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Ecotoxicological response of marine organisms to inorganic and organic sediment amendments in laboratory exposures

Gunther Rosen, James Leather, Jinjun Kan, Yolanda Meriah Arias-Thode

A S T R A C T

Experimental materials currently being investigated for use as amendments for the in situ remediation of contaminated sediments were assessed for their potential impacts on marine benthos. Laboratory toxicity tests involving lethal and sublethal endpoints were conducted on sediments amended with apatite, organoclay, chitin, or acetate, with the polychaete Neanthes arenaceodentata, the amphipod Eohaustorius estuarius, and the larval sheepshead minnow Cyprinodon variegatus. Amendments were mixed loosely into uncontaminated or metal-contaminated sediments, and also added inside experimental geotextile mats, at sediment dry weight (dw) concentrations ranging from 0.5% to 10%. The geotextile mats, containing apatite (5 or 10% dw), and/or organoclay (5%) did not result in adverse effects on any of the test organisms. Chitin and acetate, however, repetitively resulted in adverse effects on survival and/or adverse or positive effects on organism growth at concentrations of ≤ 2.5% dw. The adverse effects were attributed to water quality degradation in the exposure vessels (notably ammonia and dissolved oxygen concentration, for chitin and acetate, respectively) as a result of the microbial breakdown of the amendments. For N. arenaceodentata, growth was enhanced in the presence of chitin at concentrations as low as 0.5% sediment dw, which stimulated bacterial growth that may have provided an additional food source for the polychaete. Sediment chitin concentrations of 0.5% resulted in a statistically significant reduction in N. arenaceodentata body burdens of 61%, 29%, and 51%, relative to unamended contaminated sediment, for Cu, Zn, and Cd, respectively. The studies suggest a lack of inherent toxicity of these materials on the experimental organisms, as the adverse or positive responses observed are likely related to artifacts associated with laboratory exposure. Assessments in field settings are needed to verify this conclusion.

PUBLISHED BY ELSEVIER INC.

A R T I C L E I N F O

Available online 15 August 2011

Keywords:
Sediment amendment
Marine sediment
Sediment toxicity
Metals

1. Introduction

Heavy metal contamination is a problem in marine and freshwater environments worldwide as a result of various industrial activities. Aquatic sediments tend to be an efficient sink for cationic metals such as cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn). The mobile, soluble forms of these metals are generally considered bioavailable and potentially toxic, as they easily pass through cell walls and can bioaccumulate (Goyer, 1991; Rainbow, 1993), presenting potential for trophic transfer to higher level organisms. Particle-associated metals can also be a significant source of metal uptake in many benthic invertebrates through ingestion of sediment particles (Lee et al., 2000).

Remedies for situations in which unacceptable environmental risk has been established at sediment sites include ex situ approaches (i.e. dredging, or the removal of contaminated sediment from a site), and in situ approaches including monitored natural recovery, and passive or reactive capping (USEPA, 2005). Passive capping is a relatively economical remedy that consists of a covering or cap of clean, inert material (e.g. sand) on top of contaminated sediment to provide a physical barrier that reduces contaminant migration to subsequent deposited sediment and the overlying water column. Passive caps, however, do not completely prevent toxic contaminants from being released due to processes such as leaching and mechanical disturbance, and can lead to substantial alteration of the benthic community due to the required thickness of the caps (Knox et al., 2008). Reactive capping, in contrast, involves the use of capping materials that react with surficial sediment contaminants to reduce their toxicity or bioavailability (Millward et al., 2005; Reible et al., 2006; Knox et al., 2008). Reactive caps, therefore, are designed to provide both a physical barrier and permanent sequestration of sediment-associated contaminants.
A variety of materials show promise for enhancing sequestration of organic and metal contaminants in sediments. Activated carbon, for example, has been shown to be useful for reducing bioavailability of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs; Millward et al., 2005; Cho et al., 2007; Janssen et al., 2010), while metal sequestration has been shown to be viable in freshwater and saltwater environments with natural materials such as apatite (rock phosphate; Ma et al., 1993; Knox et al., 2003; Melton and Gardner, 2004; Knox et al., 2008; Atlas-Thode et al., 2009). Apatites are capable of reacting with heavy metals through both surface sorption to the apatite, and precipitation via the formation of metal phosphates (Fedoroff et al., 1999; Singh et al., 2001; Bailliez et al., 2004), which can form chemically stable and insoluble compounds, particularly at estuarine and saltwater pH levels (Chen et al., 1997). Organoclays (e.g. bentonite) have also been shown to remove both non-polar organic contaminants and metals from water (Alther, 2002; Knox et al., 2008).

While some experimental amendments have been added to sediments to sorb, degrade, transform or immobilize toxins and metals, others are added to stimulate growth of indigenous microorganisms to contribute to these processes (Robinson-Lora and Brennan, 2009). For example, salt marsh and marine bacteria degrade chitin and release organic acids such as acetate (Boyer, 1986; Bassler et al., 1991), which likely serve as substrates for sulfate-reducing bacteria, promoting the precipitation of metals as sulfides, rendering the metals non-bioavailable.

Regardless of the amendment composition, accurate methods for introducing reactive materials to contaminated sediments are still in development. Cho et al. (2007) described the use of a large-scale mixing device suitable for working on tidal mudflats, while Menzie (2009) demonstrated the use of a low-impact agglomerate (Sedimite™) that delivers treatment materials from the water surface. Geotextiles are porous, synthetic fabrics that could enable the accurate placement of a thin layer of highly sorptive media (e.g. activated carbon, apatite, organoclays) in the form of reactive mats at sediment sites (McDonough et al., 2007). These reactive core mats allow for the movement of water and gases through them, and therefore, may be effective in areas of groundwater discharge. They also require less material to stabilize contami-
nants than conventional chemical batch treatment because only mobile pollutants are treated.

It is imperative that materials used for in situ remediation of contaminated sediments are not only effective, but also do not pose additional risk to the benthic or pelagic communities. While Millward et al. (2005) observed significant sequestration of PCBs from sediments treated with activated carbon, they also reported reduced growth of infaunal polychaetes relative to unamended sediments. In contrast, Janssen et al. (2010) reported no adverse effects on growth in similar experiments with the same species. Accordingly, this paper focuses on the assessment of the potential for marine benthic community effects of several materials currently being considered for use in reactive caps. A series of laboratory toxicity experiments with multiple species and biotic endpoints, and a single bioaccumulation study were conducted. Because of their critical roles in these processes, bacterial communities from overlying water and sediments were also characterized from these bioassays, and are presented in greater detail in a companion to this paper (Kan et al., this issue).

2. Materials and methods

Three series of exposures were conducted (Table 1). The first exposure series examined initial toxicity assessment of amendments to three different marine test species following addition to uncontaminated sediment. The second set of exposures addressed water quality-related toxicity that was observed for two of the amendments in the first series of experiments. The third set of experiments used the results of the first and second exposure series to amended sediments were then added to the final laboratory study to assess any differences in toxicity and uptake from a metals-contaminated field sediment amended with chitin or apatite.

2.1. Exposure series #1: toxicity in uncontaminated sediment

2.1.1. Amendments used

Four different materials were investigated, either singly or in combination: Phosfill apatite (rock phosphate mined from North Carolina), PM-199 organoclay (a proprietary granular clay compound marketed by Cetco Remediation Technolo-
gies, Hoffman Estates, IL, USA), chitin (from crab shells, practical grade, coarse flakes, Sigma Aldrich, Product #C9213, CAS#1398–61–4), and anhydrous sodium acetate (~99.9% solid, Sigma Aldrich, CAS#127-09-3). Amendments were either mixed directly into sediment (see Section 2.1.2, Sediment preparation) or housed inside reactive core geotextile mat samples (Cetco) that were placed beneath a 3 cm layer of uncontaminated sandy sediment from San Diego Bay, CA, to simulate subsequent application of a thin layer cap.

Amendment concentrations were selected based on the range used in recent laboratory and field studies for various types of amendments (Ma et al., 1995; Millward et al., 2005; Cho et al., 2007). The reactive mats were similar to those used by McDonough et al. (2007), but were fabricated into circular shapes with a 3" diameter to accommodate the toxicity exposure chambers. The mat core was made from a high-loft polypropylene fiber that was needle-punched into a polypropylene woven geotextile. The high-loft fibers had an opening size of 0.85 mm (#20 mesh). The top of the mat was made from a nonwoven polypro-
pylene geotextile with similar pore size (~80 μm). Once constructed, the fabricated mats contained apatite and/or organoclay that reflected dry weight sediment concentrations shown in Table 1.

2.1.2. Sediment preparation

Amendments were mixed into sediment collected from a relatively uncontami-
nated site (3B2441; lat 32.69129, long ~117.23803) located near the mouth of San Diego Bay (SD; CA). Physico-chemical characteristics of this sediment and bulk metal concentrations are shown in Table 2. Sediment was pressed by hand (without dilution or loss of pore water) through a 2 mm sieve to remove indigenous organisms and large particles prior to use.

For the loosely mixed amendments, the appropriate amounts of sediment and amendment were added to one gallon glass jars, and initially mixed with an impeller mixer attached to a drill motor for 30 min. Following the initial mixing period, all jars were placed on a roll jar mill (US Stoneware, East Palestine, OH, USA) for 48 h for further homogenization. Amended sediments were then added to pre-cleaned 10L glass mason jars, which served as the toxicity exposure vessels. The reactive core mats were placed in uncontaminated flowing filtered seawater for 24 h prior to addition to exposure jars. Mats were placed on the bottom of the jars with the nonwoven side up. This was followed by the addition of ~3 cm of SD control sediment.

2.1.3. Toxicity exposures

Several lethal and sublethal marine toxicity endpoints were employed: 28-day polychaete (Neanthes arenerodentata) survival and growth (ASTM, 2000); 10-day amphipod (Ehrensteinia stauri) survival (USEPA, 1994a); and 7-day larval sheephead minnow (Cyprinodon variegatus) survival and growth (USEPA, 1994b). The different test species represent different trophic levels and potential routes of contaminant exposure, with N. arenorodentata being a tube building surface deposit feeder, E. stauri a free-burrowing deposit feeder, and larval C. variegatus a vertebrate species that forages in surficial sediments.
For each treatment and test species, 150 g of sediment (a depth of approximately 3 cm) was added to each of five replicate beakers. A sixth replicate was used for daily water quality (including pore water at test termination) measurements. Approximately 750 mL of uncontaminated, filtered (0.45 μm) natural seawater (adjusted to 30 psu) collected from near the mouth of San Diego Bay was added to each jar, followed by a 3-day equilibration period prior to addition of test organisms. All beakers were gently and continuously aerated at a rate of ~100 bubbles/min.

Two controls were used for each test type, one consisting of unamended San Diego Bay sediment (SD Control), and the other consisting of uncontaminated sandy sediment collected from the amphipod collection site near Yaquina Bay, OR, USA (YB Control). The latter was used to verify test acceptability (i.e. test organism health), while the former was used to assess any inherent toxicity associated with the San Diego Bay sediment, and to make statistical comparisons to the treatments with amendments.

Aqueous only reference toxicant tests using either copper or cadmium were conducted alongside all exposures and compared to laboratory control charts for batch sensitivity assessment for all bioassays.

### Table 2

Characteristics of unamended test sediments. ERM = Effects range median (Long et al., 1995). WQC = US EPA Water Quality Criterion for Saltwater (acute/chronic values). No value indicates parameter not measured. Reliable detection limit for bulk sediment metals analyzed using XRF is 50 μg/g dry weight.

<table>
<thead>
<tr>
<th>Sediment Type</th>
<th>Bulk Sediment (μg/g)</th>
<th>Pore Water (μg/L)</th>
<th>ERM (μg/g)</th>
<th>WQC (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yaquina Bay (YB)</td>
<td></td>
<td></td>
<td>7.9</td>
<td>4.8</td>
</tr>
<tr>
<td>San Diego Bay (SD)</td>
<td>2.5</td>
<td>18.5</td>
<td>0.77</td>
<td>4.14</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.90</td>
<td>&lt; 50</td>
<td>450</td>
</tr>
<tr>
<td>Mare Island (MI)</td>
<td>154</td>
<td>410</td>
<td>370</td>
<td>1100</td>
</tr>
</tbody>
</table>

2.1.3.1. Polychaete survival and growth. *N. arenaceodentata* survival and growth were assessed in 28-day static-renewal exposures (ASTM, 2000) at a temperature of 20 °C. Two-week old post emergent juveniles were obtained from lab cultures held by Dr. Don Reish (California State University Long Beach, Long Beach, CA, USA) and acclimated to test conditions at SSC Pacific for 3–5 days prior to exposure. Each replicate beaker received five worms at exposure initiation. The overlying water was not renewed during the exposure period. Upon termination of the exposure, beaker contents were sieved using a 1 mm stainless steel screen, and surviving amphipods enumerated. For each treatment and test species, 150 g of sediment (a depth of approximately 3 cm) was added to each of five replicate beakers. A sixth replicate was used for daily water quality (including pore water at test termination) measurements. Approximately 750 mL of uncontaminated, filtered (0.45 μm) natural seawater (adjusted to 30 psu) collected from near the mouth of San Diego Bay was added to each jar, followed by a 3-day equilibration period prior to addition of test organisms. All beakers were gently and continuously aerated at a rate of ~100 bubbles/min.

Two controls were used for each test type, one consisting of unamended San Diego Bay sediment (SD Control), and the other consisting of uncontaminated sandy sediment collected from the amphipod collection site near Yaquina Bay, OR, USA (YB Control). The latter was used to verify test acceptability (i.e. test organism health), while the former was used to assess any inherent toxicity associated with the San Diego Bay sediment, and to make statistical comparisons to the treatments with amendments.

Aqueous only reference toxicant tests using either copper or cadmium were conducted alongside all exposures and compared to laboratory control charts for batch sensitivity assessment for all bioassays.

2.1.3.2. Amphipod survival. *E. estuarius* survival tests were conducted following standard protocols (USEPA, 1994a) with 3–5 mm length field-collected amphipods (Yaquina Bay, Oregon, USA). Amphipods were acclimated to test conditions over 2.1 days for growth or biomass determination based on dry weight.

2.1.3.3. Sheephead minnow survival and growth. *C. variegatus* experiments followed guidance for chronic survival and growth testing with this species for whole effluent toxicity testing (USEPA, 1994b), with the exception that fish were exposed in sediment–water interface instead of water only exposures. Early life stages of *C. variegatus* are negatively buoyant, tolerant of low dissolved oxygen, and tend to dig into sediment to hide from predators or to seek refuge from particularly warm or cold water (Sakowicz, 2003). Ten, 7-day old larvae were exposed in each replicate at a temperature of 20 °C. Fish were fed twice daily with freshly hatched Artemia nauplii. Fish were recovered from the exposure beaker by gently swirling the beaker contents and pouring over a 1 mm screen, rinsed with clean seawater, survivors enumerated, and rapidly euthanized by severing the spinal cord with a scalpel. They were subsequently rinsed in deionized water to remove salts and placed in pre-weighed aluminum weigh boats. Following drying for 24 h at 60 °C, fish were weighed to the nearest 0.00001 g on a Sartorius Model 1712 balance.

2.2. Exposure series #2; dose-response experiments with chitin and acetate

Due to issues observed with water quality (ammonia and dissolved oxygen [D.O.] for chitin and acetate, respectively) during some Series #1 exposures, a second set of experiments was conducted with *E. estuarius* to confirm suspected causes of observed toxicity as well as to optimize the exposure concentration range for subsequent experiments (Exposure Series #3).

Chitin and acetate were separately mixed directly into SD sediment in four different concentrations (Table 1) using the same sediment preparation procedures and test conditions described in Section 2.1. Chitin was tested at 15 °C only, while acetate experiments were conducted at both 15 and 20 °C. The addition of the higher temperature was made to help interpret the adverse effects observed in the Series #1 results with *N. arenaceodentata* (exposed at 20 °C) with acetate, which was suspected to be associated with higher biological oxygen demand (BOD) at the higher temperature.

2.3. Exposure series #3: *N. arenaceodentata* exposures with field-contaminated sediments

Amendments in uncontaminated sediments were not expected to result in toxicity. Therefore, field sediments naturally contaminated with relatively high metals concentrations were used in this experiment to help evaluate the potential for adverse effects on marine benthos. This experiment was conducted with *N. arenaceodentata* based on its relative sensitivity to some of the amendments in Exposure Series #1 and #2. Sediment was collected from Green Sands Beach at Mare Island Naval Shipyard (lat 38.086, long -122.255) and selected based on historically high bulk sediment metal concentrations (Table 2). Exposures were conducted as described for other experiments with *N. arenaceodentata*, differing only in that only YB control sediment was used, and that surviving worms were assayed for whole body metal concentration by inductively coupled plasma mass spectrometry (ICP-MS). This involved purging the gut of the exposed worms in clean seawater overnight, followed by wet weight determination. Worms were dried in 2 mL polypropylene microcentrifuge tubes to constant weight at 60 °C (2 days) in preparation for nitric acid digestion and subsequent analysis (Rosen et al., 2008).

2.4. Water and sediment quality measurements

Overlying water quality (pH, D.O., salinity, temperature) was recorded daily in one surrogate chamber associated with each treatment. Ammonia was measured in both the overlying water and pore water at test initiation and test termination for each bioassay using an ammonia salicylate method (Method 10031, Hach Company, Loveland, CO) with a Hach DR/2400 spectrophotometer. Pore water was collected from the test chambers by decanting the overlying water of the surrogate beakers, and subsequently centrifuging a portion of the remaining sediment at approximately 4000 rpm for 20 min. Unionized ammonia was calculated based on the pH, salinity, and temperature of the overlying water and pore water samples (USEPA, 1989).
2.5. Sediment, pore water, and tissue sampling and analysis

Bulk sediment metals concentrations were measured using X-Ray Fluorescence (XRF) following EPA Method 6200. Samples were dried and 10 g of dry sediment was analyzed by XRF for As, Cd, Cr, Cu, Ni, Pb, and Zn. Pore water metal concentrations were determined following centrifugation of the bulk sediments using ICP-MS according to procedures described by Bufflap and Allen (1995). Following bioassays, overlying water from one surrogate chamber was siphoned off and placed in an anaerobic chamber where the wet sediment was sealed in a centrifuge tube and then removed from the chamber and spun at 4000 rpm for 30 min. Centrifuge tubes were returned to the anaerobic chamber where pore water was recovered and filtered (0.45 μm) before being acidified (below pH of 2), followed by analysis for metals listed above.

After weighing, dried worms underwent nitric acid digestion and whole body metals concentrations were determined using ICP-MS following procedures described in Rosen et al. (2008).

2.6. Data analysis

Comparisons between results for amendments and SD controls were made using t-tests (a = 0.05), following arcsine square root transformation of data associated with the specific endpoint. Where relevant, no-observable-effects-concentrations (NOEC) and lowest-observable-effects concentrations (LOEC), as well as median lethal (LC50) or effects (EC50) concentrations were calculated with the assistance of ToxCalc 5.0 (Tidepool Scientific). One-way analysis of variance (ANOVA) was used to make statistical comparisons among treatment effects for several of the toxicity endpoints and for polychaete body burden, with Tukey’s test used for making pairwise comparisons.

3. Results

3.1. Exposure series #1: toxicity in uncontaminated sediment

Results from the three different toxicity tests for each amendment type or combination are summarized in Table 3. Control performance was high, ranging from 89–100% in both the YB and SD sediments for all endpoints. No significant effects were observed relative to the SD control for either the reactive mats by themselves or reactive mats containing apatite and/or organoclay (P > 0.05). Similarly, loosely-mixed apatite and organoclay resulted in no adverse effects whether added individually or in combination to sediment for any endpoint (P > 0.05). Statistically significant reductions in E. estuarius survival and C. variegatus growth were observed in the presence of chitin, regardless of whether exposed singly or in combination with apatite (P < 0.05; Table 3), with the largest effects being observed for E. estuarius survival. Mean C. variegatus survival was also reduced in the presence of chitin, but this difference was not statistically significant.

In contrast, N. arenaceodentata biomass was significantly enhanced in the presence of chitin, by a factor of ~2, relative to the SD control (Table 3). Although not statistically significant (P > 0.05), N. arenaceodentata biomass in the presence of acetate was higher than the SD control by a factor of ~1.5. Unlike organoclay or apatite-amended sediments, the chitin and acetate treatments rapidly induced prominent bacterial blooms, which resulted in marked coloration changes of the overlying water paper (Kan et al., this issue).

N. arenaceodentata survival was statistically lower than the control only in the acetate treatment (P < 0.05; Table 3). Results were rather variable among replicates, however, with very low D.O. concentration occurring at times in individual beakers exhibiting reduced survival.

3.1.1. Water quality reduction

Unionized ammonia concentrations in exposure beaker overlying water and pore water are summarized in Table 4. Ammonia concentration was relatively low and similar to the SD control treatment for most amendments, but was consistently elevated in chitin treatments, sometimes to concentrations that approached or exceeded known toxicity thresholds for the various endpoints (Table 4; USEPA, 1989; Dillon et al., 1993; Kohn et al., 1994; USEPA, 1994a, 1994b).

In contrast to chitin, acetate routinely resulted in lower ammonia concentrations than those measured in the SD control (Table 4). The D.O. concentration in the presence of acetate, was also typically lower than most other treatments during the first two weeks of the N. arenaceodentata exposure (Table 4), and dipped below that tolerated by the polychaetes (~ < 1 mg/L; Dillon et al., 1993) on Day 4 of the exposure. When chambers were renewed on this day, dead polychaetes in the acetate treatment were observed on the sediment surface.

Differences in other physical parameters, including pH (8.0 ± 0.1), temperature (15.2 ± 0.3°C for E. estuarius, 20.2 ± 0.3°C for N. arenaceodentata and C. variegatus), and salinity (30.5 ± 0.6 psu), were negligible among treatments.

Table 3

Summary of toxicity testing results from Series #1 exposures involving reference site sediments (SD control) amended with various materials either contained in reactive core mats or mixed loosely in the sediments. The YB control is the collection site sediment for Eohaustorius estuarius. Values are means (± 1 s.d.). N=5 replicates per treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>E. estuarius Survival (%)</th>
<th>C. variegatus Survival (%)</th>
<th>Biomass (mg)</th>
<th>N. arenaceodentata Survival (%)</th>
<th>Biomass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YB control</td>
<td>96 (4.2)</td>
<td>98 (5.0)</td>
<td>0.777 (0.083)</td>
<td>96 (8.9)</td>
<td>24.3 (3.63)</td>
</tr>
<tr>
<td>SD control</td>
<td>89 (9.6)</td>
<td>100 (0)</td>
<td>0.666 (0.086)</td>
<td>100 (0)</td>
<td>24.9 (4.86)</td>
</tr>
<tr>
<td>Mat</td>
<td>94 (4.2)</td>
<td>100 (0)</td>
<td>0.676 (0.180)</td>
<td>92 (11)</td>
<td>28.8 (5.99)</td>
</tr>
<tr>
<td>Mat–Ap</td>
<td>94 (8.2)</td>
<td>100 (0)</td>
<td>0.740 (0.040)</td>
<td>96 (8.9)</td>
<td>26.8 (4.35)</td>
</tr>
<tr>
<td>Mat–Ap–O</td>
<td>88 (7.6)</td>
<td>100 (0)</td>
<td>0.607 (0.152)</td>
<td>100 (0)</td>
<td>28.9 (0.74)</td>
</tr>
<tr>
<td>Ap</td>
<td>92 (5.7)</td>
<td>100 (0)</td>
<td>0.773 (0.105)</td>
<td>100 (0)</td>
<td>29.2 (2.67)</td>
</tr>
<tr>
<td>O</td>
<td>90 (9.4)</td>
<td>100 (0)</td>
<td>0.697 (0.081)</td>
<td>96 (8.9)</td>
<td>25.3 (3.35)</td>
</tr>
<tr>
<td>Ap–O</td>
<td>95 (3.5)</td>
<td>100 (0)</td>
<td>0.720 (0.058)</td>
<td>100 (0)</td>
<td>27.4 (4.38)</td>
</tr>
<tr>
<td>Ac</td>
<td>86 (7.5)</td>
<td>50 (49)</td>
<td>0.467 (0.054)</td>
<td>50 (49)</td>
<td>39.0 (7.88)</td>
</tr>
<tr>
<td>Ch</td>
<td>12 (8.4)*</td>
<td>65 (41)</td>
<td>0.407 (0.168)*</td>
<td>100 (0)</td>
<td>52.0 (12.9)*</td>
</tr>
<tr>
<td>Ch–Ap</td>
<td>27 (21.7)*</td>
<td>48 (35)</td>
<td>0.401 (0.066)*</td>
<td>100 (0)</td>
<td>47.9 (8.30)*</td>
</tr>
</tbody>
</table>

* Indicates statistically different from SD control using unequal variance t-tests (a = 0.05).

3.2. Exposure series #2: dose-response chitin and acetate experiments

3.2.1. Chitin experiment

Ammonia concentration in the overlying water was positively correlated with increasing chitin concentration added to SD sediment (Fig. 1; r²=0.981). A dose-response was also observed with increasing chitin and corresponding ammonia concentrations, with statistically significant (P < 0.05) partial mortality and complete mortality occurring at chitin concentrations of 1% and 2.5%, respectively (Fig. 2). Toxicity metrics including NOEC, LOEC, and LC50s from the dose-response experiment with chitin are shown in Table 5. Unionized ammonia concentrations in the overlying water exceeded the 0.8 mg/L NOEC for E. estuarius in the 2.5% treatment (0.91 mg/L), and approached the NOEC in the 1% treatment (0.65 mg/L). Although pore water ammonia concentrations were not measured in this experiment, concentrations in the pore water in Exposure Series #1 were two-fold higher than that of the overlying water, suggesting that pore water concentrations in both of these treatments would have been toxic to E. estuarius.
3.2.2. Acetate experiments

Summary toxicity metrics (NOEC, LOEC, LC50) for the multiple concentration acetate experiments are shown in Table 5. The D.O. concentrations remained within the acceptable range (44 mg/L; USEPA, 1994a, 1994b) in the 15°C treatments through Day 7, but rapidly declined towards critical levels (1 mg/L; Dillon et al., 1993) at all concentrations greater than 0.5% acetate (Fig. 3). In the 20°C experiment, D.O. rapidly dropped to concentrations well below 4 mg/L in the toxicity test prior to organism addition, recovered to 6.6 mg/L, then steadily dropped to below 1 mg/L by Day 7, where it remained for all treatments. An exception was one replicate from the 1% treatment, which had relatively high D.O. after Day 7 (6.8 mg/L) and also had high E. estuarius survival (85%), while the other replicates resulted in no surviving amphipods.

### Table 4

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* USEPA (1994a, 1994b), for survival.
* Kohn et al. (1994).
* Dillon et al. (1993). NH3 NOEC = growth; LC50 = survival. D.O., NOEC = survival.

### Figure 1

Relationship between sediment chitin concentration and unionized ammonia concentration measured in the overlying water following 10-day toxicity exposures with amphipods (E. estuarius). N=5 for toxicity exposure, N=1 for ammonia measurement.

### Figure 2

Relationship between unionized ammonia concentration measured in overlying water and amphipod (Eohaustorius estuarius) survival following 10-day multiple concentration chitin exposure conducted in San Diego Bay sediment. Percentages refer to chitin sediment concentration (dry wt.). N=5 for toxicity exposure, N=1 for ammonia measurement.

### 3.3. Exposure series #3: N. arenaceodentata toxicity and bioaccumulation in field-contaminated sediments treated with selected amendments

Survival of N. arenaceodentata was high in all treatments (range = 92–100%; Fig. 4) and was not statistically lower for either the MI sediment nor any amendment or amendment combination in MI sediment relative to the control (P > 0.05). The relatively low (0.5%) chitin concentration used in both the MI-Ch, MI-ApCh treatments, however, resulted in statistically higher growth, by a factor of ~1.6 relative to unamended MI sediment (one-way ANOVA, Tukey's HSD test, p < 0.05; Fig. 4). Visual examination of the overlying water indicated substantial bacteria growth in both treatments containing chitin (verified in Kan et al. (this issue)).
N. arenaceodentata body burdens for Cu, Zn, Cd, and As were higher in MI sediment, sometimes substantially (e.g. factor of 6 for Cu), relative to the YB control sediment used in this experiment (Fig. 5). Apatite alone did not result in reduced body burdens for any of the four metals relative to the unamended MI sediment. In fact, Cd body burdens were statistically higher (Tukey’s HSD test, p < 0.05) in the MI-Ap treatment relative to both the control sediment and unamended MI sediment. When MI sediment was amended with 0.5% chitin or 0.5% chitin combined with 5% apatite, however, body burdens were reduced by 61–63%, 29–32%, and 54–55%, for Cu, Zn, and Cd, respectively. A 57% increase in tissue As, however, was observed in the presence of chitin, relative to unamended MI sediment (Fig. 5).

4. Discussion

4.1. Lack of toxicity associated with apatite and organoclay

Neither apatite nor organoclay resulted in statistically significant effects for any of the test endpoints evaluated, whether loosely mixed or housed in reactive core mats. Therefore, it is expected that these materials should not have negative impacts on natural benthic communities at the concentrations employed in this study. Lack of toxicity of North Carolina apatite and organoclay was also observed by Paller and Knox (2010) in laboratory exposures to freshwater and brackish sediment-dwelling invertebrates at higher concentrations (25–100%) than those used in this study. Rather, North Carolina apatite has been shown to very effectively sequester metals, particularly Cu and Zn, in both fresh and saltwater (Knox et al., 2008), reducing the bioavailability of potentially toxic metals. In marine sediments, it is expected that phosphate-solubilizing bacteria will effectively dissolve the inorganic apatite ($\text{CaPO}_4 \cdot \text{H}_2\text{O}$) and enhance the precipitation of metals in solution by forming insoluble metal phosphates, resulting in permanent sequestration (Schulz and Schulz, 2005).

It should be noted that it is probable that the test organisms used in this study had relatively little direct contact with the...
contents of the reactive core mats due to their presence 3 cm below the uncontaminated sediment layer. N. arenaceodentata, however, were observed, in some cases, clinging to the exterior of the mats themselves upon recovery from the vessels, suggesting that there was no toxicity or avoidance response associated with the geotextiles. The presence of increased bacterial numbers in the overlying water in treatments containing loosely-mixed apatite and organoclay relative to treatments where mats held the apatite and organoclay (Kan et al., this issue) is suggestive of lesser exchange of water between the amendments and the sediment–water interface in the geotextile mats under this exposure system. It is unclear, however, whether or not the difference in water exchange between loosely mixed and geotextile mat-contained amendments containing apatite and/or organoclay would be observed in the field without conducting field studies.

4.2. Water quality-related effects with chitin and acetate

Unlike apatite and organoclay, lethal and/or sublethal effects were observed for all three species in treatments where organic amendments (acetate or chitin) were present. Treatments in which effects (deleterious or positive) were observed, however, were also associated with enhanced microbial activity (Kan et al., this issue) that appears to be indirectly responsible for toxicity via a reduction in water quality (i.e. elevated ammonia and reduced D.O. concentrations), as verified by subsequent dose-response experiments.

Chitin. The substantially elevated ammonia concentration in chitin amended sediment isn’t surprising. Ammonia is a normal breakdown product of chitin (Campbell and Williams, 1951; Bassler et al., 1991), a polysaccharide that can serve as a food source for aerobic and some anaerobic bacteria (Osawa and Koga, 1991).
The breakdown of chitin is catalyzed by bacteria-produced enzymes (chitinases), which result in cleaving of the glycosidic bonds and conversion of chitin to simple sugars (such as acetate) and ammonia. The dramatic increases in unionized (the fraction that is generally considered to be the most toxic) ammonia concentration observed in all exposures with chitin explain the observed adverse effects on *E. estuarius* survival. The overlying water and pore water ammonia concentrations measured in the initial chitin experiments, which employed a chitin sediment concentration of 2.5%, were in excess of published thresholds for *E. estuarius* ([USEPA, 1994a, 1994b; Kohn et al., 1994]). The subsequent dose-response chitin experiment conducted with *E. estuarius*, indicate that toxicity was correlated with the ammonia concentration, providing strong evidence that the observed toxicity was a result of the breakdown of chitin and not the chitin itself. However, it should be noted that the 0.5% chitin NOEC derived in this study is specific to the unique physico-chemical characteristics of the SD sediment used and the laboratory conditions used in this study.

In addition to impacts on *E. estuarius* survival, reduced growth of *C. variegatus* for 2.5% chitin treatments corresponded with increased unionized ammonia concentrations (as high as 0.99 mg/L), but ammonia thresholds for *C. variegatus* growth are unknown. The reported unionized ammonia 96-h LC50 for this species is 2.72 mg/L ([USEPA, 1989]), but it is likely that chronic (i.e., growth) effects occur at substantially lower concentrations. For example, *Menidia beryllina* (inland silverside) growth is significantly reduced at concentrations 21 times lower than the 96 h LC50 ([USEPA, 1989]). It is also likely that *C. variegatus* larvae were exposed to a mix of overlying and pore water ammonia concentrations, thus resulting in a greater exposure to ammonia than that reported here. Visual observations indicated that the fish spent considerable time at the sediment–water interface, which is expected due to their negative buoyancy ([Sakowicz, 2003]).

The lack of ammonia-related effects on survival or growth of *N. arenaceodentata* in the presence of chitin is likely due to the lower concentrations detected relative to the static *E. estuarius* exposures (Table 4), the fact that ammonia concentrations associated were only marginally in excess of published thresholds for these endpoints in the overlying water, and, unlike the free-burrowing amphipods that are exposed directly to pore water, *N. arenaceodentata* were likely protected from direct pore water exposure by their mucoid tubes ([Dillon et al., 1993]).

Elevated ammonia concentration is not a unique finding in toxicity tests used to evaluate sediment amendments, and raises concerns with how future assessments of their biological effects should be conducted. Burgess et al. (2009) reported that some types of coal fly ash increased both ammonia concentration and pH in the overlying water of toxicity test chambers to levels in excess of reported ammonia LC50s for the species that were exposed. Although the pH increases were not suspected to directly cause the observed toxicity to amphipods in their study, the increase in unionized ammonia concentration associated with the pH corresponded with mortality of marine invertebrates. Real world deployments might result in improved understanding of how the microbial decay of chitin might affect water quality and subsequent effects on the biota.

**Acetate.** Acetate serves as a carbon source to stimulate indigenous bacterial communities that might be able to reduce metals and immobilize them from contaminated systems ([Istok et al., 2004]). It is also a product from the breakdown of chitin ([Bassler et al., 1991]), and was thus explored in this study as a means of stimulating the growth of sulfate-reducing bacteria, which could in turn reduce metal bioavailability by the creation of metal-sulfides.

Sharp declines in D.O. concentration were repetitively associated with reduced survival of *N. arenaceodentata* and *E. estuarius*, at acetate concentrations greater than 0.5%. Of interest was the observation of high within treatment variability, with some replicates having D.O. concentrations as low as 0 mg/L, while others were near saturation (~8 mg/L). It is suspected that very high biological oxygen demand (BOD) associated with the utilization of acetate by indigenous bacteria as a food source, even in the presence of continuous aeration, resulted in frequent D.O. concentrations below those required to maintain *N. arenaceodentata* and *E. estuarius* survival. Enhanced toxicity to *E. estuarius* at 20 °C, relative to the 15 °C exposures, was expected due to the greater bacteria concentrations at the higher temperature ([Kan et al., this issue]). The more extreme D.O. declines at the higher temperature, particularly early on in the exposure, were likely due to the increased metabolism rate and microbial activities of ambient bacterial groups ([Vinolas et al., 2001]). This helps explain why *N. arenaceodentata*, which was tested at the higher temperature, suffered from mortality in the presence of acetate, while *E. estuarius* (tested at the standard condition of only 15 °C) did not, in Exposure Series #1 at the initial dose rate of 5%.

High BOD and depressed D.O. concentrations from deicing products containing sodium acetate have been reported to cause adverse effects in toxicity tests with rainbow trout, with lethality occurring at aqueous concentrations of 16.1 g/L (Bang and Johnston, 1998). In the present study, initial acetate concentrations (5%) in the water column from *C. variegatus* exposures would have achieved a maximum concentration of 6.6 g/L, but no mortality was observed. It is unclear as to whether or not the reduced growth of *C. variegatus* is due to the presence of acetate in the water column, or due to stress associated with the relatively low D.O. concentrations (5.7 ± 0.9 mg/L) associated with the acetate treatment.

4.3. Polycheate toxicity and bioaccumulation in amended Mare Island sediment

**Growth.** Individual food rations for these experiments were 4 mg ground Tetramin/worm/week, which was below that used in other experiments with *N. arenaceodentata* ([Pesch et al., 1986; Bridges et al., 1997]), presumably resulting in relatively greater exposure to contaminated sediment than in those studies. No toxicity was observed in the *N. arenaceodentata* exposures with MI sediment. Rather, statistically significant bioaccumulation and growth were observed in some treatments. Increased *N. arenaceodentata* growth (factor of ~1.5) was observed in MI contaminated sediment amended with chitin relative to both YB control and unamended MI sediments, while apatite-amended sediment did not affect growth, consistent with observations previously made from exposures with SD sediment. The increased growth with chitin was not surprising considering the added food source that was provided by both waterborne and sediment-dwelling bacteria ([Kan et al., this issue]), as well as the possibility of ingestion of organic matter associated with the chitin, in addition to organic matter associated with the sediment itself. Enhanced growth (~1.5 times control) of *N. arenaceodentata* was also observed in acetate treatments, and although the increase was not statistically significant, this once again is likely due to the added nutrition from feeding on the relatively high bacterial population in this treatment. These results contrast with reduced *N. arenaceodentata* weight in exposures to activated carbon amendments, as observed by Millward et al. (2005). Those authors suggested that ingested organic carbon likely reduced nutrient uptake due to its sorbent properties, however, there was no indication that bacterial growth was induced with activated carbon.

**Body Burden.** While the addition of chitin resulted in increased *N. arenaceodentata* growth, whole body Cu, Zn, and Cd residues were significantly lower in the treatments that contained...
0.5% chitin relative to the unamended MI sediment. Even at the lowest dosing level, chitin treatments resulted in marked bacterial blooms early on in the exposures. The reduced uptake of Cu, Zn, and Cd could be due to several reasons, including metal sorption to the chitin particles or dissolved organic carbon, the formation of insoluble metal-sulfides (or metal phosphate in the case of chitin and apatite combinations), and/or preferential feeding on the microorganisms inhabiting the overlying water column, thus avoiding contaminated sediment ingestion. Yang and Zall (1984) demonstrated effective sorption of Cu, Zn, Cd, Cr, and Pb to chitin, resulting in significant precipitation removal of these metals from aqueous solutions. In sediments, chitin has been shown to stimulate activity of sulfate-reducing bacteria, resulting in metal-sulfides with low bioavailability (Robinson-Lora and Brennan 2009). While sulfides weren’t quantified in these experiments, chitin and chitin/apatite mixtures possessed a particularly strong sulfide odor upon exposure breakdown. It is, therefore possible that, in addition to ammonia, sulfides may have contributed to observed toxicity associated with the exposures that included chitin.

Pore water metal concentrations (data not shown) did not show consistent trends and did not correspond with observed bioaccumulation differences. Lee et al. (2000, 2001) stress that feeding and sediment ingestion, as opposed to pore water, may be the primary uptake pathway for metals for N. arenaceodentata. In addition, N. arenaceodentata, in feeding from its mucoid tube, may have to some extent avoided interaction with the pore water in favor of feeding on bacteria present in the overlying water.

In this study, there was no apparent reduction of Cu, Zn, or Cd tissue concentrations in apatite-only treatments. It is possible that apatite concentrations greater than 5% might be required to reduce metal uptake by marine organisms from contaminated sediments. Although data for apatite effects on bioaccumulation were not found, Faller and Knox (2010) demonstrated that organoclay concentrations of 50% significantly reduced uptake of PAHs in freshwater oligochaetes, while 15% formulations did not. The 5% dosing selected for this study was based on the approximate target doses that have been employed in pilot-scale studies where amendments have been tilled directly into the contaminated sediments (Millward et al., 2005, Cho et al., 2007).

Further examination of the effectiveness of apatite to sequester cationic metals in marine sediments is therefore needed.

Cd body burdens were higher as a result of amendment with apatite, relative to unamended sediment. The concentration of Cd in the presence of apatite, however, was below that reported in another control sediment (Moore and Dillon, 1993). It is possible that elevated uptake of Cd in the presence of apatite could be attributable to uptake of bioavailable Cd impurities reported in the mixed phosphate material (Knox et al., 2006).

5. Conclusions

This study demonstrated that no inherent lethal or sublethal toxicity associated with the inorganic amendments (North Carolina apatite and organoclay) was observed. Adverse effects from the organic amendments (chitin and acetate) were observed, but are likely associated with a microbially-induced decline in water quality. Evaluated sediment concentrations of 5% for apatite and organoclay, whether mixed directly in contaminated sediments or contained in geotextile reactive core mats, are therefore, not expected to pose an environmental risk to marine benthos. The presence of elevated ammonia and depressed dissolved oxygen concentrations in chitin and acetate treatments, respectively, are attributable to bacterially mediated processes in closed systems (i.e. laboratory bioassay chambers). It is unclear from these laboratory experiments, however, whether these organic amendments would react similarly in open marine systems. This conclusion is also valid for the laboratory-associated reduction of copper, zinc, and cadmium (but higher arsenic) body burdens with chitin exposure. Therefore, it is advised that further investigation into the use of these materials as a means of in situ sediment management for marine environments include incorporation of field (in situ) studies.

Acknowledgments

The Strategic Environmental Research and Development Program (SERDP) funded this research under project #ER-1551. The authors thank Jennifer Podegracz, Sarah Douglass, Ryan Halonen, Kyle Miller, Joel Guerrero, Ignacio Rivera, Brandon Swayne, Pat Earley, Anna Obraztsova, and Yanbing Wang for technical support.

References


The authors thank Jennifer Podegracz, Sarah Douglass, Ryan Halonen, Kyle Miller, Joel Guerrero, Ignacio Rivera, Brandon Swayne, Pat Earley, Anna Obraztsova, and Yanbing Wang for technical support.
Marine microbial community response to inorganic and organic sediment amendments in laboratory mesocosms

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ARTICLE INFO

Article history:
Received 8 January 2011
Received in revised form 5 May 2011
Accepted 18 June 2011
Available online 23 July 2011

Keywords:
Bacterial community
Amendments
Marine sediments
Mesocosms

ABSTRACT

Sediment amendments provide promising strategies of enhancing sequestration of heavy metals and degradation of organic contaminants. The impacts of sediment amendments for metal and organic remediation including apatite, organoclay (and apatite and organoclay in geotextile mats), acetate, and chitin on environmental microbial communities in overlying water and sediment profiles are reported here. These experiments were performed concurrent with an ecotoxicity evaluation (data submitted in companion paper) and X-ray absorption spectroscopy of zinc speciation post apatite amendments. X-ray absorption spectra showed that a modest modification of zinc speciation occurred in amended treatments. Significant changes in both bacterial cell densities and populations were observed in response to amendments of apatite–organoclay, chitin, and acetate. The enriched bacteria and breakdown of these amendments were likely attributed to water quality degradation (e.g. ammonia and dissolved oxygen). Molecular fingerprints of bacterial communities by denaturant gradient gel electrophoresis (DGGE) showed that distinct bacterial populations occurred in overlying waters from different amendments: apatite–organoclay led to the dominance of Gammaproteobacteria, acetate enriched Alphaproteobacteria, and chitin treatment led to a dominance of Bacteroidetes and Alphaproteobacteria. In amended sediments, Firmicutes, Bacteroidetes, and Deltaproteobacteria (Desulfovibrio) were commonly found with chitin and apatite–chitin treatments. Finally, sulfate-reducing bacteria (e.g. Desulfovibrio) and metal-reducing bacteria were also recovered with most probable number (MPN) analyses in treatments with acetate, chitin, and apatite–chitin. These geochemically important bacteria were stimulated by amendments and may play critical functional roles in the metal and organic contaminant remediation process for future investigations of contaminated sediments.

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1. Introduction

Many contaminated sites of concern may be contaminated with both metals and organic contaminants. While sediment dredging and disposal remain important components of current contaminated sediment managements, the use of amendments in situ has been proven to be a potential approach to enhance removal of metal and/or organic contaminants from soil, groundwater, and sediments (Anderson et al., 2003; Brown et al., 2004; Istok et al., 2004; Melton and Gardner, 2004; Seager and Gardner, 2005; Werth et al., 2005; Cho et al. 2009). Currently, promising sediment amendments include inorganic (e.g. activated carbon, apatite, organoclay, and geotextile mats containing apatite and organoclay) and organic materials, some of which are also nutrients (e.g. short chain fatty acids and chitin). Organoclay and activated carbon reduced the availability of organic contaminants such as phenols, PCBs, etc. (Mortland et al., 1986; Cho et al., 2009) while phosphates (apatite) helped to immobilize several toxic metals (Ma et al., 1995; Melton and Gardner, 2004; Cao et al., 2009; Paller and Knox, 2010). Geotextiles are porous, synthetic fabrics that could enable the accurate placement of a thin layer of highly sorptive media (i.e., activated carbon, apatite, and organoclay) in the form of reactive mats at sediment sites (McDonough et al., 2007).

Because bacteria are known to influence sediment geochemistry, or assist in the decomposition of many organic contaminants, some researchers have attempted to stimulate indigenous
bacteria or specific indigenous bacterial populations. Acetate and chitin have been applied for the bioreduction of petroleum hydrocarbon (Kleikemper et al., 2002), degradation of trichloroethylene (Werth et al., 2005), and groundwater dechlorination (Ver a et al., 2001). Acetate has been used often for the bioremediation of metals (Anderson et al., 2003; Istok et al., 2004; Chang et al., 2005; Lukas and Hollibaugh, 2001; Lear et al., 2007). Chitin has been used in a few instances for the removal of metals in aqueous solutions (Benguella and Benaissa, 2002; Zhou et al., 2005).

Inorganic amendments mainly involve physical and chemical processes including absorption, adsorption, and transformation. In marine sediments, transition metals form relatively stable insoluble complexes with hydrogen sulfide (HS⁻) and carbonate, and/or are sequestered with phosphates and form more stable metal–phosphate complexes, and thus decrease the bioavailability of these metals in natural environments (Brown et al., 2004). Efficient metal immobilization using phosphate relies on increasing the solubility of the phosphate. This process is likely facilitated by phosphate solubilizing bacteria (PSB), which solubilizes phosphate from the undissolved apatite fractions to sequester bioavailable metals. PSB have been extensively studied for agricultural purposes in freshwater systems (reviewed by Rodriguez and Fraga (1999)), but very few studies have examined the microbial capacity for phosphate solubilization in marine systems (Ayyakkannu and Chandramohan, 1971). One reason for this is that marine systems (in general) are not phosphate limited. Recently, remarkable percentages of isolated attached and free-living marine bacteria have been shown to be able to solubilize phosphate compounds with glucose as carbon source (Uzair and Ahmed, 2007). Therefore, we hypothesize that amendment of apatite along with organic carbon source will induce growth of PSB, and subsequently enhance phosphate solubilization and metal immobilization in marine environments.

In contrast to inorganic amendments, organic amendments such as acetate and chitin serve food sources for many microorganisms. Acetate has been used to induce indigenous microbes capable of bioreduction (use metals as electron acceptors) and/or biologically mediated immobilization (e.g. uptake, absorption, etc.) of toxic metals (Anderson et al., 2003; Istok et al., 2004; Chang et al., 2005; Lukas and Hollibaugh, 2001; Lear et al., 2007). Diverse groups of microorganisms have shown interactions with metals and lead to decreased metal solubility and mobility (Brierley, 1990; Tebo, 1995). For instance, iron reducing bacteria (FeRB) and sulfate-reducing bacteria (SRB) have been noted for their capabilities in metal precipitation and immobilization, primarily due to their metabolic end-products, such as Fe(II) and sulfide (Lovley, 1993; Barnes et al., 1994; Nealson, 1997; Barton and Fauque 2009). So far, FeRB and SRB have been proven responsible for reduction of many metals including uranium, chromium, manganese, iron, selenate, and arsenate (Tebo and Obraztsova, 1998; Arias and Tebo, 2003a; Anderson et al., 2003; Istok et al., 2004; Chang et al., 2005; Lear et al., 2007). In addition, other bacterial groups, such as Bacillus sp. and Streptomyces sp. have also been applied in the field and significantly reduced cadmium potentially available for plants (Jezequel and Lebeau, 2008).

Furthermore, in an acetate and selenate amendment experiment, Lukas and Hollibaugh (2001) showed even broader phylogenetic bacterial groups responded to added acetate and were responsible for selenate reduction. Thus, indigenous bacteria and enriched bacterial groups may play key roles in the mobilization and immobilization of metals.

Despite the potential importance of the usage of sediment amendments in natural marine systems, there have been few concurrent systematic studies of typical sediment amendments used in marine remediation on the combined responses of the marine invertebrate benthic community, microbial population structures along a horizontal gradient, and corresponding metal speciation to the various amendments. Therefore, the aims of this study were to evaluate the following: (1) the risk or harm to the environment and ecotoxicological effects on macro-invertebrates (Rosen et al., companion submission); (2) what groups of bacteria are enriched through the amendments, and how they potentially influence metal solubility/ecotoxicity; (3) how the amendments affect the metal speciation under natural environments. San Diego Bay reference sediments (Table 2) were used to study the impacts of inorganic (geotextile mats, apatite, and organoclay) and organic (acetate, chitin) amendments on cell densities and population structures of bacterial communities in mesocosm experiments. Planktonic and sedimentary microorganisms play central roles in water quality and also, bioremediation. Therefore, bacterial communities were monitored in both overlying waters and in sediments. X-ray absorption spectra were used to examine the metal speciation in San Diego Bay reference sediments under amendment treatments. The information obtained from this current study provides necessary information to aid in decisions for the usage of sediment amendments as remediation strategies in contaminated sediments.

## 2. Materials and methods

### 2.1. Amendments and amendment concentrations used

<table>
<thead>
<tr>
<th>Treatment ID</th>
<th>Treatment description</th>
<th>Amendment concentration (% sed. wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Unamended control</td>
<td>-</td>
</tr>
<tr>
<td>Mat</td>
<td>Mat only</td>
<td>-</td>
</tr>
<tr>
<td>Mat–Ap</td>
<td>Mat + Apatite</td>
<td>10</td>
</tr>
<tr>
<td>Mat–Ap–O</td>
<td>Mat + Apatite + Organoclay</td>
<td>5 + 5</td>
</tr>
<tr>
<td>Ap</td>
<td>Apatite only</td>
<td>5</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetate only</td>
<td>5</td>
</tr>
<tr>
<td>Ch</td>
<td>Chitin only</td>
<td>2.5</td>
</tr>
<tr>
<td>Ap–Ch</td>
<td>Apatite + Chitin</td>
<td>2.5 + 2.5</td>
</tr>
<tr>
<td>O</td>
<td>Organoclay only</td>
<td>5</td>
</tr>
<tr>
<td>Ap–O</td>
<td>Apatite + Organoclay</td>
<td>2.5 + 2.5</td>
</tr>
</tbody>
</table>

Table 1

**Sediment amendments and the concentrations used.**

<table>
<thead>
<tr>
<th>Percentage (μg/g)</th>
<th>San Diego Bay (SD) bulk sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity</td>
<td></td>
</tr>
<tr>
<td>% Silt ( &lt; 62 μm)</td>
<td>33%</td>
</tr>
<tr>
<td>% TOC (%)</td>
<td>0.47</td>
</tr>
<tr>
<td>Cu</td>
<td>45.6</td>
</tr>
<tr>
<td>Zn</td>
<td>132</td>
</tr>
<tr>
<td>Cr</td>
<td>41.8</td>
</tr>
<tr>
<td>As</td>
<td>6.67</td>
</tr>
<tr>
<td>Cd</td>
<td>0.26</td>
</tr>
<tr>
<td>Pb</td>
<td>24.8</td>
</tr>
<tr>
<td>Ni</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Table 2

**Physical and chemical characteristics of San Diego Bay reference sediments. Data provided here from Sample SB2433 in Katz (2007).**
Amendment concentrations were selected based on the range used in recent laboratory and field studies for various types of amendments (Ma et al., 1995; Millward et al., 2005; Cho et al., 2009). The reactive mats were similar to those used by McDonough et al. (2007), but were fabricated into circular shapes with a 3 in. diameter to accommodate the ecotoxicity exposure chambers. The mat core was made from a high-loft polypropylene fiber that was needle-punched into a polypropylene woven geotextile. The high loft fibers had an opening size of 0.85 mm (#20 mesh). The top of the mat was made from a non-woven polypropylene geotextile with similar pore size (~80 μm). Once constructed, the fabricated mats contained apatite and/or organoclay that reflected different dry weight sediment concentrations. The amendment concentrations in the geotextile mats were containing apatite only (10%) and the mats containing organoclay plus apatite (5% of each). The loose amendments concentrations were as follows: apatite in sediment (5%), organoclay in sediment (5%), apatite plus organoclay in sediment (2.5%) each, acetate in sediments (5%), chitin in sediments (2.5%), and chitin plus apatite in sediments (2.5%) each.

2.2. Sediment preparation

Amendments were mixed into sediment collected from a San Diego Bay reference site (Katz, 2007), and physico-chemical characteristics are summarized in Table 2. Sediment was pressed by hand (without disturbance from loss of pore water) through a 2 mm sieve to remove indigenous organisms and large particles prior to use. For the loosely mixed amendments, the appropriate amounts of sediment and amendment were added to 3.8 L (one-gallon) glass jars, and initially mixed with an impeller mixer attached to a drill motor for 30 min. Following the initial mixing period, all jars were placed on a roll jug (US Stoneware, East Palestine, OH, USA) for 48 h for further homogenization. Amended sediments were then added to pre-cleaned, acid-washed, 1 L glass mason jars, which served as the ecotoxicity exposure vessels. The reactive core mats were leached in flowing filtered seawater (20 μm) for 24 h prior to addition to exposure jars. Samples were placed on the bottom of the jars with the non-woven side up. This was followed by the addition of ~3 cm of SD control sediment. The jars were set up in replicates of five and continually aerated (~100 bubbles/min) with filtered air. The experiments were conducted at 20°C (±1°C) with light and dark (18 and 6 h) cycles. Indicators of overlying water quality (pH, dissolved oxygen, ammonia, salinity, and temperature) were monitored daily (see more details in Rosen et al., companion submission).

2.4. Water and sediment quality measurements

Overlying water quality (pH, D.O., salinity, and temperature) was recorded daily in one surrogate chamber associated with each treatment. Ammonia was measured in both the overlying water and pore water at test initiation and test termination using an ammonia salicylate method (Method 10031, Hach Company, Loveland, CO) with a Hach DR/2400 spectrophotometer. Pore water was collected from the test chambers by decanting the overlying water of the surrogate beakers, and subsequently centrifuged a portion of the remaining sediment at approximately 4000 RPM for 20 min. Unionized ammonia was calculated based on the pH, salinity, and temperature of the overlying water and pore water samples (USEPA, 1989).

2.5. Most probable number (MPN) analysis

MPN analyses of overlying water and sediment horizon samples (see Section 2.4) were done by using an epifluorescent microscope (Zeiss, Germany) using 100× Antiflex Neofluo oil objective lens.

2.6. Cell number determination by epifluorescence microscopy

Overlying water samples were stained by SYBR Gold and observed following the protocol described by Chen et al. (2001) for counting microbial cells. Briefly, 0.5 mL of water was fixed with 0.5% of 4 paraformaldehyde for 24 h and filtered onto a 0.2 μm pore-size A250 Anodic 25 mm membrane filter (Whatman) with an approximately 10 kPa vacuum. The membranes were stained with ~2.5 × SYBR Gold solution (final concentration) in the dark. The stained membrane filters were mounted on glass slides and covered with cover slips. The total bacterial cells were observed and counted under blue excitation (485 nm) on a Zeiss Axioplan epifluorescence microscope (Zeiss, Germany) using 100× Antiflex Neofluo oil objective lens.

2.7. DNA extraction, PCR, and DGGE

The overlying water samples were filtered by 0.2 μm filters (47 mm diameter) and DNA was extracted with lysozyme, Proteinase K, SDS concomitant with phenol–chloroform extraction, and isopropanol precipitation as previously described (Kan et al., 2006). Three horizons of sediment samples (0.3 g each) were extracted by UltraCleanTM Soil DNA Kit (MO BIO Laboratories) following the manufacturer’s instructions. DNA concentration was estimated based on 260 nm absorbance using a Spectrophotometer ND-1000 (Nanodrop). PCR amplification was performed in a 50 μl reaction containing approximately 25 ng of template DNA, 25 μl PCR Mastermix (Qiagen), 0.5 mM (each) primer, and water (double distilled). PCR program was performed with a Mastercycler (Eppendorf). PCR primers used were 341F (GC) and 907F and the PCR program followed the protocol described by Scäfer and Muyzer (2002). Agarose gel electrophoresis was used to detect and estimate the size of PCR amplicons. In order to reduce sample numbers, PCR amplicons from replicates of water and sediment were pooled before loading on DGGE. Prior to pooling, however, a DGGE was run on the individual samples to ensure acceptable reproducibility (results not shown). DGGE was performed as previously described (Kan et al., 2006); except the linear denaturant gradient was 40–70% instead of 40–65%, briefly. DGGE was performed using a DCodeTM Universal Mutation Detection System (Bio-Rad) and similar amount of PCR products were loaded on a 1.5 mm-thick vertical polyacrylamide gel with a linear gradient of the denaturants urea and formamide. Electrophoresis was performed at 40°C in 1× TAE buffer, and a voltage of 75 V was applied for 16 h. The DGGE gel was stained with SYBR Gold and photographed (Ihrevais et al., 1997) with a CCD camera mounted on a UV transilluminator (UVP).

2.8. DGGE band sequencing and phylogenetic analysis

Because DGGE is a semi-quantitative approach, only band presence and absence were assessed for comparison and Qualitari software by Gelcompar II (Applied Math). Dominant bands were excised from DGGE gels and incubated in diffusion buffer (0.25 M ammonium acetate, 10 mM magnesium chloride, and 0.1% SDS) at 50°C for 30 min. One μl supernatant was used to reamplify the band. PCR products were purified by ExoSAP-IT (USB) and sequenced with primer 341 f (no GC) using Bigdye-terminator software by ABI PRISM3100 Genetic Analyzer (Applied Biosystems).

All sequences were compared with GenBank database using BLAST, and the closest matched sequences were obtained and included in the downstream phylogenetic analysis. Phylogenetic trees were constructed using MacVector 10.0 software package (MacVector Inc.). Briefly, sequence alignment was performed with the program CLUSTAL W. Evolutionary distances were calculated using Jukes–Cantor method (Jukes and Cantor, 1969) and distance trees were constructed using the neighbor-joining algorithm (Saitou and Nei, 1987). Bootstrap values were obtained based on the analysis of 1000 resampling datasets. Sequences of the partial 16S rRNA genes of representative DGGE bands have been deposited in the GenBank database under accession numbers GU938714–GU938760.

2.9. X-ray absorption spectroscopy (XAS) speciation analysis

Experiments were conducted at the Materials Research Collaborative Access Team’s (MRCAT) beamline 10-ID, Sector 10 located at the Advanced Photon Source (APS), Argonne National Laboratory (ANL), Argonne, IL. The electron storage ring
operated at 7 GeV in top-up mode. A liquid N₂ cooled double crystal Si(1 1 1) monochromator was used to select incident photon energies and a platinum-coated mirror was used for harmonic rejection. The beam energy was calibrated by assigning the first derivative inflection point of the K₂-absorption edge of a zinc metal (9659 eV) foil. The samples were prepared as thin pellets with a hand operated IR pellet press and the samples were secured by Kapton tape. The zinc references were diluted with boron nitride to 1000 mg kg⁻¹ and formed into pellets. Reference materials examined include hopeite (Zn₃(PO₄)₂4H₂O), smithsonite (ZnCO₃), Zn–Al layered double hydroxide with nitrate and silicate interlayers, Zn(OH)₂, ZnO, sphalerite (ZnS), zinc sorbed to ferrihydrite, zinc sulfate, aqueous zinc nitrate, franklinite (ZnFe₂O₄), willemite (ZnSiO₄), hemimorphite (Zn₅Si₄O₁₄(OH)₂H₂O), and galnate (ZnAl₂O₄). Five XAS spectra were collected in fluorescence mode at room temperature from ~200 to 1000 eV relative to the absorption edge position of Zn with a Canberra multielement detector. Thegeo chamber was filled with N₂ while the i détector (for reference materials) contained approximately 60:40 Ar:N₂.

The collected spectra were analyzed using the Athena software program in the computer package IFEFFIT (Ravel and Newville, 2005) for data reduction and data fitting. The five individual spectra for each sample were averaged followed by subtraction of the background through the pre-edge region using the Autobk algorithm and normalized to an atomic absorption of one. The data were converted from energy to photoelectron momentum (k-space) and weighted by k². Identification of zinc phases in the sediment samples was accomplished by principal component analysis (PCA) and linear combination fitting (LCF) of the sediment XAS spectra relative to the known reference spectra.

3. Results

3.1. Stimulation of bacterial growth and microbial cell densities in amendments

Bacterial cell counts were performed on Days 0, 10, and 28 to coincide with the ecotoxicology sampling. Cell counts and fluorescence microscopy observations indicated that among all the treatments, with the exception of those containing acetate, chitin, apatite + chitin, and apatite + organoclay, microbial cell numbers did not change significantly over time (Fig. 1A and B). From Fig. 1B, the bacterial cell densities became stable by day 10 in all the amendments, continuing through day 28.

The acetate treatment stimulated the largest bacterial bloom with the final cell density of ~1.34 × 10⁶ cells/mL, which was 3 orders magnitude higher than the control (2 × 10⁵ cells/mL). Chitin only and apatite + chitin treatments reached the cell density of ~7.56 × 10⁵ cells/mL and ~5.64 × 10⁵ cells/mL, respectively (Fig. 1A). The apatite + organoclay treatment also induced bacterial growth, with a final cell density of 1.76 × 10⁶ cells/mL, slightly less than 10 times the control. In contrast, the cell numbers from other amendments remained similar to the controls, with a final cell density of ~2.6 × 10⁴ cells/mL.

In addition, different treatments stimulated distinct species of microbes based on the morphology as shown in Fig. 1B. In the acetate treatment, most of the microorganisms were rod-shaped single cells, while in chitin and chitin + apatite treatments, multicellular filamentous forms like trichomes were present (Fig. 1B). One interesting difference was observed between chitin only and chitin + apatite treatments: linear multicellular filaments consisting of long rod-shaped cells in chains were observed in chitin only but were not detected in chitin + apatite amended sediment.

3.2. Microbial community in overlying waters

To determine if the sediment amendments had a great effect on an overlying water column (for example, a static water environment such as a holding pond), the bacteria were counted in the overlying water. As expected, the inorganic amendments applied alone in this study had little or no impact on the microbial populations in overlying water. For example, the mat only, dispersed organoclay, and dispersed organoclay + apatite were not significantly different from the control samples. Worthy of note, the inorganic amendments of mat material containing apatite versus dispersed apatite had a few distinct differences. The mat + apatite and apatite only had a common dominant band of Rugeria sp. (Fig. 2, Mat–Ap and Ap, bands 2 and 7) observed in the control. However, the mat + apatite contained a Roseobacter (Figs. 2, band 3) as a second dominant band relative to the Sulfurovibrio (Fig. 2, band 4) observed in the dispersed apatite. The mat + apatite + organoclay (Fig. 2, Mat–Ap–O) differed in that the dominant band was Pseudanthenonas spp. (bands 5 and 6). In the mesocosms with different geotextile amendments, distinct bacterial groups were observed in the water column, indicating that the amendments diffused out of the mat material and were capable of causing an effect on the microorganisms in the water column.
In contrast, the acetate and chitin amendments (Ac and Ch in Fig. 2) shifted the bacterial population structures significantly. For example, in the acetate treatment, two bands commonly found in controls, 16 and 17, disappeared while two new bands, 19 and 20, for uncultured Alphaproteobacteria became dominant. Chitin also introduced significant changes with bands 9, 10, and 11 becoming the dominant bands (Fig. 2). These bands represent Bacteroidetes and two unidentified Alphaproteobacteria. The same three bands were seen when apatite and chitin were used together (Ap–Ch). Combined amendments impacted bacterial communities and induced some bacterial groups to appear or disappear in corresponding treatments (Mat–Ap, Mat–Ap–O, and Ap–O in Fig. 2).

Sequencing of the selected DGGE bands and phylogenetic reconstruction confirmed that amendments stimulated distinct bacterial groups from the controls. The control treatments mainly contained bacterial groups of *Sulfito bacter* sp. (band 17) and *Ruegeria* sp. (bands 16 and 18) (Fig. 3). Besides the shared *Sulfito bacter* and *Ruegeria* groups, *Pseudoalteromonas* and *Halomonas* (Gammaproteobacteria) and *Groseibacter* (Bacteroidetes) were obtained in apatite-organoclay and mat-apatite-organoclay amendments (bands 5, 13, 14, and 15, Figs. 2 and 3). In contrast, acetate only amendment induced Alphaproteobacteria (roseobacter- and bacteroides) (bands 19 and 20), while chitin and apatite-chitin stimulated *Phaeobacter, Roseobacter* (bands 10 and 11), and Bacteroidetes (band 9).

3.3. Bacterial communities in sediments versus overlying water

Due to the significant effects of amendments on microbial population structures within overlying water, we chose acetate only, chitin only, and apatite-chitin treatments to compare the bacterial communities between water and 3 sediment horizon depths. DGGE band patterns indicated that the stimulated bacteria in overlying water and in sediment were similar (Alphaproteobacteria and Bacteroidetes) for acetate amendments (Fig. 4, Ac), but were quite different for chitin and apatite-chitin treatments (Fig. 4, Ch, Ap–Ch). For example, Alphaproteobacteria and Bacteroidetes were dominant in overlying waters from chitin and apatite+chitin. However, Deltaproteobacteria (*Desulfovibrio*, bands 25, 27, and 37), Firmicutes (bands 21, 24, 33, 34, and 36), and Bacteroidetes (bands 29, 30, 40, and 41) were the primary retrieved phylotypes within the 3 sediment horizons analyzed (Figs. 4 and 5).

3.4. MPN analysis

Compared to the control and other treatments, SRB and MRB were significantly increased in all three horizons of sediments amended with acetate, chitin, and apatite-chitin. The MPN analysis confirmed that the three measured sediment horizons (0–1, 1–2, and 2–3 cm) showed a 10–100 fold increase in numbers of MRB and SRB versus the control. SRB and MRB were also recovered in overlying water samples from the treatment with acetate, where the water levels near the sediment/water interface became anaerobic (Fig. 3 in Rosen et al., companion submission) during the experiments.

3.5. X-ray absorption spectroscopy speciation analysis

Fig. 6A showed the XAS spectra of the reference, relatively uncontaminated sandy sediment samples from San Diego Bay, CA, with unamended, apatite, apatite+chitin, and chitin treatments. XAS was focused on metal analysis; therefore, XAS was not performed for the organoclay or geotextile mats amendments. Also, because acetate would not be used in a field due to its expense, but chitin may be used due to its formation of acetate as a by-product (Bassler et al., 1991), chitin was evaluated as a surrogate of an acetate treatment.

The low concentration of Zn in the sediment material is reflected in the relatively low edge step (absorption intensity) (Table 3). Principal component analysis (PCA) identified five suitable components for linear combination fitting (LCF) validity. These components include hopeite (Zn₃(PO₄)₂·4H₂O), smithsonite (ZnCO₃), zinc sorbed to ferrihydrite, franklinite (ZnFe₂O₄), and gahnite (ZnAl₂O₄) (Fig. 6B). The accuracy of LCF results is estimated to be ±10% (Scheckel et al., 2005). However, given the relative concentration of the samples presented here, ±15% accuracy may be a better estimate.

In all cases, the two dominant forms of Zn in the unamended and amended sediments are gahnite and Zn sorbed to an iron oxide (ferrihydrite) (Table 3). With apatite as an amendment, a small portion of the Zn-phosphate mineral, hopeite, was observed. Likewise, the chitin amendment, which demonstrated a positive response to the microbial community, was noted to have an increase in ZnCO₃ species.

4. Discussion

This work was performed alongside a companion paper (Rosen et al., this issue) to address the potential effects of sediment amendments used to decrease organic and inorganic pollutants in sediments on the macro-benthic marine communities. In the Rosen paper, effects such as decreased oxygen levels in the system or increased growth of the polychaete worm were likely caused by the microbiology in the mesocosms. This paper demonstrates how the sediment amendments affected the bacteria, which in turn had an effect on the benthic biota, and the metal speciation under natural environments.

4.1. Apatite amendment and phosphate solubilizing bacteria (PSB)

The inorganic amendments did influence the microbial cell densities or community structures when combined with other
amendments. The chitin + apatite and apatite + organoclay significantly increased the microbial cell densities in water columns (Fig. 1A) and chitin-rich apatite enriched different bacterial groups, including Gammaproteobacteria, Deltaproteobacteria, Firmicutes, and Bacteroidetes in sediments (Figs. 4 and 5). Certain groups of bacteria have been proved to be capable of producing sulfides (e.g., Deltaproteobacteria (Barnes et al., 1994; Barton and Fauque, 2009)) and carbonates (e.g., Gammaproteobacteria and Firmicutes (Rivadeneyra et al., 1994a,b)) that quickly bind bioavailable metals. Under the chitin amendment, the XAS analysis demonstrated an increase in the smithsonite, ZnCO₃ species (Table 3 and Fig. 6), which was also observed by others (Ma et al., 1995; Laperche et al., 1996). The XAS speciation results for Zn in unamended and apatite amended sediments for this study also showed occurrence of mineral Zn-phosphate (hopeite) (Table 3 and Fig. 6), which were in line with previous observations (Scheckel et al., 2011). Metal phosphates are typically more thermodynamically stable than the metal sulfides (Nriagu, 1974), so over time there should be a conversion of transitory metal sulfides to metal phosphates where these mixed amendment systems helped provide a more efficient and permanent metal sequestration.

Recent evidence suggests bacteria such as Beggiatoa sp. and Thiomargarita sp. may promote the precipitation of apatite minerals via the enhancement of phosphate gradients (Schulz and Schulz, 2005). Furthermore, additions of organic materials (as carbon sources) have been shown to enhance the bacterial growth rate and phosphate solubilization (De Souza et al., 2000). Bacterial involvement has been invoked to explain the formation of phosphorites (natural apatite mineral deposits) in both present (Nriagu, 1974) and ancient (Reimers et al., 1990; Leather, 1993) environments. In freshwater systems, strains of Bacillus, Rhizobium, and Pseudomonas have been identified as abundant phosphate solubilizers (Rodriguez and Fraga, 1999). More recently diverse bacterial groups (i.e., Serratia, Shewanella, Escherichia coli, Vibrio, and Proteus) have been shown to be capable of solubilizing phosphate compounds that were previously considered to be insoluble (Uzair and Ahmed, 2007). Some of these groups of bacteria (Pseudomonas, Shewanella, and others (Ma et al., 1995; Laperche et al., 1996). The XAS speciation results for Zn in unamended and apatite amended sediments for this study also showed occurrence of mineral Zn-phosphate (hopeite) (Table 3 and Fig. 6), which were in line with previous observations (Scheckel et al., 2011). Metal phosphates are typically more thermodynamically stable than the metal sulfides (Nriagu, 1974), so over time there should be a conversion of transitory metal sulfides to metal phosphates where these mixed amendment systems helped provide a more efficient and permanent metal sequestration.

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In contrast, chitin is an abundant structural polysaccharide present in both water and sediments. Amendments of acetate, chitin, or both water and sediments. Amendments of acetate, chitin, or chitin plus apatite (Ap–Ch) were purchased as waste-product from the crab and shrimp industry; acetate kinase and phosphotransacetylase) of the acetate metabolic pathway to metals via dissimilatory reduction of metals. Therefore, SRB could be beneficial in bioremediation of toxic metals via metal reduction and metal sequestration by HS–SRB could be beneficial in bioremediation of toxic metals via metal reduction and metal sequestration by HS. SRB are also capable of mediating electron flow to metals via dissimilatory reduction of metals. Therefore, SRB could be beneficial in bioremediation of toxic metals via metal reduction and metal sequestration by HS–SRB could be beneficial in bioremediation of toxic metals via metal reduction and metal sequestration by HS (Arias and Tebo, 2003a; Muyzer et al., 1993; Muyzer and Stams, 2008; Barton and Fauque, 2009). In our DGGE analysis, a group of SRB (Desulfovibrio) was present in chitin treatments but no SRB was recovered in acetate treatments. This observation agrees with a previous study that SRB were detected in petroleum hydrocarbon contaminated aquifers by fluorescence in situ hybridization (FISH) and sulfur isotope fractionation, but not via DGGE (Kleikemper et al., 2002). In fact, we cannot completely rule out the occurrence of SRB other than Desulfovibrio in amendments because PCR–DGGE only reveals the dominant populations and misses the minor groups that have never reached densities to be detected by PCR–DGGE (Muyzer et al., 1993; Kan et al., 2006). Given the fact that minor groups of microbes could have a major geochemical impact, the results underscore the importance of more detailed cultivation-linked and cultivation-independent molecular techniques such as cloning, pyrosequencing, and metagenomics for future investigations.

4.2. Effects of acetate and chitin amendments

Acetate and chitin are two commonly used organic amendments. Acetate is a two-carbon short chain fatty acid and serves as a general food source for most of the microbes including bacteria, archaea, and even eukaryotic microbes. The key enzymes (i.e., acetate kinase and phosphotransacetylase) of the acetate metabolic pathway are widely distributed in bacteria and thus acetate serves as the best carbon source to stimulate the growth of indigenous bacteria in natural environments (Ingram-Smith et al., 2006). In contrast, chitin is an abundant structural polysaccharide produced by many marine organisms and it is a (1→4)-β-linked homopolymer of N-acetylglucosamine (NAG). The primary breakdown products of chitin are acetate and fructose, both of which are excellent carbon sources for anaerobic and facultative microorganisms capable of metal reduction (Bassler et al., 1991). Chitin can be purchased as waste-product from the crab and shrimp industry; therefore, it serves as an excellent cost-effective source to stimulate bacteria that grow in response to acetate.

Acetate and chitin induced significant increase in bacterial cell numbers and shifted the bacterial community composition in both water and sediments. Amendments of acetate, chitin, or chitin–apatite induced more diverse groups of Roseobacter in overlying waters, which were not dominant in the control (e.g., bands 3, 10, 11, 19, and 20 in Figs. 2 and 3). In marine environments, Roseobacter was a major phylogenetic group (about 5–30% of total) and they were widely distributed across a wide gradient of environments (reviewed in Buchan et al. (2005)). Members of Roseobacter have been found to be free-living, particle-associated, or in a symbiotic relationship with other living organisms (Buchan et al., 2005). One interesting physiological feature of Roseobacter was transformations in the biogeochemical cycling of sulfur. Roseobacter spp. harbored the ability to transform both organic (degradation) and inorganic (oxidation) forms of sulfur, including elemental sulfur, sulfide, sulfate, and thiosulfate (Moran et al., 2003). Many metal ions react with different forms of sulfur (e.g. sulfide) and form relatively stable compounds such as metal sulfides. Although little is known about the direct physiological roles of Roseobacter on heavy metals or organic contaminants, comparative genomic studies have shown that Roseobacter genomes contain common genes associated with metal toxicity, such as ABC-type transporter genes, copper resistance genes (copA, copB), and arsenate reductase gene (arsC) (Moran et al., 2007). These observations and the fact that organic amendments stimulated the growth of Roseobacter suggest that the versatile physiological features of this group of Alphaproteobacteria deserve further study.

Organic amendments also stimulated distinct bacterial communities in sediments in comparison to overlying water. For instance, Firmicutes and Bacteroidetes were dominant in the three horizons of chitin-amended sediments. Firmicutes and Bacteroidetes are two widely distributed bacterial groups, which are common to soils, sediments, seawater, and animal guts. To date, it is not clear if these two bacterial groups are centrally involved in metal bioremediation, a fact that is partially attributed to the difficulty of cultivation of environmental microbes in the laboratory. For instance, our DGGE band sequences primarily matched with uncultured bacterial phylotypes in the GenBank (Fig. 6). However, the high occurrence of these two groups of bacteria under heavy metal environments (Akob et al., 2006; Garau et al., 2007) suggests that (1) these two groups of microorganisms may be well adapted to contaminated sites, and (2) if not directly, these bacteria may cooperate with other microorganisms to facilitate the process of heavy metal immobilization or bioremediation in natural environments.

Based on MPN analyses, it is clear that the abundance of SRB was increased by both acetate and chitin amendments compared to controls. SRB are anaerobic microorganisms that commonly use sulfate as the terminal electron acceptor. Besides high production of sulfide, SRB are also capable of mediating electron flow to metals via dissimilatory reduction of metals. Therefore, SRB could be beneficial in bioremediation of toxic metals via metal reduction and metal sequestration by HS (Arias and Tebo, 2003a; Muyzer and Stams, 2008; Barton and Fauque, 2009). In our DGGE analysis, a group of SRB (Desulfovibrio) was present in chitin treatments but no SRB was recovered in acetate treatments. This observation agrees with a previous study that SRB were detected in petroleum hydrocarbon contaminated aquifers by fluorescence in situ hybridization (FISH) and sulfur isotope fractionation, but not via DGGE (Kleikemper et al., 2002). In fact, we cannot completely rule out the occurrence of SRB other than Desulfovibrio in amendments because PCR–DGGE only reveals the dominant populations and misses the minor groups that have never reached densities to be detected by PCR–DGGE. Given the fact that minor groups of microbes could have a major geochemical impact, the results underscore the importance of more detailed cultivation-linked and cultivation-independent molecular techniques such as cloning, pyrosequencing, and metagenomics for future investigations.

**Fig. 4.** DGGE fingerprints of overlying waters and sediment bacterial communities collected on day 28. Amendments: Ac, acetate only; Ch, chitin only; Ap-Ch, apatite+chitin. Water samples (W) and three horizons of sediments were included in the analysis. T = top horizon, 0–1.0 cm; M = middle horizon, 1.0–2.0 cm; B = bottom horizon, 2.0–3.0 cm. Bands 21–47 were excised for sequencing.
4.3. Water quality and ecotoxicology

In addition to the microbial effects described here, the studied sediment amendments, especially the organics acetate and chitin, affected the survival or growth rates of various macro-organisms under controlled laboratory exposure (Rosen et al., companion submission). Significant lower survival rates and growth rates of tested organisms indicate both mortality and chronic toxicity effects from the amendments such as acetate, chitin, and chitin-apatite. Reduced dissolved oxygen (acetate treatments) or excess ammonia (chitin treatments) produced by increased microbial cell densities or microbial degradation of the amendments aided in explaining the toxicological effects. It was likely the increased microbial cell numbers and/or microbial community shift that deteriorated the overlying water quality, which subsequently affected the survival and growth rates of the macro-organisms. Meanwhile, we could not exclude the possibility that microbial pathogens were enriched by the amendments and directly infected the tested animals. However, this was out of the scope of current work and thus was not included in further discussion.

5. Conclusions

The most probable number technique demonstrated that geochemically important bacterial groups including SRB and MRB were present in the sediments under amendment conditions and could play important roles in metal bioremediation and organic degradation processes. XAS analysis demonstrated that under chitin conditions, there was a modest modification of zinc speciation (increase in ZnCO₃), very likely due to the induced bacterial population. DGGE analysis showed that typical organisms responsible for these processes were not always dominant in the sediments. However, the DGGE analysis can be a useful tool to
visualize the distinct bacterial populations in overlying water and sediments that were enriched by organic amendments including acetate and chitin. This data was corroborated with the bacterial cell counts and the XAS analysis. DGGE analyses of the sedimentary bacteria did make evident that treatments with organoclay, apatite + organoclay, acetate, and chitin induced significant changes in the bacterial population and reshaped the microbial population structures in both the overlying water and in the sediment horizons. These results emphasize the benefit of performing cultivation-dependent and cultivation-independent concurrent analyses.

Due to the potential water quality and toxicity effects on the macro-benthic communities (see Rosen et al., companion submission), the tendency to be easily dissolved upon exposure to seawater, and cost effectiveness, acetate is not recommended for further investigation as a sediment amendment for contaminated sediments. In contrast, chitin, a naturally occurring and abundant structural polysaccharide in crustaceans, provides a potential for practical application in sediment amendments for organic and inorganic contaminants. However, we recommend further efforts on investigating the environmental safety effects of chitin amendments and optimizing the concentration to apply in marine sediments. Naturally, there will be differences based on processes such as current and flow, shallow or deep, and stagnant waters versus fast-moving water bodies. In conclusion, incorporation of apatite and chitin sediment amendments at select sites may be one of the preferred options of in situ remediation of heavy metal and organic contaminated sediments.

Acknowledgment

This work was funded by The Strategic Environmental Research and Development Program (SERDP), award no. 07ER-1551 and has been subjected to internal review. The authors would like to thank Joel Guerrero and Jennifer Podegracz for laboratory assistance. MRCAT operations are supported by the Department of Energy and the MRCAT member institutions. A portion of this research was conducted by the National Risk Management Research Laboratory of the U.S. Environmental Protection Agency Office of Research and Development. The paper has not been subjected to USEPA internal review; therefore, the opinions expressed are those of the authors and do not necessarily reflect the official positions and policies of the USEPA.

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