Toxicity of density separation media to Escherichia coli and Mycobacterium strain PC01: implications for density-separation of soils and sediments

Karl J. Rockne \textsuperscript{a},*; Wenhsin Liang \textsuperscript{b}, L.Y. Young \textsuperscript{b}, Gary L. Taghon \textsuperscript{c}

\textsuperscript{a} Department of Civil and Materials Engineering (M/C 246), University of Illinois at Chicago, 842 West Taylor Street, Chicago, IL 60607-7023, USA

\textsuperscript{b} Biotechnology Institute for Agriculture and the Environment, Rutgers, The State University of New Jersey, New Brunswick, NJ 08854, USA

\textsuperscript{c} Institute of Marine and Coastal Sciences, Rutgers, The State University of New Jersey, New Brunswick, NJ 08854, USA

Received 21 May 2002; received in revised form 28 August 2002; accepted 4 September 2002

First published online 22 October 2002

Abstract

Separation of low-density sediment detritus in CsCl solution is a promising technique for benthic studies. The known toxicity of CsCl suggests the possibility of toxicity in the separated sediment. Fluoranthene biodegradation by Mycobacterium strain PC01 was used to probe microbial activity in sediment following density separation. Complete inhibition of biodegradation occurred in sediment previously contacted with CsCl media, but washing eliminated inhibition. Washing may not be preferable for subsequent study of the separated sediment, suggesting the need for less toxic media. We studied how various density separation media affected the viability of Escherichia coli to quantify toxicity. Although all media decreased viability, Nycodenz\textsuperscript{b} and metrizamide were one to three orders of magnitude less toxic to bacteria than CsCl or Na\textsubscript{2}WO\textsubscript{4}. Toxicity was generally related to ionic strength. These results demonstrate that iodinated benzoic acids are superior to metal salts solutions for preserving biological activity on separated sediment or soil.

O%NNz 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Density separation; Centrifugation; Isopycnal; Toxicity; Low density; Soil; Sediment

1. Introduction

Sediment and soil particles provide abundant surface area for attached bacterial growth and metabolism of organic matter. Depending on the availability of substrate and nutrients, sediment and soil particles can harbor >10\textsuperscript{4} times the biomass of the water-column (on a volume basis). In some organic-rich sediments, cell counts can reach >10\textsuperscript{10} cells g\textsuperscript{-1} (dry weight) [1]. Reports in the literature demonstrate that density separation can be a useful technique to harvest low-density sediment and soil particles (\(\rho < 1.7 \text{ g cm}^{-3}\)) of potentially great ecological importance from a contaminant exposure and transport standpoint [2–5]. For example, we characterized the properties of low-density sediment detrital fractions [3,5] and found greatly elevated (>10 higher) sediment organic matter (SOM) and nitrogen levels than in the bulk sediment. We also found that the vast majority (up to 85%) of polycyclic aromatic hydrocarbons (PAHs) were sequestered in this sediment fraction, playing a key role in overall PAH bioavailability. These findings suggest the potential for greatly elevated microbial biomass in the low-density phase but as yet, little is known of the biological characteristics of these particles.

A significant impediment to studying the microbial ecology of low-density particles is that most of the methods of separating particles by density cannot achieve liquid densities above 1.25 g cm\textsuperscript{-3} and most media are potentially toxic to the cells on the particles. Density separation is typically performed by isopycnal centrifugation in a high-density liquid [6]. The process typically takes over 90 min from addition of sediment to the density media to washing. It is difficult to achieve liquid densities higher than 1.25 g cm\textsuperscript{-3} using sucrose or Percoll\textsuperscript{b}, media routinely used in cell component separations [6]. Pure glycerol...
has a density of 1.25 g cm\(^{-3}\), making it impossible to investigate higher media density. In addition, high concentration sucrose and Renograin\(^{\circledR}\) (a radiological image enhancer) solutions have very high osmolarity, with the potential for rupturing cell membranes. Although Ficoll-400\(^{\circledR}\) solution does not have the high osmolarity of sucrose, the practical achievable density limit is 1.2 g cm\(^{-3}\) [6]. In contrast to these five commonly used gradient media, solution densities above 1.7 g cm\(^{-3}\) are easily reached with both CsCl and Na\(_2\)WO\(_4\). However, both of these metal salts are potentially toxic, as suggested by toxicity studies in mammalian systems [7,8], although the data are few and in some cases inconclusive [9]. Other developments with higher achievable density media have led to the use of iodinated aromatic compounds like metrizamide and Nycodenz\(^{\circledR}\) [10].

Given the demonstrated ecological importance of low-density sediment fractions, understanding the bioavailability of PAHs in this material is critical. It is impossible to quantitatively measure the PAH biodegradation kinetic rate and PAH bioavailability from this material without separating it from the whole sediment and subjecting it to either a biodegradation or uptake experiment. Because this low-density material is preferentially ingested by detritivores (an important process affecting PAH fate and transport in sediments), a better understanding of PAH uptake, degradation, and depuration by detritivores is also needed. In order to investigate these biological activities in the low-density fractions of real sediments and soils, a technique for separating sediment particles by density fractionation without toxicity to sediment biota is needed. We describe here model systems to assess the toxicity of several different density media in a simulated density separation.

2. Materials and methods

2.1. Effect of sediment washing on bacterial activity

We measured biodegradation activity as a specific probe for bacterial activity in sediments to determine whether toxicity remained following separation by CsCl centrifugation. We used sediment from the New York/New Jersey harbor estuary that was highly contaminated with PAHs [3]. PAH biodegradation activity by a bacterial isolate from this sediment, designated Mycobacterium strain PC01, was used to measure bacterial activity in sediment that had previously been separated by isopycnal centrifugation in CsCl solution (1.80 g cm\(^{-3}\)), as well as in sediment suspended in seawater growth media as described in [11]. To test the efficacy of sediment washing, identical biodegradation experiments were performed with CsCl-exposed sediment washed by rotary shaking (200 rpm, 10 min) in seawater (5 ml g\(^{-1}\) of sediment) and centrifuged (500 \times g, 10 min) for a total of five cycles. Biodegradation of fluoranthene previously present on Piles Creek sediment (fluoranthene \(C_0 = 4000\) ng g\(^{-1}\) dry weight) by Mycobacterium strain PC01, a bacterium capable of biodegrading a variety of PAHs under a wide range of salinities [11], was monitored in duplicate incubations. Strain PC01 was pre-grown in mineral salt basal medium (MSB) salinity adjusted to 25\% salinity with 0.675 g (per liter) of MgCl\(_2\)-6H\(_2\)O, 0.075 g of CaCl\(_2\), and 20 g of NaCl with yeast extract (1.8 g l\(^{-1}\) medium) on a rotary shaker operating at 200 rpm at 30°C for 5 days. Cells were washed twice with MSB medium to remove residual yeast extract. After each washing, the cells were collected by centrifugation (3200 \times g for 15 min). Biomass concentration in the cell suspension was determined using the bicinchoninic acid (BCA) protein assay as described in [11]. The washed and CsCl-exposed sediment was added to 50 ml serum bottles as a slurry (1.5 g wet weight per 4 ml seawater). Washed cells of PC01 were added in suspension (1 ml containing 0.5 mg protein) to the sediment and the bottles were sealed with Teflon-coated butyl rubber stoppers and placed on a shaker table (200 rpm). Part of the headspace (15 ml) was replaced with pure oxygen to ensure sufficient oxygen was present in the headspace for complete biodegradation of the PAHs. At time points, a 0.5 ml portion of slurry was transferred to a 1.5 ml gas chromatography vial and the aqueous supernatant was removed with a pipette after centrifugation (3200 \times g, 20 min) with a Beckman GS-6 centrifuge (Beckman Instrument, Palo Alto, CA, USA). Acetonitrile was then added to the vial to restore the original volume and extracted using a hot acetonitrile extraction procedure as described previously [3]. All chemicals except for growth media were obtained from Sigma Chemicals (Saint Louis, MO, USA) at ACS grade or better.

2.2. Media preparation

We used Escherichia coli to assess toxicity and provide comparability to other studies using this bacterium. Pre-grown cultures were incubated in a NaCl solution (3.0 M) and four different density separation media (\(\rho = 1.36\) g cm\(^{-3}\)). The NaCl solution was of similar high ionic strength to the two ionic density media: 2.9 M CsCl and 1.4 M Na\(_2\)WO\(_4\). Two non-ionic iodinated media were used: 0.84 M Nycodenz\(^{\circledR}\) solution and 0.83 M metrizamide solution. Viability (as defined by culturability) in the five solutions was compared to an ‘iso-osmotic control’ solution (0.086 M NaCl, \(\rho = 1.004\) g cm\(^{-3}\)) that was iso-osmotic with the tryptic soy broth growth medium (Fisher Scientific, Pittsburgh, PA, USA). The ionic strength of each solution was computed from the media recipe using standard methods [12]. The osmolarity of each solution was calculated from data in [13] or [10]. All media were buffered to pH 7.2 with K\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\) buffer. Selected physical and chemical properties of the media are shown in Table 1.
2.3. Culture preparation and incubation

E. coli strain HB101 was provided by Dr. Gerben Zylstra from the Biotechnology Center for Agriculture and the Environment at Rutgers University. Cells were grown up overnight in tryptic soy broth (pH 7.2, Fisher Scientific) to a density of $1.5 \times 10^8$ cells ml$^{-1}$. The cells were harvested by centrifugation (1000×g) and washed in sterile iso-osmotic solution prior to the start of the experiment.

The density media were filtered (0.2 μm) and dispensed into 20 ml test tubes, previously sterilized by autoclaving at 120°C for 20 min, and capped. To maintain accurate media densities, all media were filter-sterilized directly into the test tubes with no heat sterilization. All manipulations of the cultures were performed in a laminar flow hood. The cells were diluted in sterile iso-osmotic solution and aliquots (0.1 ml) were dispensed in a 10-fold dilution series ($10^{-2}$–$10^{-6}$) into the previously prepared test tubes (this resulted in a 0.3% decrease in final density). At time points (0, 30, 60, 120 min), the contents of the test tubes were aseptically transferred to a sterile filter apparatus containing a sterile fecal coliform counting filter (Fisher Scientific) and filtered under vacuum. The filters were aseptically transferred to plates prepared with M-Endo LES coliform counting agar (Fisher Scientific), placed in a humidifier (> 98% R.H.) and incubated (35°C, 48 h). Following incubation, colonies were counted and most probable numbers (MPNs) were computed from standard tables [14]. Experiments were performed in triplicate and data are shown with 95% confidence interval. Statistical significance ($P < 0.001$) was determined by two-tailed $t$-test using standard methods.

3. Results and discussion

3.1. Residual toxicity in CsCl-exposed sediments

To assess the potential for residual bacterial toxicity by CsCl following density separation of the sediment, we investigated bacterial activity in CsCl-contacted sediments compared to both non-CsCl-contacted sediment and CsCl-contacted sediment that was subsequently washed. In sediment previously separated in CsCl media, the residual toxicity was total: no biodegradation of fluoranthene was observed (Fig. 1). In contrast, there were no significant differences in the biodegradation rate or extent between the sediment that had been pre-exposed to density separation media and washed versus sediment that had not been exposed to the media (Fig. 1). Although these results show that subsequent washing removes toxicity, the washing step may not be practical if the objective is to investigate the native biota in the separated sediment. Therefore, given the demonstrated toxicity of CsCl in unwashed separated sediments, it would be preferable to utilize a less toxic density separation medium under conditions where sediment washing is not desirable. To assess toxicity, we measured viability (defined by cell culturability) in several different density separation media. Given that identical experimental conditions were used for each medium, the relative responses of the test organism to the different media can then be used to compare toxicity.

3.2. Cell culturability in density media

There was a significant decrease in viability (all $P < 10^{-4}$, except for Nycodenz® with $P = 0.0015$) for all density separation media after 2 h incubation compared to the iso-osmotic control (Fig. 2). Over the same time period, there was a slight ($P = 0.18$, not significant) increase in cell numbers in the iso-osmotic control, demonstrating that toxicity occurs within a short time frame. For all media but metrizamide, there were only minor decreases in culturability (if any) after 30 min, and there was a slight but significant increase (as shown by 95% C.I.) in culturability in the tungstate media. Both non-ionic media had approximately a 90% decrease in culturability, much less than other media (including the NaCl). The trend in culturability followed most closely the trend in ionic strength of the media, with tungstate having the

Table 1

Physical and chemical properties of the incubation media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Density* (g cm$^{-3}$)</th>
<th>Ionic strength* (μ)</th>
<th>Osmolarity (Os l$^{-1}$)</th>
<th>Conductivity (mS cm$^{-1}$)</th>
<th>Molarity* (mol l$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso-osmotic control</td>
<td>1.00</td>
<td>0.01</td>
<td>0.16</td>
<td>8.2</td>
<td>0.086</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.12</td>
<td>3.0</td>
<td>6.4</td>
<td>180</td>
<td>3.0</td>
</tr>
<tr>
<td>Nycodenz®</td>
<td>1.36</td>
<td>N.I.</td>
<td>0.91</td>
<td>N.I.</td>
<td>0.84</td>
</tr>
<tr>
<td>Metrizamide</td>
<td>1.36</td>
<td>N.I.</td>
<td>N.D.*</td>
<td>N.I.</td>
<td>0.83</td>
</tr>
<tr>
<td>CsCl</td>
<td>1.36</td>
<td>2.9</td>
<td>5.0</td>
<td>280</td>
<td>2.9</td>
</tr>
<tr>
<td>Na$_2$WO$_4$</td>
<td>1.36</td>
<td>4.1</td>
<td>3.2</td>
<td>150</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*Measured at 25°C.

*Calculated from molarity of solution.

*Measured. Does not include phosphate buffer.

*Non-ionic.

*Unable to interpolate data due to disagreement between data in table and figure in Rickwood [10]. Presumably osmolarity is similar to Nycodenz®.
The greatest decrease in culturability and the highest ionic strength (Table 1). Ionic strength was not the only factor in the decrease in culturability, however, as demonstrated by comparing the NaCl and CsCl results. Both media were of similar ionic strength and osmolarity, but there was an order of magnitude greater decrease in culturability in the CsCl media. Although conductivity was highest in the CsCl media, the tungstate media had a similar conductivity to the NaCl media but had the lowest cell counts.

3.3. Implications for density fractionation

Although subsequent washing removes toxicity, the washing step may not be practical if the objective is to investigate the native biota in the separated sediment. Washing may remove and/or alter the microbial composition on the sediment. In these situations, iodinated benzoic acid solutions are superior to metal salts solutions for preserving biological activity on low-density sediment and soil particles when post-separation sediment washing is not preferable. If this is not a concern, metal salts media may be preferred because of their ease of use coupled with the apparent lack of residual toxicity after washing. It is important to consider that the magnitude and duration of bacterial exposure to the toxic media is likely greater in our simplified model system than would be experienced by bacteria attached to sediment particles during density separation. A full isopycnic centrifugation process typically requires preparation of the gradient media–sediment slurry, a 20 min shaking period for equilibration, and centrifugation for 20 min followed by filtration. With preparation time this likely would result in a 1–1.5 h exposure, less than the 2 h exposure in the viability experiment. It is apparent, however, that although all media were toxic, the decrease in culturable cell counts was lowest by more than two orders of magnitude in the non-ionic media. It would therefore be preferable to use the non-ionic density media rather than the metal salts media for density fractionation if toxicity is a concern.

Although results for Nycodenz® and metrizamide were similar, there are significant differences in preparation that suggest Nycodenz® would be a better choice as a density medium. Unlike Nycodenz®, metrizamide is not stable at autoclaving temperatures, so filter sterilization techniques must be employed. In addition, we had difficulty preparing metrizamide solutions with densities greater than 1.4 g cm⁻³, whereas Nycodenz® solutions of higher density were relatively easy to prepare. Because of the large amount of media needed to achieve high densities coupled with the cost of these chemicals, media recycling is preferable following fractionation. It is much easier to recycle the metal salts by evaporation followed by combustion at 550°C. Metrizamide and Nycodenz® would be destroyed by this process, so an alternative recycling technique must be used. Nycodenz® can be recycled by dialysis; however, there is no rapid means of separating dissolved organic carbon (DOC) from the soil or sediment from contaminating the Nycodenz®. Therefore, Nycodenz® recycling may be limited, particularly in sediments or soils with large amounts of DOC.

Acknowledgements

This work was supported in part by Grant R825303.
from the National Center for Environmental Research and Quality Assurance section of the U.S. Environmental Protection Agency and Grant NA97OR0338 from the National Oceanographic and Atmospheric Administration through the Cooperative Institute for Coastal and Estuarine Environmental Technology. The authors wish to thank Julie Johnson, David Mercando, and Dr. Randhir Makkar for assistance in the laboratory and discussions on the text.

References