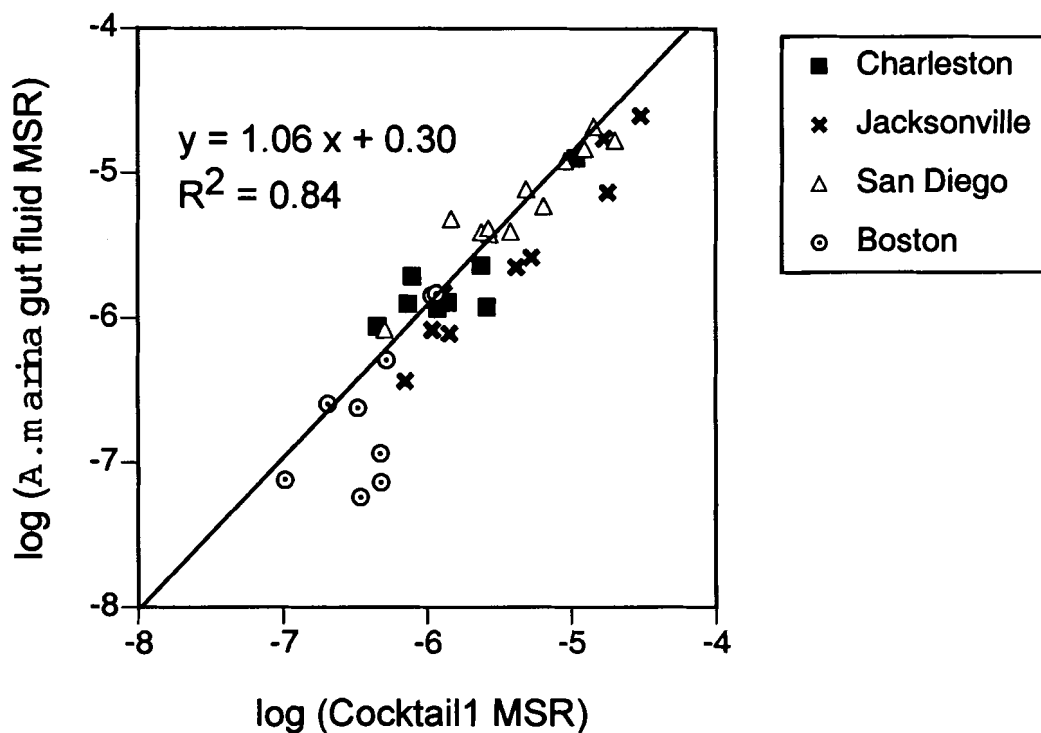
5.5. Discussion

The cocktail offers a means to apply a standard measure of bioavailability to disparate sediments, which after an initial investigation with cocktail can be prioritized for additional investigation. *In vitro* incubations with cocktail can use any sediment, even though sediments have wider ranges of characteristics, e.g. grain size, total organic carbon, enzymatically-hydrolyzable amino acids (food), and salinity than are acceptable to any single species that could be used in bioaccumulation tests. Though other measures of bioavailability have been proposed, e.g. equilibrium partitioning theory (EqP), we believe that gut fluid extractions are more relevant for deposit feeders – animals that are exposed to the brunt of sedimentary contaminants. Deposit-feeder exposure is via a micellar fluid, whereas EqP calculates exposure due to contaminants solvated by water (DiToro et al., 1991).

For a deposit feeder, the process of lipid bioaccumulation can be broken down into a series of steps. Ingested sediment and digestive fluids mix in the gut, where lipids are desorbed from the sediment and perhaps attacked by digestive enzymes. Solubilized lipids are transported via diffusion and advection to the gut wall, and compounds are absorbed (assimilated) into tissues. Our "cocktail" development has focused on the solubilization step of digestion. For vertebrates, lipid assimilation into tissues is passive, directly controlled by the concentration of lipid solubilized by digestive fluids; lipids must be solubilized by micelles in order to be assimilated (reviewed by Shiau, 1986). We suspect the same importance of solubilization for invertebrates based upon work with organic contaminants. Similarities between gut fluid solubilization and bioaccumulation of hydrophobic compounds like PAH and chlorinated organic compounds suggest a

transfer efficiency approaching 100% in deposit feeder guts (Weston & Mayer, 1998; Weston et al., 2001; Ahrens et al., 2001). *In vitro* incubations do not account for biotransformation and subsequent excretion of contaminants, which is common in benthic invertebrates (DiGiulio et al., 1995; Stegeman 2000; McElroy et al., 2000). Therefore, gut fluid and cocktail techniques are metrics for acute digestive exposure to contaminants for deposit-feeders and not net body burden.

We calculated molar solubilization ratios (MSR) to normalize the amount of lipid solubilized in gut fluid and cocktail solutions to the amount of surfactant. For sediment assessment, interest is in the concentration of contaminant to which an animal is exposed, not an MSR. Therefore, in practice a cocktail must be adjusted to match the digestive surfactancy of *A. marina*, which is plastic and likely varies with many factors including age (Mayer et al., 1997; Ahrens et al., 2001) and diet (Bock & Mayer, 1999). We have found surfactant concentrations to range from no evidence of micelles (< 2 mM) to 25 mM (Voparil, pers. obs.). Our experience with *A. marina* shows that individuals usually have surfactant concentrations of 6 to 13 mM – corresponding to CMDs of 15 - 30%. To mimic the median of this range, a cocktail should consist of 10 mM sodium taurocholate and 5 g L^{-1} BSA in ASW.

Most contaminated sediments contain mixtures of hydrophobic contaminants, not just a single compound. Our cocktail has been tested using multi-lipid systems, e.g. the binary lipid (Fig. 5.3) and contaminated sediments (Fig. 5.9) experiments. Synergistic and antagonistic interactions occur among multiple lipids solubilized in micelles (Carey & Small, 1970; Guha et al., 1999; Chapter 3), interactions that do not occur among monomers in aqueous solutions (Banerjee 1984; Burris & MacIntyre, 1986; Munz &

Roberts, 1987). Not all surfactants handle pairs of lipids similarly. For example, the solubility of a nonpolar hydrocarbon is enhanced by fatty alcohols in aliphatic detergents, but not in bile salt micelles (Carey & Small, 1970). Therefore, our cocktail, when compared to other surfactants and equilibrium partitioning theory, will more accurately assess digestive bioavailability after the modification by these mixture interactions.

5.5.1. Other cocktails to measure "bioavailability"

Other extractants have been used to predict the bioavailability of hydrophobic organic contaminants from soils and sediments. Ethanol, tetrahydrofuran, cyclodextrin, Triton X-100, and supercritical CO_2 extractions have been proposed to assess bioavailability (Tang et al., 2002; Reid et al, 2000, Volkering et al., 1998; Loibner et al., 1998). These studies have found correlations between the amounts of contaminants in extractants and other standard measures of bioavailability, but are not quantitatively accurate predictors of exposure.

More mechanistic models have been developed to describe the mobilization of hydrophobic contaminants from soils accidentally ingested by humans. Hand to mouth behavior in children is the primary route of human exposure to soil-bound contaminants (Stanek et al., 1998). In cocktails used to predict human exposure, conditions in the small intestine are approximated using mixtures of bile salts, suites of lipids that represent ingested lipids and their digestive products, and often digestive enzymes (Laher & Barrowman, 1983; Hack & Selenka, 1996; Oomen et al, 2000; Holman et al., 2002). Sodium taurocholate is a bile salt constituent. These formulations use concentrations of bile salts that are similar to that developed here for *A. marina* digestive exposure – e.g.,

Laher & Barrowman (1983) used sodium taurocholate concentrations up to 12 mM and

Holman et al. (2002) used 20 mM of bile salts to mimic fed stages of human digestion.

In humans, bile salt micelles play the central role in releasing hydrophobic organic

compounds such as PCB and PAH from soils, making the compounds bioaccessible

(Hack & Selenka, 1996; Oomen et al., 2000). With some fine tuning of the

concentrations of bile salts and the addition of lipids presumably present in the gut during

in vivo digestion, a taurocholate cocktail may thus be applicable to both vertebrates' and

invertebrates' digestive exposure to lipids.

5.5.2. Similarities between sodium taurocholate and *A. marina* gut fluid

The functional similarities for lipid solubilization by sodium taurocholate and *A.*

marina gut fluid surfactants are striking. Both micellar systems have evolved to transport

ingested nutritional lipids, though the composition of ingested material and associated

lipids is presumably quite different for humans and marine deposit feeders. Both

vertebrate and invertebrate surfactants contain a lipid moiety conjugated via an amide

bond to an amino acid. Amide bonds tend to make surfactants more stable in alkaline

solutions (Mizushima et al., 1999) and form smaller micelles (Folmer et al., 2001). In

vertebrates, the lipid moiety is sterol-based (Small 1971) whereas in invertebrates it is

fatty acid-based (Vonk, 1969; Smoot et al., in prep.). Lester et al. (1975) suggested that

this substitution was due to crustaceans' inability to synthesize cholesterol. However,

invertebrate polychaetes such as *A. marina* can synthesize cholesterol (Goad, 1978),

suggesting that the use of fatty acid-based surfactants is due to some, as yet

undetermined, aspect of their performance in saline solution.

The universal use of amide-linked amino acids as the hydrophilic end of biosurfactants may serve to limit the loss of these compounds via adsorption or precipitation. For example, all commercial surfactants tested here, except sodium taurocholate, precipitated when dissolved in ASW containing calcium. Biosurfactants are also less likely to denature proteins and cause malfunction of digestive proteins. Commercial surfactants like SDS are often used to denature proteins (Jones, 1996). At concentrations similar to that in the gut (> mM), SDS readily forms complexes with proteins in a very predictable manner – approximately one SDS molecule per two amino acid residues (Reynolds & Tanford, 1970).

Our empirical comparison of cocktail and gut fluid suggests that digestive enzymes such as esterases and lipases have little effect on PAH digestive availability from contaminated sediments, though these enzymes are present in gut fluids (Mayer et al., 1997). One could envision a situation where the lack of enzymes in this cocktail would cause problems for sedimentary contaminant risk assessment. For example, fatty alcohols are products of the digestive cleavage of wax esters (Place, 1992) and may serve as co-surfactants that increase BaP solubilization and hence digestive bioavailability in *A. marina* gut fluids (Chapter 3). However, our test sediments are probably richer in nutritional lipids than most marine sediment, so co-surfactant formation is not likely a concern with contaminated sediments.

We stress that bioavailability depends on a species-specific interaction between a particular organism and the geochemical characteristics of sediment. *A priori*, our approach of mimicking digestion likely won't predict suspension-feeder bioaccumulation, because these animals' exposure is mostly from the freely-dissolved

phase (DiToro et al., 1991). While our cocktail was developed with focus on a particular deposit-feeding polychaete, *Arenicola marina*, we realize the potential interest in applying the cocktail to predict exposure in other deposit feeders. *Arenicola marina* gut fluids from > 30 individuals solubilized BaP at a range of concentrations with a mean equal to the median amount solubilized by gut fluids from 17 of 18 other species of benthic invertebrate deposit feeders across several phyla (Mayer & Weston, 2001), indicating that our test animal may be a good general model for benthic macroinvertebrates. However, justification of our cocktail's application to other animals requires additional work in identifying and quantifying the digestive surfactants of other animals of interest. Surfactant identification would enable CMC calculations from CMD measurements, which are easily obtainable with contact angle titrations, and hence MSRs to compare the abilities of the different animals' surfactants to solubilize test compounds.

The cocktail provides a simple, cost-effective method to rapidly screen and rank sediments for associated, potentially bioavailable organic contaminants. This approach is a predictive tool for contaminant bioavailability, potentially substituting for longer, more expensive bioaccumulation tests, and can be incorporated into the early stages of a tiered framework for sediment assessment. By mimicking the natural constituents of digestive fluid with commercially available substances, we intend to create a readily available protocol that can be applied to a wide range of sediments and hydrophobic contaminants to address their potential to cause biological impacts.

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