

BENZO[*a*]PYRENE AND ZINC SOLUBILIZATION BY DIGESTIVE FLUIDS OF BENTHIC INVERTEBRATES—A CROSS-PHYLETIC STUDY

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**Abstract**—Contaminant bioavailability via digestive exposure was examined for 18 species of marine benthic invertebrates, using incubation of digestive fluids with sediments that were spiked with either radiolabeled benzo-*[a]*-pyrene (BaP) or zinc. Interphyletic trends in contaminant solubilization were compared with measures of digestive biochemistry, including enzyme activities, surfactancy, pH, and fluid phase organic carbon, amino acids, and lipids. Contaminant solubilization ranged from values equal to that of a seawater control to as much as an order of magnitude higher but were lower than those obtained with commonly used chemical extractants. Digestive fluids from echinoderms and a cnidarian tended to be relatively weak, those from polychaetes and echiurans were relatively strong, and those from taxa such as sipunculans and mollusks were intermediate. These trends correlated strongly with concentrations or activities of digestive biochemicals but not with pH. These correlations are consistent with previous work on mechanisms of digestive solubilization of polycyclic aromatic hydrocarbons (PAH) and metals, though strong covariance among digestive parameters does not allow this approach to be used for identification of specific mechanism(s).

**Keywords**—Bioavailability    Contaminants    Sediment    Invertebrates    Digestion

## INTRODUCTION

Dietary exposure is significant for contaminant bioaccumulation in some contaminant–organism combinations [1–7], and in such cases the rate and extent of contaminant uptake can be strongly influenced by digestive processes [8]. Because of preferential association of many contaminants with particles, dietary bioaccumulation is of particular interest for the many invertebrate species that ingest and digest sedimentary particles. While this route can serve as the major source of uptake for some contaminants in these organisms, it is also clear that only a fraction of the sediment-associated contaminant is bioavailable [7].

From a chemical perspective, bioavailability requires solubilization of the contaminants. For uptake by gills, this solubilization is generally a function of environmental conditions controlling contaminant dissolved speciation, while for digestive uptake the solubilization often occurs from particles within the organism's gut. To understand bioavailability via digestive exposure, it is necessary to relate characteristics of the unique biochemical milieu found in animal guts, which vary greatly among species [9], to the processes of contaminant solubilization.

Digestive release of contaminants has been studied by our group over the last several years, using incubations of gut fluids with sediments, to reach an understanding of those chemical factors that govern the dissolution step. We have noted variation in the ability of different species to solubilize contaminants from sediments [10–12], though the phyletic coverage has been low. We have addressed the chemical mechanisms of solubilization, identifying various digestive biochemicals in gut fluids as contributing to Cu or PAH solubilization [13,14]. The concentrations and activities of these digestive biochemicals have been studied in larger scale, cross-phyletic, physiological studies [9]. Finally, we have shown that our *in vitro* incubations of sediment with gut fluid provide a measure of

bioavailability that is consistent with other more traditional approaches to quantifying bioavailability of sediment-associated contaminants to deposit-feeding organisms [15].

The ability to solubilize contaminants from a sedimentary matrix has important implications for the potential effects of contaminants on any given species and, from an interspecific perspective, may influence the benthic community structure of pollution-stressed environments. The purpose of this study is to address large-scale, cross-phyletic patterns of contaminant solubilization both to establish the degree of variability in digestive bioavailability of contaminants among taxa and to interpret this variability in the context of measured concentrations and activities of digestive biochemicals. Our approach has been to collect 18 species of benthic animals, measure a wide range of digestive biochemical concentrations and activities, and correlate these results with measurements of contaminant solubilization under constant incubation conditions.

## MATERIALS AND METHODS

*Animal and digestive fluid collection*

A total of 18 species spanning seven phyla were available for the interphyletic comparison (Table 1). Polychaetes were the best represented (six species), followed by echinoderms (five species), echiurans (two species), mollusks (two species), a sipunculan, a priapulid, and an anthozoan (all one species each). The animals were collected from Alaska, Washington, California, or Maine, USA, during the summer and fall of 1997. Most animals were collected in the intertidal zone using a shovel at low tide, although a few (*Molpadia*, *Brisaster*, and *Travisia*) were collected from subtidal environments using a biological dredge.

The animals were held in seawater and dissected within 3 to 20 h after collection. An experiment with *Arenicola brasiliensis* (described in the following) tested for change in gut fluid biochemistry and contaminant solubilization with time held in seawater, up to 31 h. Dissections and gut fluid removal

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Table 1. Species used in this study, along with putative feeding strategy, location collected, gut segment sampled and method of sampling, typical gut fluid yield per individual, and number of individuals composited per sample

Species	Major taxon	Feeding strategy	Collection location	Gut segment sampled	Recovery method <sup>a</sup>	Typical ml fluid/individual	No. individuals per sample
<i>Abarenicola pacifica</i>	Annelida: Polychaeta	Deposit feeder	Puget Sound, Washington	Midgut	A	0.04	14
<i>Abarenicola vagabunda</i>	Annelida: Polychaeta	Deposit feeder	Puget Sound, Washington	Midgut	A	0.9	1
<i>Archidoris montereyensis</i>	Mollusca: Gastropoda	Predator on sponges	Seldovia, Alaska	Stomach	D	0.4	2-3
<i>Arenicola brasiliensis</i>	Annelida: Polychaeta	Deposit feeder	San Francisco, California	Midgut	A	1.4	Usually 1
<i>Arenicola marina</i>	Annelida: Polychaeta	Deposit feeder	Various sites, Maine	Midgut	A	1.0	>30
<i>Brisaster latifrons</i>	Echinodermata: Echinoidea	Deposit feeder	Puget Sound, Washington	Entire gut	C	1.6	1
<i>Chirdota</i> sp.	Echinodermata: Holothuroidea	Deposit feeder	Seldovia, Alaska	Entire gut	C	0.2	4
<i>Echiurus echiurus</i>	Echiura	Deposit feeder	Seldovia, Alaska	Foregut	A	0.1	1
				Midgut	A	1.1	1
				Hindgut	C	0.1	1
<i>Eupentacta quinquesimita</i>	Echinodermata: Holothuroidea	Deposit feeder	Seldovia, Alaska	Entire gut	B	0.1	10
<i>Katharina tunicata</i>	Mollusca: Polyplacophora	Herbivore	Seldovia, Alaska	Stomach/intestine	B	0.2	4
<i>Molpadia intermedia</i>	Echinodermata: Holothuroidea	Deposit feeder	Puget Sound, Washington	Entire gut	C	2.8	1
<i>Nephtys discors</i>	Annelida: Polychaeta	Omnivore	Seldovia, Alaska	Entire gut	A	0.8	2-4
<i>Parastichopus californicus</i>	Echinodermata: Holothuroidea	Deposit feeder	Puget Sound, Washington	Foregut	C	1.0	1
				Midgut	D	7.0	1
				Hindgut	C	10.0	1
<i>Priapulus caudatus</i>	Priapula	Carnivore	Seldovia, Alaska	Entire gut	B, D	2.0	1-2
<i>Siphonosoma ingens</i>	Sipuncula	Deposit feeder	Bodega Bay, California	Entire gut	A, B	1.0	1
<i>Travisia foetida</i>	Annelida: Polychaeta	Deposit feeder	Puget Sound, Washington	Entire gut	C	0.4	1
<i>Urechis caupo</i>	Echiura	Suspension feeder	Various sites, California	Foregut	A	0.1	1
				Midgut	A	1.3	1
				Hindgut	A	0.7	1
				Rectum	A	2.0	1
<i>Urticina crassicornis</i>	Cnidaria: Anthozoa	Carnivore	Seldovia, Alaska	Oral cavity	E	No data	1

<sup>a</sup> Mode of digestive fluid recovery: A, pierced gut wall with pipette and withdrew fluid; B, gut cut into segments, centrifuged, and supernatant collected; C, sediment slurry removed from gut, centrifuged, and supernatant collected; D, gut punctured and drained to vials; E, pipette inserted into oral cavity of live anemone at low tide.

were performed under air to avoid contamination by seawater. Methods of digestive fluid collection varied among the species, depending on the size and contents of the gut. For large organisms with obvious free liquid in the gut, a pipette was inserted through the gut wall to withdraw fluid, or in some cases, the gut was simply punctured and fluid drained to a vial. If the fluid contained visible sediment particles, it was centrifuged at 160 *g* for 5 to 10 min and the supernatant recovered. For those organisms with substantial amounts of sediment in the gut, the entire gut contents were removed, centrifuged at 360 *g* for 5 min, and the supernatant was recovered. For a few species, it was necessary to cut the gut into small segments and centrifuge both the gut wall and its contents at 360 *g* for 5 min. For the anthozoan *Urticina crassicornis*, a pipette was inserted into the oral cavity when the animals were exposed at low tide, and 5 ml of fluid were withdrawn. The fluid was centrifuged at 160 *g* for 5 to 10 min and the supernatant recovered.

Digestive fluid from the sipunculan *Siphonosoma ingens* was collected by two methods. In some individuals the gut wall was punctured with a pipette and fluid withdrawn. While the fluid recovered in this manner was unambiguously digestive fluid, it was possible to obtain sufficient fluid only for biochemical characterization and not for pollutant extraction from sediment. Therefore, in other individuals the gut and its contents were centrifuged, and the supernatant was collected. This supernatant, including some coelomic fluid contamination, was used for both biochemical characterization and pollutant extraction. The following results distinguish between the two *S. ingens* fluids by mode of collection (pipette or centrifuge).

Sampling focused on midguts, in which digestive agents are typically maximal, but in some species fluid was also recovered from other gut segments from which sufficient quantities were available. The preferred strategy was to collect fluid from morphologically distinct gut sections, keeping each fluid segregated for later analysis. This approach was possible only for *Parastichopus californicus* (foregut, midgut, and hindgut fluids), *Echiurus echiurus* (foregut, midgut, and hindgut fluids), and *Urechis caupo* (foregut, midgut, hindgut, and rectal fluids). For some species, free liquid was evident in only one portion of the gut (e.g., the midgut in arenicolid polychaetes). For those species with fluid throughout the gut but for which no one gut segment would provide enough fluid for analysis, contents of the entire gut were combined.

Collections deliberately targeted large invertebrates, providing enough fluid from a single individual to use for both pollutant extraction experiments (~600–1,000  $\mu$ l) and measurement of fluid characteristics (~50–150  $\mu$ l). For most species, single individuals provided sufficient fluid for all analyses without the necessity of compositing multiple samples. However, in a few cases, because of the small volume of fluid available from each individual or in order to reduce variability within the species, gut fluid from multiple individuals was combined to obtain samples for later pollutant extraction and fluid characterization (Table 1).

#### *Sediment collection and preparation*

The sediment used in this experiment was collected from the Central California coast, wet sieved on a 1.0-mm screen to remove the coarser material, then spiked with either [ $^{14}$ C]benzo[*a*]pyrene (BaP) in acetone or  $^{65}$ ZnCl<sub>2</sub> in 0.5 N HCl. The  $^{14}$ C-BaP (Sigma Chemical, St. Louis, MO, USA; specific

activity = 26.6 mCi/mmol) had a radiochemical purity of 97.9% as determined by thin-layer chromatography immediately before use. The  $^{65}$ Zn (Amersham, Arlington Heights, IL, USA; specific activity = 2.71 mCi/mg) had a radiochemical purity of 99% as determined by the supplier.

Sediments were spiked with  $^{14}$ C-BaP and  $^{65}$ Zn (in separate treatments) by adding the label and its carrier (BaP in 0.13 ml acetone; Zn in 6.4 ml 0.5 N HCl) to 400 g wet sediment and 450 ml seawater. Sodium hydroxide solution was added to the zinc treatment so as to neutralize the acid. Complete mixing was accomplished by either placing the jar on a rolling table for 8 h (Zn) or mixing the slurry in a blender for 2 min (BaP). After mixing, the sediment was stored at 4°C for 60 d before decanting the overlying water and using the wet sediment (about 70% solids by weight) for extraction with digestive fluid.

#### *Digestive fluid preparation and extraction*

Digestive fluid was frozen at –80°C after collection and held for up to 10 months until use for extraction of BaP or Zn. The digestive fluid was allowed to thaw at room temperature, and then the pH and volume of each fluid sample were measured. Sediment and fluid were placed in glass centrifuge tubes at a ratio of 0.8 ml digestive fluid to 0.5 g wet sediment. The vials were placed on a reciprocating shaker in a darkened room at 17°C for 2 h. This time period was chosen to keep incubation period constant among species and to choose a period intermediate among the varying gut residence times of the species studied. The vials were then centrifuged for 10 min at 4000 *g*, and the overlying fluid was recovered for analysis of BaP or Zn.

In order to compare pollutant extractability by digestive fluid with that of more traditional extractants, BaP-spiked sediment was also extracted using seawater, methanol, and acetonitrile. These solvents were selected because of their miscibility with water incorporated within the wet sediments. The Zn-spiked sediment was extracted using seawater and hydrochloric acid in 0.5-, 1.0-, and 4.0-N solutions. All extractions with conventional extractants were done following the methods described previously for comparison with the digestive fluids, without the addition of energy (e.g., heat, microwaves) as might be used in some conventional chemical extractions.

The  $^{14}$ C-BaP-spiked sediment and fluids used for extraction were placed in glass scintillation vials with Hionic-Fluor scintillation cocktail (Packard Instruments, Meriden, CT, USA). The  $^{14}$ C activity was determined using a Beckman LS6500 liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA) with corrections for background and quenching (by external standard method). The  $^{65}$ Zn-spiked sediment and the fluid samples were counted for radioactivity using a gamma counter Beckman Gamma 5500 (Fullerton, CA, USA) with corrections for background, counting efficiency, and decay.

#### *Characterization of gut fluid biochemistry*

We examined a variety of concentrations and activities of biochemically important substances in these gut fluids. Total organic carbon was measured on a Shimadzu 5000A TOC analyzer (Shimadzu, Columbia MD, USA) before and after sparging to correct for inorganic carbon concentrations. Total amino acids were measured after 6-N HCl hydrolysis overnight at 110°C under N<sub>2</sub>, using orthophthaldialdehyde as a fluorescent reagent to quantify the total concentration of resultant

amino acid monomers [9]. Lipid concentrations were analyzed by extracting the gut fluids using a 1:2 chloroform:methanol mixture overnight, separating the resulting lipids into chloroform by adding chloroform and water to a final chloroform:methanol:water ratio of 1:1:1, and storing at 4°C [16]. Analysis of compound classes was performed by thin-layer chromatography with a flame ionization detector (TLC-FID) on a Mark 5 Iatroscan (Bioscan, Washington, DC, USA) [17]. Standards for the various classes were nonadecene for hydrocarbons, hexadecyl palmitate for wax esters, palmitic acid for free fatty acids, tripalmitin for triglycerides, hexadecanol for alcohols, cholesterol for sterols, and dihexadecanoyl lecithin for acetone-mobile polar lipids.

Digestive enzyme activities were measured by examining the rate of hydrolysis of fluorescently tagged monomers of various substrates (Sigma). These substrates included the methylumbelliferyl (MUF) or methylcoumarinyl (MCA) fluorophores conjugated via the appropriate bond to alanine (protease), butyrate (esterase), palmitate (lipase),  $\beta$ -D-N,N'-diacetylchitobioside (chitinase), and glucose ( $\alpha$ - and  $\beta$ -glucosidase). The reactions were carried out in microplate wells, and the time course of fluorescence appearance was measured in a Fluostar microplate reader (BMG, Durham NC, USA).

Surfactancy was assessed, as in [9], by measuring the contact angle between a 2- $\mu$ l droplet of gut fluid and Parafilm, using an image analysis system. To assess the presence/absence of micellization of surfactants, we titrated this droplet with clean seawater and monitored the contact angle during the titration. A two-phase line resulting from this titration indicates the presence of surfactant micelles; the degree of gut fluid dilution at this slope change is termed the critical micelle dilution (CMD) factor, with greater concentration of surfactants leading to lower CMDs.

## RESULTS

### Sediment characteristics

The sediment used in the extractions was primarily sand, with 18% of the weight consisting of silt and clay particles. Total organic carbon content was 2.1%; total nitrogen content was 0.17%. Both BaP and Zn were quantified based on radioactivity but when adjusted for specific activity were equivalent to concentrations of 93  $\mu$ g/kg for BaP and 0.078  $\mu$ g/g for Zn. Such spike concentrations may or may not be relevant to many contaminated sediments, but our experimental design emphasizes interphyletic variability in contaminant extraction rather than the actual percentage of contaminant extracted by any species. These concentrations are independent of any unlabeled BaP or Zn existing in the sediment prior to collection. Those concentrations were not measured but are anticipated to be low as the collection site was on the Pacific Coast in an area with minimal anthropogenic influence. A low standard deviation of concentrations from 10 subsamples (1.3  $\mu$ g/kg and 0.001  $\mu$ g/g for BaP and Zn, respectively) indicated homogeneity had been achieved during sediment spiking.

### Effects of nonphyletic variables on results

Our study tests the role of taxon on contaminant solubilization but is potentially subject to a variety of confounding factors. We therefore tested for the potential influences of animal holding time, geographic source of organisms, and gut section within species.

The activities or concentrations of digestive agents thought

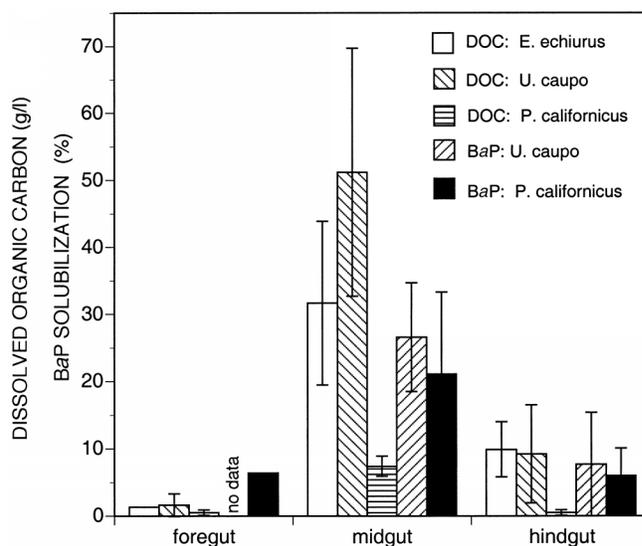


Fig. 1. Solubilization of BaP (as percentage of total sediment spike that was solubilized) and organic carbon (DOC) concentrations (g/L) in gut fluids of foregut, midgut and hindgut sections of *Parastichopus californicus* and *Urechis caupo*.

to be responsible for contaminant solubilization (see the following discussion) typically reach maximal levels in the midgut, though exceptions are known [9]. To test for the effect of gut segment on fluid characteristics and contaminant solubilization, fluids were available from different longitudinal sections of the gut in three species (*E. echiurus*, *U. caupo*, and *P. californicus*). All species showed consistent longitudinal patterns in gut fluid composition and pollutant solubilization potential. Figure 1 illustrates longitudinal trends for organic carbon, but similar results were found for virtually all digestive agents measured. Midgut fluids had organic carbon concentrations approximately an order of magnitude higher than either the foregut or the hindgut fluids in all species tested. Fluid from midgut sections also showed the greatest BaP solubilization (Fig. 1), and a smaller number of experiments with Zn solubilization gave similar results. Chen and Mayer [12] showed that highest levels of dissolved trace metals were found in the midgut, corresponding to the highest concentrations of metal-solubilizing agents, and the results for Zn here correspond to the model of longitudinal solubilization patterns that they presented. Midgut fluids, when available, were therefore routinely used for solubilization experiments throughout the study, and all subsequent results discussed for *E. echiurus*, *U. caupo*, and *P. californicus* are based on midgut fluids.

Digestive physiology is potentially affected by sampling and holding the animal before extracting its digestive fluid. We tested for holding time by allowing different individuals of *A. brasiliensis* held in seawater to evacuate their guts for 4- versus 31-h periods and then extracting gut fluids. For nearly all parameters measured (all enzyme classes, amino acids, total organic carbon, pH, contact angle, contaminant solubilization), there were negligible differences in characteristics of the gut fluids extracted after 4 versus 31 h. For example, BaP solubilization was  $17.6 \pm 3.6\%$  after 4 h and  $15.6 \pm 4.8\%$  after 31 h. However, there was no micellization in 4 of the 10 individuals evacuated for 31 h, as indicated by CMD values of 100% (Fig. 2), which implies surfactant concentrations below the critical micelle concentration. Fluid from these same individuals tended to be among the weakest in extraction of

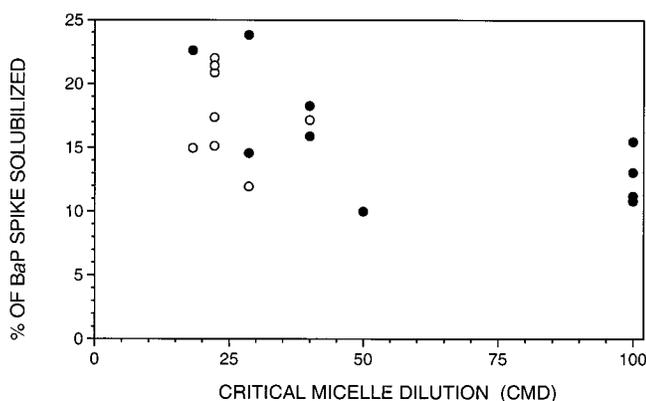


Fig. 2. Percentage of BaP spike solubilized versus critical micelle dilution (CMD, that dilution of pure gut fluid at which micelles disappear) by gut fluids of *Arenicola brasiliensis* after 4-h (open circles) and 31-h (closed circles) evacuations.

BaP. This loss of micelles on sediment evacuation is consistent with previous work with *Nereis virens* showing that micellization occurs only in the presence of sediment in the gut [18].

The effect of geographic variance was assessed by sampling *U. caupo* from three sandy beach sites along 250 km of the California coast. All populations were sampled within a 30-d period and the samples processed identically. For nearly all gut fluid parameters measured, there was no significant difference among the three populations (Kruskal–Wallis one-way analysis of variance,  $p > 0.05$ ). The only exception to this homogeneity was for micellization; only one of the three populations consistently demonstrated CMD values of less than 100% in the gut fluid (11 of 13 individuals vs 0 of 12 individuals from the other two populations). Contaminant solubilization was essentially constant across all the populations; for example, the proportion of BaP solubilized was comparable across the populations ( $25.8 \pm 8.8\%$ ,  $22.1 \pm 4.1\%$ ,  $32.3 \pm 8.3\%$ ), as was Zn solubilization ( $15.8 \pm 1.5\%$ ,  $12.6 \pm 3.0\%$ ,  $17.4 \pm 2.8\%$ ).

These tests on the influence of nonphyletic variables such as source population of test organisms, gut evacuation time, and gut segment were not exhaustive and could not be performed on every species collected. Data from the few species tested suggest that variation in gut evacuation times over the 24-h range was not likely to affect results substantially, nor was the site of collection for a given species. However, the choice of gut segment from which fluid was available does have a major influence on fluid traits ascribed to that species.

#### Digestive fluid characteristics

Gut fluid characteristics of the various taxa are presented here (Table 2) and discussed briefly, but more extensive interphyletic analyses and discussion will be the subject of a later publication on digestive physiology. Another interphyletic analysis can also be found in Mayer et al. [9].

The most noteworthy pattern in the present gut fluid data is the extremely broad range in enzyme activity, surfactancy, and most other fluid characteristics among the taxa. Moreover, the parameters tend to covary; a taxon with a low value for enzymatic activity or concentration of amino acids, organic carbon, or lipids tended to be low in all measures. All these indicators showed negative correlation with contact angle, an inverse indicator of surfactancy, which is in turn related to some combination of surfactant strength and concentration. The Cnidaria and Echinodermata generally showed the weakest digestive intensity, as

indicated by both enzyme activity and surfactancy (low esterase activity; concentrations of amino acids, dissolved organic carbon [DOC], and lipids; and high contact angle). With one exception (*P. californicus*), there was no evidence of micelle formation in the gut fluids of these taxa. At the other extreme, the Annelids and Echiura tended to show the strongest digestive intensity. Esterase activity, for example, was two to three orders of magnitude higher than in the weakest taxa. Annelids and echiurans tended to show greater surfactancy (contact angles of  $40\text{--}60^\circ$ ) and the presence of micelles. Representatives of the phyla Mollusca, Priapulida, and Sipuncula tended to fall between the two extremes in digestive intensity.

Two taxa differed from these cross-phyletic patterns. Among the annelids, *T. foetida* was remarkable in that its digestive chemistry was rather weak in comparison to the other polychaetes. Among the echinoderms, *P. californicus* had enzymatic activity and amino acid concentration typical of the Echinodermata but greater surfactancy than other echinoderm species, reflected by both the low contact angle and the CMD.

The only parameter that showed little cross-phyletic variation was gut fluid pH. Values were near neutral in all taxa (6.2–8.6). We measured gut fluid pH just prior to sediment incubation so that values could be related to in vitro contaminant solubilization potential, but these pH values are not necessarily equivalent to pH in vivo.

#### Extent of contaminant solubilization

The extent of contaminant solubilization from the test sediment showed order-of-magnitude differences among species. The BaP solubilization ranged from 6 to 45% among the taxa, with phyletic position exerting a strong influence (Fig. 3). The gut fluids of most echinoderms and the cnidarian solubilized no more BaP than the seawater reference (about 6%), with *P. californicus* midgut fluid being the only notable exception (21%). Gut fluids from the annelids, excluding *T. foetida*, echiurans, and the priapulid, on the other hand, solubilized up to seven times more BaP than the seawater control. Fluid from the polychaete *N. discors* was the most effective BaP extractant of all gut fluids tested (45% BaP solubilized). Digestive fluid from the two mollusks (the nudibranch *A. montereyensis* and polyplacophoran *K. tunnicata*) and the sipunculan (*S. ingens*) occupied intermediate positions (12–23% BaP solubilization).

The two water-miscible organic solvents tested extracted far more BaP than did any of the natural digestive fluids. Methanol extracted 75%, and acetonitrile extracted virtually all the BaP.

Zinc was, in general, less extractable than BaP but also exhibited order-of-magnitude differences among the taxa (Fig. 4). The echinoderm and cnidarian gut fluids, again, showed relatively low solubilization (0.9–1.7% Zn), comparable to that of the seawater reference (0.9%). Annelid, echiuran, and priapulid gut fluids showed higher levels of solubilization, all within a relatively narrow range (8.7–15.4%). The vast majority of the Zn (>85%) was not soluble in any of the digestive fluids tested.

These results can also be compared with the Zn solubilized by HCl solutions of various strengths. The 0.5-N HCl solution solubilized 2.6% of the added Zn, at the low end of the range of gut fluid results, while the stronger HCl solutions solubilized virtually all the Zn spike (82 and 95% for 1 N and 4 N HCl, respectively). It is possible that better agreement between the 1-N HCl and the gut fluid results would have obtained if a correction for acid-volatile sulfide (AVS) concentrations was determined [19], especially because some AVS-bound metals appear to be unavailable to gut fluid extraction [20, but also see 21]. Never-

Table 2. Selected gut fluid characteristics (mean with standard error in parentheses) of digestive fluid for all test species. Only esterase activity, the dominant enzyme class measured in most tested species, is shown, although other enzymes were measured; total lipids are shown although individual classes were also quantified (Table 3). Lipid data not available for all species. Percentage of individuals with micelles refers to fraction of population with two-phase titration plot of contact angle versus dilution

Taxon	Esterase (nmol/ml/min)	Amino acids (g/L)	DOC <sup>a</sup> (g/L)	Total lipids (mg/L)	pH	Contact angle (degrees)	% of individuals with micelles
Cnidaria							
<i>Urticina crassicornis</i>	6.1 (9.9)	1.5 (2.1)	0.7 (1.0)	139 (122)	8.3 (0.5)	96 (4)	0
Echinodermata							
<i>Brisaster latifrons</i>	1.0 (0.8)	3.2 (0.2)	4.8 (1.3)	274 (84)	7.9 (0.0)	60 (5)	0
<i>Chirdota</i> sp.	0.7 (0.4)	3.5 (0.9)	5.8 (1.6)	—	6.9	62 (7)	0
<i>Eupentacta quinquesimata</i>	4.7 (8.2)	9.3 (12.0)	9.3 (10.6)	—	7.1 (0.0)	69 (3)	0
<i>Molpadia intermedia</i>	0.0 (0.0)	0.4 (0.2)	0.9 (0.3)	58 (36)	8.0 (0.3)	66 (8)	0
<i>Parastichopus californicus</i>	1.7 (0.3)	2.0 (0.8)	7.4 (1.5)	469 (328)	6.6 (0.5)	49 (5)	100
Mollusca							
<i>Archidoris montereyensis</i>	45 (15)	29 (1)	17 (0)	—	6.2 (0.3)	77 (5)	0
<i>Katharina tunicata</i>	50 (18)	54 (23)	37 (13)	—	7.1	74 (8)	0
Annelida							
<i>Abarenicola pacifica</i>	499 (183)	47 (12)	48 (13)	—	7.0 (0.5)	43 (2)	100
<i>Abarenicola vagabunda</i>	342 (124)	63 (11)	67 (13)	—	7.9 (0.2)	36 (3)	93
<i>Arenicola marina</i>	290 (203)	52 (47)	28 (18)	—	—	48 (9)	60
<i>Arenicola brasiliensis</i>	231 (103)	31 (12)	43 (9)	2,860 (640)	7.6 (0.3)	42 (3)	84
<i>Nephtys discors</i>	685 (191)	54 (11)	57 (15)	6,510 (970)	8.0 (0.2)	54 (4)	80
<i>Travisia foetida</i>	119 (86)	19 (9)	16 (6)	—	6.9 (0.3)	65 (12)	0
Priapula							
<i>Priapulus caudatus</i>	25 (7)	36 (26)	67 (23)	3,500 (4,230)	8.3 (0.4)	48 (1)	67
Echiura							
<i>Echiurus echiurus</i>	1,070 (670)	26 (10)	32 (12)	653 (342)	8.6 (0.2)	56 (7)	0
<i>Urechis caupo</i>	3,350 (1,390)	49 (29)	51 (19)	2,400 (1,540)	7.7 (0.5)	50 (6)	44
Sipuncula							
<i>Siphonosoma ingens</i> (pipette sample)	116 (94)	10 (10)	8.2 (9.0)	405 (445)	—	78 (14)	0

<sup>a</sup> DOC = dissolved organic carbon.

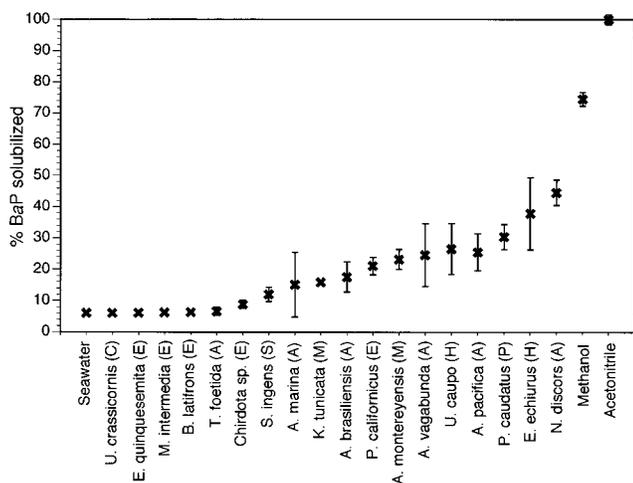


Fig. 3. Percentage of BaP solubilized by 3-h midgut fluid extraction by various species. Phylum is in parentheses following species name (C = Cnidarian, E = Echinoderm, A = Annelid, S = Sipunculid, P = Priapulid, H = Echiuran, M = Mollusk). Data for *Echiurus echinurus* are averaged across individuals from three different sites. Error bars indicate standard deviations.

theless, it is clear that a single-value measurement of simultaneously extractable metals-acid volatile sulfide (SEM-AVS), often proposed to explain bioavailable concentrations of trace metals [19], would not capture the considerable variability in solubilization of Zn by gut fluids of different species.

The BaP and Zn solubilization experiments were typically carried out on different subsets of the gut fluid samples within a given species because of a lack of sufficient gut fluid from an individual animal to carry out all experiments and analyses. Hence, the correlation between BaP and Zn solubilization can be assessed only by comparing species' means. Via this analysis, the extent of BaP solubilization correlated very strongly with that of Zn solubilization (Pearson product-moment correlation;  $n = 12$ ;  $r = 0.711$ ;  $p < 0.01$ ). The most noteworthy exception to this correlation was midgut fluid from the hol-

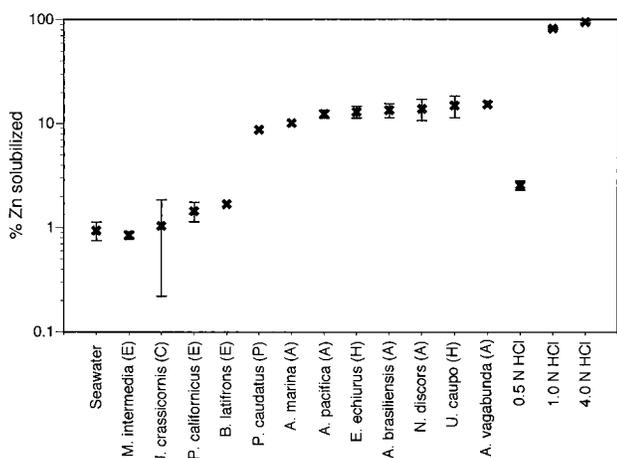


Fig. 4. Percentage of Zn solubilized by 3-h midgut fluid extraction by various species. Phylum is in parentheses following species name (C = Cnidarian, E = Echinoderm, A = Annelid, S = Sipunculid, P = Priapulid, H = Echiuran, M = Mollusk). Data for *Echiurus echinurus* are averaged across individuals from three different sites. Error bars indicate standard deviations.

Table 3. Significance ( $p <$  value shown) of Pearson pairwise correlations between Zn or benzo[*a*]pyrene (BaP) solubilization and various digestive biochemical activities or concentrations

Gut fluid property	BaP	Zn
$\alpha$ -glucosidase	0.008	0.0003
$\beta$ -glucosidase	0.35	0.0000
Lipase	0.002	0.0000
Chitinase	0.0000	0.0001
Protease	0.0000	0.0000
Esterase	0.0000	0.0000
Amino acids	0.0000	0.0000
Total lipids	0.06	0.29
Hydrocarbons	0.3	0.79
Wax esters	0.15	0.45
Triglycerides	0.37	0.69
Free fatty acids	0.69	0.46
Alcohols	0.58	0.46
Sterols	0.35	0.59
Acetone-mobile polar lipids	0.04	0.77
Phospholipids	0.36	0.31
Total dissolved organic carbon	0.0000	0.0000
pH	0.13	0.4
Contact angle	0.0000	0.0000

othuroid *P. californicus*, which was a moderately good extractant for BaP but similar to seawater for Zn.

#### Correlation with digestive biochemistry

Among species, both BaP and Zn solubilization showed very strong positive correlations with indicators of digestive activity or concentrations of organic materials in digestive fluid (Table 3). Equally strong but negative correlation was found with contact angles because of the negative relationship between surfactant concentration and contact angle. No significant correlations were found with pH. The significance level was low for most lipid class parameters, although demonstration of significance for lipids was more difficult because of the lower numbers of samples (typically 5–20 vs 40–130 for most other parameters). The BaP solubilization showed better correlation with most lipid classes than did Zn solubilization, though the only significant correlation was with the acetone-mobile polar lipid fraction (which averaged 18% of total analyzed lipids). This last class of compounds can contain a variety of relatively polar compounds whose composition is uncertain [17].

The BaP solubilization showed one of its best correlations with the total organic carbon concentrations of the gut fluids (Fig. 5B). Only above DOC levels of about 3 g/L was there an increase in BaP solubilization beyond that of the seawater control. Because the DOC concentration includes many components that may contribute to BaP solubilization (see the following discussion), there are phyletic differences in BaP solubilization that are not explained by DOC alone. For example, the echinoderm *P. californicus* showed higher BaP solubilization than other taxa with similar DOC levels.

The Zn solubilization closely followed the amino acid concentration of the gut fluids (Fig. 5B), consistent with our previous demonstration of the role of amino acids in dissolution of Cu and other metals from sediments [13,22]. This plot clearly shows minimal solubilization of Zn until a threshold amino acid level of about 1 g/L, above which there is a monotonic increase in Zn solubilized. The logarithmic transformation of the x-axis, shown here to accentuate the 1-g/L threshold, obscures a plateau of 12 to 15% Zn solubilization that occurred above 35 g/L.

No interphyletic correlations between contaminant solu-

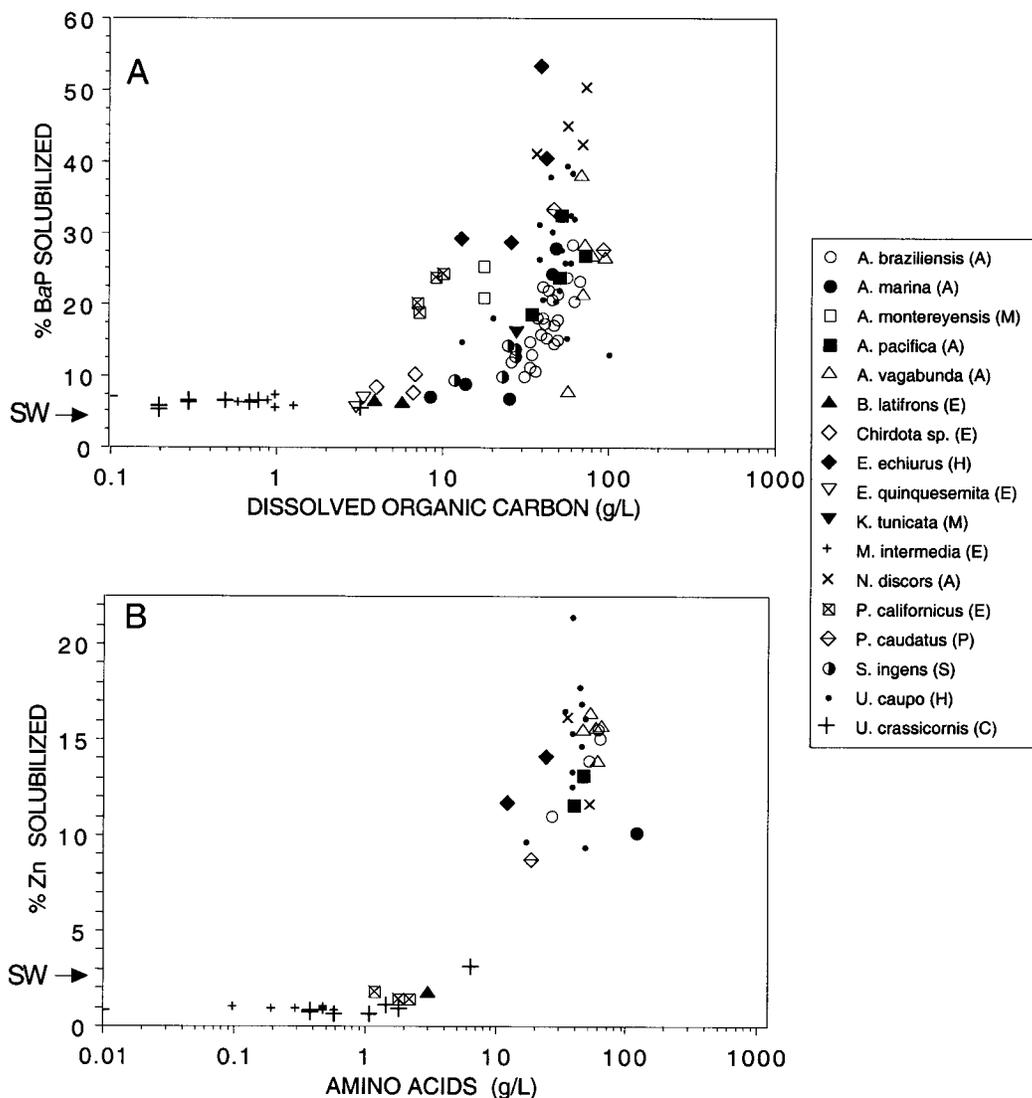


Fig. 5. Percentage of (A) BaP spike solubilized versus the organic carbon concentration (g/L) and (B) Zn spike solubilized versus the amino acid concentrations (g/L). Phylum given by letter in parentheses following species name (C = Cnidarian, E = Echinoderm, A = Annelid, S = Sipunculid, P = Priapulid, H = Echiuran, M = Mollusk). "SW" refers to percentage of spike solubilized by the seawater control. X-axis values plotted on log scale to show detail at low concentrations. Only midgut sections, which showed maximum solubilization, are plotted.

bilization and CMD are presented because CMD cannot be compared interphyletically. This parameter allows comparison of surfactant concentration among samples only if the surfactants are the same among those samples. This congruence likely does not apply interphyletically. Applying nonparametric statistics to intraphyletic data sets showed a significant inverse relationship between CMD and BaP solubilization for the *A. brasiliensis* data (Fig. 2; Spearman rank correlation,  $p < 0.01$ ).

Intraspecies correlations between contaminant solubilization and other digestive biochemical parameters were not as strong as those found in the cross-phyletic data set because of narrower data ranges and smaller data subsets. Even within the most extensive species data subsets (*A. brasiliensis* BaP solubilization and *U. caupo* BaP and Zn solubilization, all with  $n$  of 11–18), correlations with digestive biochemical parameters were infrequent and inconsistent. The *A. brasiliensis* BaP solubilization correlated only with DOC (Pearson correlation,  $p < 0.05$ ). The *U. caupo* BaP solubilization correlated only with chitinase and phospholipids. The *U. caupo* Zn sol-

ubilization correlated with  $\alpha$ -glucosidase, acetone-mobile polar lipids, phospholipids, total lipids, and pH.

## DISCUSSION

### Bioavailability

These results provide a striking and systematic exposition of the species dependence of bioavailability via the digestive pathway. Numerous past efforts to quantify bioavailability by approaches such as uptake kinetics [23], steady-state body burden [24], or absorption or assimilation efficiencies [5,8] have generally been done with a single species. Very few studies have contrasted bioavailability of contaminants from a given sediment to even a few species, much less the 18 used in the present study, and such studies often find variation due to different uptake pathways. Our results show that the amount of sediment-associated contaminant that is bioavailable to an organism from ingested material varies markedly depending on the species.

Bioavailability via digestion also varies among individuals within a species. Digestive fluid composition within species

varies because of many factors, including age [9] and diet composition [18]. Even among a group of individuals collected at the same time and place, we find differences of up to a factor of three in the amount of contaminant that is solubilized by their digestive fluids [11]. While these intraspecific differences should not go unrecognized, they are relatively small compared to the differences we find across species.

Many attempts have been made to quantify bioavailable contaminant by a selective chemical extraction. All these attempts suffer from the obvious conceptual difficulties illustrated by our cross-phyletic analysis and their failure to define the taxa for which bioavailability is being approximated. Beyond this limitation, our data suggest other difficulties with such an approach. Mild organic solvents (e.g., methanol) have been suggested to extract the bioavailable fraction [25,26], but both methanol and acetonitrile extracted far more BaP than any of the digestive fluids of these experiments. For zinc, both 1 N and 4 N HCl far overestimated bioavailability. A 0.5-N HCl extraction has been advocated as a measure of trace metal bioavailability [27], and it did extract a fraction of zinc within the range of zinc solubilized by digestive fluids.

The contaminant solubilization results presented here do not necessarily equate to actual or overall contaminant exposure, even that due to dietary exposure, for several reasons. Contaminant solubilization varies according to several parameters that were held constant in this study, such as solid-to-fluid ratio, incubation time, and others [11,12,15]. We also have not addressed intestinal absorption of contaminants once dissolved in the gut. Assessments of overall contaminant exposure in vivo must take into account such other factors.

It is also important to recognize that digestive solubilization of contaminants does not necessarily equate to the likelihood of toxicity. Our approach provides a measure of bioavailability of a contaminant, which can then be excreted, immobilized in tissue, or cause toxic effects. However, this solubilized contaminant is not necessarily the dose that would elicit a toxic response. For example, we have found in separate work that copper can inactivate digestive enzymes if released in concentrations sufficient to bind to enzymatically active sites on the enzyme proteins [28]. However, this susceptibility is inversely related to the amino acid concentration in the gut because of its dependence on the ratio of dissolved copper to amino acid concentration (Chen et al., personal communication). A species with low enzyme concentrations is at risk of enzymatic inactivation at dissolved Cu concentrations lower than a species with greater digestive enzyme levels. Thus, the potential for enzyme inactivation, though only one form of toxicological exposure, is inversely related to the amount of copper likely to be solubilized.

#### *Relationships with solubilization mechanism(s)*

The chemical mechanisms by which contaminants are solubilized from a sedimentary matrix by gut fluids are only partially understood. We have previously established that complexation with the amino acid histidine, presumably associated with secreted digestive proteins as well as hydrolyzed food proteins derived from sediment, is important in the dissolution of copper [13]. Strong correlations between amino acids and other metals among various benthic species from uncontaminated environments are also consistent with amino acid complexation [22], as are experiments showing protein solutions to mimic gut fluid solubilization of various metals from contaminated sediments [12].

For PAH, we have found that surfactant micelles play an important role in their solubilization in *Arenicola marina* digestive fluids [14]. Our observations of digestive solubilization by *P. californicus* in the present study supports the importance of surfactants in BaP solubilization. This species shares the traits of low enzymatic activities and organic carbon and amino acid concentrations with other echinoderms, but it was unique among tested species in this phylum in having surfactant micelles as indicated by a low CMD. The Zn solubilization by its digestive fluid was comparable to seawater, but BaP solubilization was far higher than other echinoderms and at a level comparable to the annelids. Previous work has shown that other compounds, such as globular proteins with hydrophobic interiors, may also contribute significantly to solubilization of PAH [14]. The results presented here for the 31-h evacuation of *A. brasiliensis* corroborates a role for other solubilizing agents because loss of micellization lowers BaP solubilization but not to a value as low as that of the seawater control.

Thus, the results of the cross-phyletic data presented in this study, with strong correlations between contaminant solubilization and properties such as amino acids or contact angles, are consistent with our previous work on mechanism. We attempted to identify the strongest correlations among the variables studied here to confirm the exact solubilization mechanisms for Zn and BaP. However, these attempts were fruitless, likely for some combination of three reasons. First, the concentrations and/or activities of digestive agents covary so strongly with one another that the correlations of contaminant solubilization with any of them are likely to be similar. Thus, the strong correlations with enzyme activities likely have no causal basis because enzyme activities correlate well with the concentration of agents that are the likely causes of solubilization, such as amino acids [12,13] or surfactants [14]. Second, we did not measure digestive biochemical parameters with sufficient compositional resolution to focus on detailed mechanisms; for example, our assay of contact angle and CMD is not an accurate or precise way to measure surfactant concentration or activity. The fact that the correlations that were highly significant at an interphyletic level tended to be less so intraphyletically suggests that we were not quantifying the exact biochemical parameters responsible for solubilization. Rather, our correlative approach differentiates only among taxa with strong versus weak digestive capabilities. Third, it is possible that mechanisms of solubilization vary among species, so that cross-phyletic correlations will be misleading.

While the strong covariance among digestive parameters confounded our attempts to identify solubilization mechanisms by correlation, it does suggest that a predictive approach could be extended to many other taxa. Our work used only large invertebrates from which we could obtain sufficient fluid for in vitro extraction of contaminants (600–1,000  $\mu$ l). However, the much smaller amounts of fluid needed to quantify digestive biochemicals can be feasibly collected from many more taxa. Because it was possible to predict the extent of contaminant solubilization by gross chemical parameters of digestive intensity, it should be possible to estimate digestive contaminant bioavailability even for those taxa that provide insufficient gut fluid for direct measurement. In particular, it would be interesting to extend this work to the Crustacea, a group of considerable toxicological significance in monitoring and regulatory applications that was not represented among our tested taxa.

Color of digestive fluid alone, in fact, appeared to be a good predictor of contaminant solubilization potential. Those taxa with weak extraction capabilities had fluid that was clear. As solubilization capability increased, color intensified through light amber and finally to dark amber or black. Color correlated with all measures of digestive intensity because of the high covariance among them, but casual observation suggested a particularly strong correlation with surfactancy.

Gut fluids appear to consist of seawater to which secreted digestive agents and hydrolyzed food products have been added [22]. These added organic compounds serve as the solubilizing agents for contaminants. Gut fluids of different taxa vary in the amounts of these added organic compounds, which accounts for most of the variance in contaminant solubilization. The reasons for the differing levels of added organic compounds among the taxa are not well understood. Likely, those species with high levels of extra organic material are engaged in faster digestion than those with lower levels. For example, measurements to date suggest that deposit-feeding annelids tend toward shorter gut residence times than deposit-feeding echinoderms [29]. The higher levels of organic material may also be related to varying fluid dynamics among species. Annelids, for example, apparently retain gut fluids in place during gut solid passage [9], allowing more accumulation of secreted digestive agents and hydrolyzed food concentrations than would be possible if the fluid spent no more time in the gut than the transiting sedimentary solids.

We found a clearer separation among phyla for Zn than for BaP solubilization (compare Fig. 5A vs B). This clearer phylogenetic distinction may be due to a simpler solubilization mechanism for Zn than for BaP. If Zn solubilization is due to amino acid complexation only, then the clear phylogenetic distinctions for amino acid concentrations (Fig. 5B) clearly explain the relatively tight correlations between Zn solubilization and amino acid concentrations, with factors such as level of digestive enzyme secretion or fluid retention thus controlling amino acid concentration and hence Zn solubilization. On the other hand, if multiple solubilizing agents are responsible for BaP solubilization, then distinctions among taxa may blur. For example, while strong differences may exist in globular protein concentrations among taxa (as evidenced by distinctions in amino acid concentrations), much smaller differences may exist in surfactant concentrations among these species (as evidenced by lesser distinctions in contact angles). More mechanistic studies on the nature of solubilizing agents for hydrophobic organic compounds and their concentrations in different taxa will be necessary to understand these cross-phylogenetic trends.

The gut fluids from several species, especially echinoderms and cnidarians, showed no solubilization of either Zn and BaP above that of the seawater control. Presumably the concentrations of solubilizing agents in these gut fluids were too low to draw extra contaminant into solution. To the extent that the seawater control represents the amount of available contaminant that would be predicted by equilibrium partitioning calculations [30], then these species may not show enhanced contaminant bioavailability above that predicted by equilibrium partitioning considerations. In other words, such species may not exhibit enhanced contaminant exposure because of ingestion of sediments. There have been several attempts to quantify the relative importance of ingestion and digestion as a route of contaminant uptake [4,7,31]. Our results indicate that these estimates are likely to be as taxa dependent as our own and generalizable only to other species with similar digestive capabilities.

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