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A MOLECULAR-BASED APPROACH FOR EXAMINING RESPONSES OF EUKARYOTES IN MICROCOSMS TO CONTAMINANT-SPIKED ESTUARINE SEDIMENTS

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Abstract: Ecotoxicological information for most contaminants is limited to a small number of taxa, and these are generally restricted to comparatively hardy organisms that are readily extractable from test media and easily identifiable. Advances in DNA sequencing can now provide a comprehensive view of benthic invertebrate diversity. The authors applied 454 pyrosequencing to examine the responses of benthic communities in microcosms exposed to sediments with elevated concentrations of triclosan, the endpoint being eukaryl communities that have successfully vertically migrated through the manipulated sediments. The biological communities associated with the 3 treatments (control triclosan, low triclosan [14 mg/kg], and high triclosan [180 mg/kg]) clustered into 3 groups: control/low ($n = 6$ controls and 4 low), moderate ($n = 2$ low), and high ($n = 5$ high). One sample was discarded as an outlier. The most pronounced change as a response to triclosan was the loss of number of metazoan operational taxonomic units (OTUs), indicative of the control/low and moderate groups, with this being most evident in the range of taxa associated with the classes Chromadorea and Bivalvia and the phylum Kinorhyncha. The authors also describe a range of other taxa that aided discrimination between the groups; compare findings with traditionally obtained meio- and macrofaunal communities obtained from the same experiment; and illustrate some of the advantages and limitations associated with both the molecular and traditional approaches. The described approach illustrates the capacity for amplicon sequencing to provide ecologically relevant information that can be used to strengthen an understanding of how sedimentary communities respond to a range of environmental stressors. *Environ Toxicol Chem* 2014;33:359–369. © 2014 SETAC

Keywords: Triclosan Metabarcoding Environmental DNA (e-DNA) Next generation sequencing 18S rRNA

INTRODUCTION

Routine community-level ecotoxicological studies of estuarine and marine sediments only examine a minute fraction of the benthic biodiversity. With the choice of biotic endpoint being constrained by the time, costs, and difficulties associated with isolating and identifying a vast majority of taxa, our understanding of the community-level ecological impacts of even the most well-studied contaminants is primarily restricted to a limited number of benthic macrofauna and, to a lesser degree, meiofauna and some protists and alga (http://cfpub.epa.gov/ecotox/). The concern is that pivotal decisions regarding the potential ecological impacts of environmental contaminants are determined by the responses of a small number of taxa encompassing a relatively narrow breadth of life cycles and ecological niches representing a minute fraction of the biodiversity that resides within sediments [1,2]. Clearly, the inclusion of a vastly wider breadth of biota would assist in identifying whether current approaches are indeed suitably sensitive, while enabling downstream decisions regarding the management of the environment to be based on a more holistic view of biodiversity.

Driven by the advent of high-throughput genome sequencers, the rapid expansion of online molecular databases (e.g., GenBank), and bioinformatics, recent advancements in the molecular sciences now make it theoretically possible to obtain ecological information from all forms of life, including bacteria, Archea, and Eukaryota, albeit at varying levels of taxonomic resolution and certainty. As it is currently not feasible to study the complete genomic constituents of an environment (i.e., the genomes of all sampled taxa from every biological sample), a gene-centric approach is used to examine single or multiple genes of interest. The approach is referred to by various terms, including metagenetics, tagged-pyrosequencing, metabarcoding, and amplicon analysis—or, more broadly, ecogenomics and environmental DNA (e-DNA). In studies examining a range of eukaryotes, the most common approach is to target a region of the gene encoding the 18S ribosomal RNA (rRNA). This gene can be used to extract phylogenetic and divergence relationships, and in many cases, relevant taxonomic and annotated information can also be obtained from online repositories such as GenBank's nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

While there are various permutations in the way high-throughput sequenced eukaryotic data can be obtained, the underpinning commonalities involve: 1) the sequencing of multiple samples (multiplexing), each comprised of numerous

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variants of the targeted genes (amplicons); 2) processing the sequenced data through a bioinformatic pipeline to remove ambiguous sequences and to minimize sequencing and polymerase chain reaction (PCR) artifacts; 3) comparing (or hitting) the processed sequences, now referred to as operational taxonomic units (OTUs), to a relevant sequence database; 4) assigning applicable taxonomy to the OTUs; and 5) statistical analyses and ecological interpretation.

Regardless of the approach used to measure biodiversity, a limitation of field studies is that observed responses are correlative, with any direct links to causality potentially confounded by the influence of environmental covariates such as salinity, bathometry, granulometry, or additional unmeasured stressors [3]. Recolonization experiments can overcome some of these limitations [4,5]; such studies generally require considerable resources (e.g., installation and collection of containers), however, with success being constrained by numerous factors such as the retention and compaction of sediments within treatments, weather, overlying physicochemical conditions, seasonal patterns in recruitment, and vandalism [6,7].

The aim of the present study was to examine the viability and ecotoxicological relevance of a novel approach that uses a laboratory-based, whole-sediment microcosm bioassay combined with high-throughput sequencing of a targeted region of the 18S rDNA gene to monitor the responses of estuarine benthic assemblages exposed to enriched concentrations of the antibacterial agent triclosan. The microcosm bioassay technique was modified from that described by Chandler et al. [8] and utilized intact cores containing entire field-collected benthic communities from a relatively uncontaminated estuarine environment [9]. The composition of the benthic communities was examined before and after a uniform layer of sediment, comprised of either a reference or contaminant-spiked treatment, was applied to the top of the intact cores. The premise of the approach is that the aerobic benthic organisms will migrate vertically to first colonize the new sediment layer; then, after 2 wk, a second, new layer of DNA-free sediment is applied to allow the surviving organisms to again migrate vertically. One week later, this layer of sediment is sampled for DNA analysis.

The present study also aimed to examine the congruencies and discrepancies between the molecular and concurrently obtained traditional macro- and meiobenthic data that were gathered from the same experiments [9]. The present study was methodologically focused, and was not designed to establish an environmental threshold for triclosan. The nominal triclosan concentrations used were 30 mg/kg and 300 mg/kg, significantly higher than the values reported in contemporary field studies (typically 0.8 mg/kg) [9]. However, the increasing use of triclosan as an antibacterial agent in a number of personal care products does pose an ecological concern because it is environmentally persistent, bioaccumulates in humans, and potentially photodegrades to dioxin [10–13].

METHODS

Collection of assemblages and application of treatments

The methodology for the collection and maintenance of the benthic assemblages and the spiking of the treatments with triclosan are comprehensively described in Ho et al. [9]. Briefly, in June 2010, benthic assemblages from an estuarine environment were captured in 24 microcosm cores (height: 15 cm; diameter: 15 cm) collected from the John H. Chafee National Wildlife Refuge at Pettaquamscutt Cove (Narrow River), Narragansett, Rhode Island, USA (41°26.92′N, 71°27.51′W).

After the cores were returned to the laboratory, they were maintained in an aerated, flow-through, filtered seawater system (average 6.4-h turnover rate; 30 ppt; 20 °C; 16:8-h light:dark cycle). Every 4 d, the core mesocosms were fed a live algal mixture of *Isochrysis galbana*, *Dunaliella tertiolecta*, and *Tetraselmis suecica* [9]. Three days after collection, the cores were randomly assigned to 4 treatment groups, each containing 6 replicates. The treatments were field control with no sediment added, control triclosan with clean reference sediment added from Long Island Sound (NY, USA), low triclosan (30 mg triclosan/kg dry Long Island Sound sediment), and high triclosan (300 mg triclosan/kg dry Long Island Sound sediment). An additional treatment, Narrow River, which consisted of samples from the field controls prior to commencement of the experiment, was used to compare the unperturbed (no additional layer of sediment) biotic assemblages at the start and end of the experiment. In all modified treatments, a layer (~2 cm) of reference sediment (triclosan control) or triclosan-spiked sediment (low triclosan and high triclosan) was carefully applied over the surficial sediment of each microcosm using a clean, plastic pastry piping bag. After 2 wk, a second, DNA-free layer of sediment slurry (2×305-mL aliquots of ~60% DNA-free sediment, ~40% seawater) was added to the top of the modified treatments. The DNA-free sediment was autoclaved prior to use and was confirmed to be DNA free by PCR amplification using the 18S rDNA primers described below. One week after the addition of the DNA-free sediment, 3 1-g subsamples of surficial sediment were obtained from the center and 2 opposing edges of each core to obtain vertically migrated biota for molecular analysis. It was assumed that organisms that did not migrate into the upper 2 cm of DNA-free sediment had not survived the exposure to the contaminated and anoxic layers below and that the DNA of the dead organisms did not move into this upper layer.

Physical and chemical measurements

Water flow rates and measurements of temperature, pH, salinity, and dissolved oxygen were made during the experiments. The triclosan concentrations of subsamples of the sediments were determined as described previously [9].

Sample preparation and sequencing

The DNA was extracted and purified from 0.25 g of each subsample of surficial sediment following the Power Soil® DNA Isolation Kit protocol (MO BIO). In addition, genomic DNA was extracted from 25 mg of Atlantic killifish (*Fundulus heteroclitus*) caudal fin tissue according to the QIAGEN DNeasy protocol for animal tissue to serve as an internal control. All DNA extracts were quantified using the picogreen assay for double-stranded DNA (Invitrogen). The 18S ribosomal RNA gene was targeted with primers 18S SSU_F04 (5′-GCTTGTCTCAAAGATTAAGCC-3′) and 18S SSU_R22 (5′-GCCTGCTGCCTTCCTTGGA-3′) as reported in Creer et al. [14]. The PCR amplifications were performed in 50 reactions containing 5 μL 10× PCR buffer, 2.5 mM $MgCl_2$, 200 μM deoxyribonucleotide triphosphate (dNTP) mix, 1 U FastStart High Fidelity polymerase (Roche), 0.4 μM of each primer, and 20 ng template DNA. The thermal profile for amplification consisted of an initial denaturation step at 94 °C for 2 min, 30 cycles of 94 °C for 1 min, 50 °C for 1 min 15 s, and 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 5 min. Amplification products were purified using the QIAquick PCR purification kit (QIAGEN) and quantified. Equal amounts of DNA from the 3 subsamples from each microcosm core (center, right edge, and left edge) were pooled, and 30 ng of each

amplicon pool was subjected to a second, abbreviated round of amplification (10 cycles) to attach 454 fusion primers and multiplex identifiers (barcodes). Amplicons were again purified and quantified. Equimolar amounts of each amplicon were then combined into 1 of 2 pools, each totaling 2 μg of DNA, ethanol-precipitated, resuspended in 50 μL of molecular grade H_2O, and submitted to the Duke Institute for Genome Sciences and Policy (Durham, NC, USA) for sequencing on a Roche 454 GS FLX sequencer. A single plate, divided into 2 sectors (1 per pool), was sequenced.

All 454 data were cleaned and processed using the QIIME pipeline [15]. Raw sequence reads were initially demultiplexed and quality trimmed using the *split_libraries.py* script in QIIME with default parameters. Approximately 970 000 sequences (those remaining after demultiplexing and cleaning) were used for downstream bioinformatics analyses. The OTUs were defined using a de novo OTU picking approach in UCLUST [16] with a 96% pairwise sequence identity cutoff. This approach was chosen based on previous evidence suggesting that a 96% cutoff enables the most accurate recovery of species richness in high-throughput, eukaryotic rRNA datasets [17]. Chimera checking was carried out using the *identify_chimeric_seqs.py* script in QIIME (blast_fragments method) with default parameters, with all potentially chimeric OTUs subsequently removed prior to downstream analyses.

Taxonomic assignment

Initial taxonomic assignments were obtained for each OTU using MegaBLAST to retrieve the top-scoring hit existing in GenBank (excluding uncultured sequences) as of 5 August 2012. Using corrected percentage similarity (percentage homology multiplied by the fraction of sequence length coverage), OTUs were assigned to interim taxonomic groups with the following cutoffs: genus (>99%), family (>97%), order (>95%), class (>92%), phylum (>90%), kingdom (>80%), and unknown eukaryote (80% or less). Validation of taxonomic allocation and reassignment of OTUs (when required) were performed using an approach modified from Chariton et al. [18]. Briefly, OTUs were aligned, generally at the level of order using the computer program MUSCLE [19], with the groupings of the OTUs visually examined using phylogenetic trees and Linnaeus Blasts. The OTUs that did not concur within their initial allocations were placed into increasingly coarser taxonomic groups until they were visually deemed to be suitably allocated. All blasting and phylogenetic tree reconstructions were performed using the bioinformatics package Geneious 5.6 (Biomatters). When OTUs could not be placed into formally recognized taxonomic groups (kingdom, phylum, class, order, family, and genus) as defined by the US National Center for Biotechnology Information (NCBI)'s Taxonomy site (http://www.ncbi.nlm.nih.gov/taxonomy), taxonomic aggregation was derived from the lowest formally assigned taxonomic level and denoted by the bracketed prefix uc. For example, Ancyromonadidae were assigned to Ancyromonadidae(ucO), indicating that OTUs from the family Ancyromonadidae could not be formally assigned to an order. The OTUs that could not be confidently assigned to finer taxonomic levels were denoted by "uk" for unknown. For example, Tubulinea, an OTU that could not be confidently assigned to below phylum, would be aggregated to the class Tubulinea(ukC).

Statistical analyses

Tests of differences in the OTU richness between the 2 nonindependent samples (Narrow River vs field control) were performed using a paired *t* test. To examine whether the addition of the sediment layer altered communities, mean OTU richness of the field control and triclosan control treatments were compared using a single-factor analysis of variance. Residuals were assessed using D'Agostino's tests for skewness, kurtosis, and omnibus normality [20], with homogeneity of variances examined using a modified Levene's equal variance test [21]. All univariate analyses were performed using NCSS v7.1.3.

In contrast to automatically assigning each sequenced community to its a priori defined treatment (control triclosan, low triclosan, and high triclosan), for the present study we used an unsupervised approach to examine the natural clusters of sequenced communities and the key taxa associated with each cluster. Prior to computation, data were transformed to presence/absence with all ubiquitous OTUs removed. Using a procedure modified from Ben-Hur and Guyon [22], scaled and centered principal component analysis based on a singular value decomposition was performed on the transformed biological matrix comprising of 849 OTUs. The first 18 principal component scores (PC1–18) were used as variables for agglomerative hierarchical clustering variables based on Euclidean distance and Ward's minimum variance method, a criterion that forms groups by finding the minimum within-group variance (sums of squares) [23]. The clustering method was repeated using increasingly fewer principal components to identify the minimum number of components required to produce stable clusters [22]. Chi-square analysis was then performed to examine the association between treatment group and the groups produced by the best clustering result (PC1–3). All ordinations and clustering were performed in R using raw code [24].

Key OTUs contributing to each natural cluster, and combinations of clusters, were determined using the R indicator analysis package *Indispecies*. Subsequent to running the package's *multipatt* function, the *signassoc* function was performed to test whether the occurrence of OTUs in each cluster group was random, with all *p* values derived from the original *multipatt* analysis being corrected for multiple testing using the Sidak method. To identify multiple OTUs potentially associated with the same taxa, Spearman–Rank correlations were performed on OTUs that returned identical NCBI accession numbers. The OTUs that were consistently copresent (i.e., $r = 1.00$) were considered to be derived from the same taxon or strongly associated taxa.

RESULTS

Sediment and porewater chemistry

The spike recoveries for triclosan averaged 110% with a standard deviation of 4.9% ($n = 2$). Prior to the addition of the triclosan-enriched sediment to the surficial sediment of the microcosms, triclosan concentrations for the 2 treatments were between 63% and 68% of nominal for the low and high concentrations, respectively [9]. After 3 d of equilibration in the mesocosm, triclosan concentrations in the overlying treated sediment declined to 13.9 mg/kg (low triclosan) and 181 mg/kg (high triclosan), which were 46% and 60% of the nominal spiked concentrations, respectively [9]. Porewater concentrations of triclosan for the low and high treatments were calculated at 10 μg/L and 140 μg/L, respectively [9]. Trace concentrations of triclosan were also observed in the triclosan control (Long Island Sound sediment; 0.0014 ± 0.001 mg/kg) and the field control (Narrow River sediment; 0.06 ± 0.04 mg/kg). At the conclusion

of the experiment, after 7 d of equilibration with the DNA-free layer, the unspiked surficial layers of the low triclosan and high triclosan treatments contained 1.19 ± 0.20 mg/kg and 1.43 ± 0.81 mg/kg, respectively. The physicochemical properties of the overlying waters varied little over the duration of the experiment, with a mean salinity of 30.3 ppt $\pm$ 0.43 standard deviation (SD) and a mean pH of 8.00 ± 0.05 SD [9].

Sequencing results and quality assurance

The Roche 454 FLX Titanium pyrosequencing run produced more than 1 037 000 reads with an average read length of 353 base pairs (bp). Subsequent to the removal of short, poor-quality, and possibly chimeric sequences, 970 000 potentially viable reads remained, containing 4993 unique 18s rDNA sequences, hereafter referred to as OTUs. A vast majority (82%) of these OTUs were present as 1 read or 2 reads per sample and contained a total read abundance of less than 17 reads (below 3 standard errors [SE] of the mean) across all samples, and were consequently removed [25]. This decision was supported by scrutinizing the results of the 2 *F. heteroclitus* reference samples, with the taxonomic assignments of OTUs with low counts (<17) frequently shown to be erroneous (results not presented). The remaining dataset (>17 reads per OTU) contained 615 706 reads derived from 1260 OTUs. The data have been deposited in the Commonwealth Scientific and Industrial Research Organisation Data Access Portal and are accessible at http://dx.doi.org/10.4225/08/518136D7EF1A8.

Further examination of the *F. heteroclitus* reference samples (Supplemental Data, Table S1) showed that, for the most part (71%), the bioinformatic pipeline assigned the OTUs to the correct species. Some OTUs, however, including the most abundant one, aligned poorly with those retained within GenBank, resulting in a low similarity ($<87\%$) and consequently could only be assigned to Eukaryota. As the reference samples contained 42 OTUs derived from the 18S rRNA gene of one organism (*F. heteroclitus*), clearly OTU richness is not analogous with traditional true alpha richness.

Experimental artifacts

Paired comparisons between the untreated assemblages sampled at the commencement (Narrow River sediment) and completion (field control sediment) of the experiment clearly showed that OTU richness increased during the experiment ($t = -4.12$, $p < 0.001$). A majority of the field control's 298 OTUs previously unobserved in the Narrow River samples were derived from Rhizaria (38%), miscellaneous metazoans (18%), stramenopiles (17%), and unknown eukaryotes (16%)—and not the larger metazoans such as annelids, arthropods, and nematodes. As the filtered water used for the flow-through setup was the most likely source of a large proportion of these OTUs, they were treated as exogenous sequences and filtered from the dataset. All subsequent analyses were performed using the filtered data.

On analysis of the filtered data, it was evident that the addition of the sediment layer had a significant impact in all treatments, with the mean OTU richness of the triclosan control (292 ± 21 SE) being almost 50% of that of the unmanipulated field control (545 ± 33 SE; t test: $t = 4.03$, $p = 0.002$). The most pronounced differences between these 2 treatments were the complete absence of 32 OTUs in the triclosan control that were ubiquitous in the field control. Approximately 79% of the OTUs missing in the triclosan controls were associated with diatoms (Bacillariophyta), fungi, and cercozoans, which have little ability to vertically migrate.

Comparisons between triclosan-spiked treatments

Of the 890 OTUs sampled in the control triclosan, low triclosan, and high triclosan treatments, approximately 44% of these were observed in all 3 treatments. In all treatments, a majority of the OTUs were observed in 3 or more replicates, with 6% to 8% being sampled in all 6 replicates (Supplemental Data, Figure S1).

As illustrated in Figure 1, the molecular analysis produced OTUs associated with a diverse range of biota, encompassing representatives from 19 phyla and a similar number of coarse unclassified taxonomic groups. Approximately 70% of the variance in the biological data derived from the control, low, and high triclosan treatments was explained by PC1–7, with the principal component analysis being truncated to 18 components (residual variance $<$ 3.3e-15). The hierarchical clusters produced from the first 7 principal components are provided in Figure 2. The underpinning trend from the analyses was that a majority of the control triclosan and low triclosan were clustered together, with a majority of the high treatments also forming a separate cluster. While only explaining approximately 33% of the total variance, the hierarchical clustering of PC1–3 was deemed best, producing clusters that reflected the original treatments, albeit with the aggregation of a majority of the low triclosan assemblages with the control triclosan assemblages (Figure 2). The association between the blind clusters and the treatment groups were statistically significant (Chi-square $= 17.6$; $p = 0.0015$). When examined collectively (Figures 3 and 4), the ordination plots derived from PC1–2 and PC–3 infer 3 principal component analysis cluster groups, hereafter referred to as: control/low group (6 controls and 4 low triclosan samples); high group (5 high control samples); and moderate group (2 low triclosan samples), with this group being more similar to the control/low group than the high group (Figure 4). One sample derived from the original high triclosan treatment was an outlier (Figure 4), and consequently was excluded from subsequent analyses and interpretation.

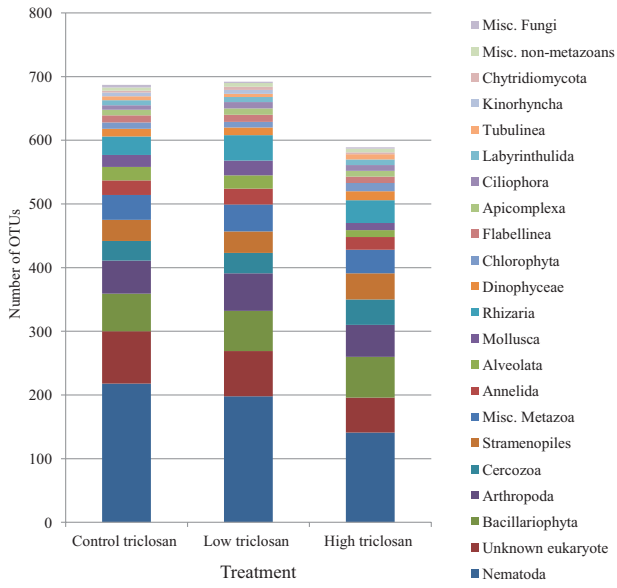

Figure 1. The biotic composition and richness of phyla (and other coarse taxonomic groups) sequenced from the surficial sediments of the 3 modified treatments at the completion of the experiment. To aid visual interpretation, data have been aggregated at the level of phylum and higher. OTU = operational taxonomic unit.

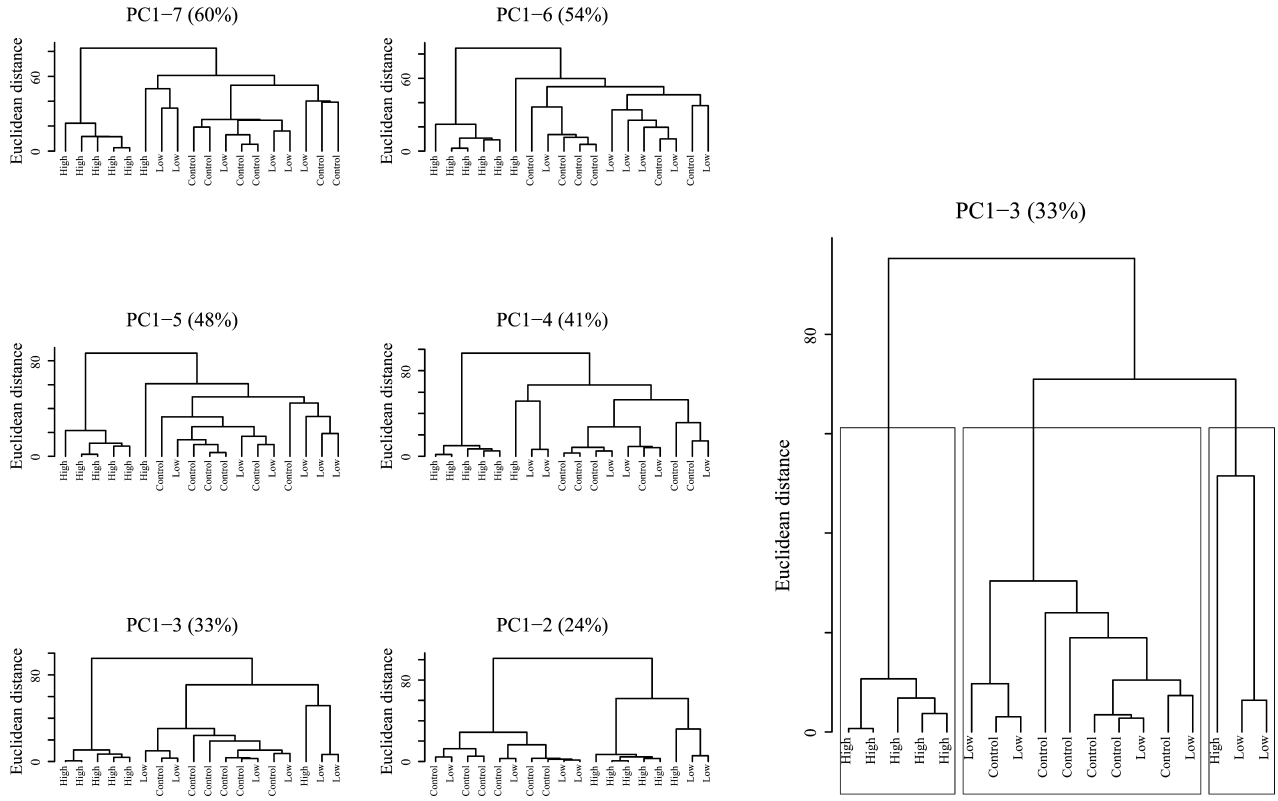

Figure 2. Comparison of hierarchical clustering using 2 to 7 principal components (PCs). The right-hand figure shows the cluster diagram created from PC1 to PC3, which was used for subsequent analysis. Control = triclosan control; Low = low triclosan treatment; High = high triclosan treatment.

The indicator analysis results are summarized in Table 1. With the exception of a single OTU from the harpacticoid genus *Stenhelia*, the OTUs indicative of the control/low group were all from the nematode families Comesomatidae and Xyalidae. While the co-occurrences of the multiple *Sabatieria* OTUs were significantly correlated ($r = 0.62–0.78$, $p < 0.05$), there was no evidence (i.e., $r = 1.00$) to suggest that these OTUs were derived from the same organism. Only 5 OTUs were identified as characteristic of the high group (Table 1). This included a different genus of harpacticoid from that of the control/low group, with the remainder being small microbiota (Cercozoa, Aleolata, and Tubulinea). Not surprisingly, no OTUs were identified as specifically indicative of the moderate group; however, a large number of indicator OTUs were identified when the control/low and moderate groups were examined collectively, emphasizing the greater similarity between these groups, than between the moderate and high groups. The biota characteristic of the less contaminated treatments (control/low and moderate groups) were predominately metazoan, containing a range of nematodes, as well as Kinorhyncha, harpacticoids, and bivalves, although there were some cercozoan exceptions. Two of the bivalve *Nutricola* OTUs were perfectly correlated ($r = 1.00$), indicating that these may have been intrataxon variants. Only 2 OTUs from taxonomically distinct cercozoan lineages were identified as indicative of both the moderate and high groups. Collectively, these findings indicate that the

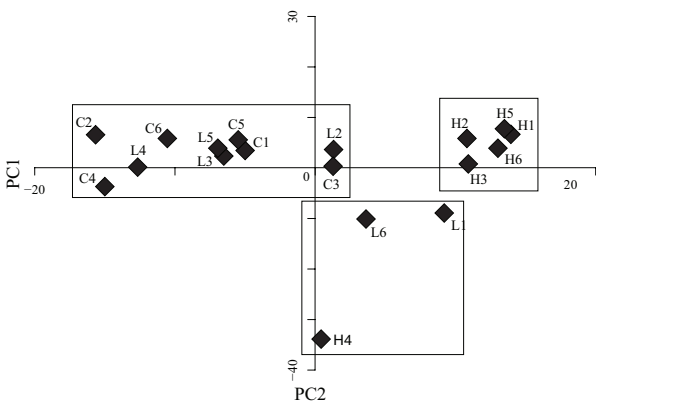

Figure 3. Samples plotted by principal components (PC)1 to 2 and grouped by principal component analysis cluster groups. Dashed rectangles show outlier groups (L1, L6, and H4). C = triclosan control; L = low triclosan treatment; H = high triclosan treatment.

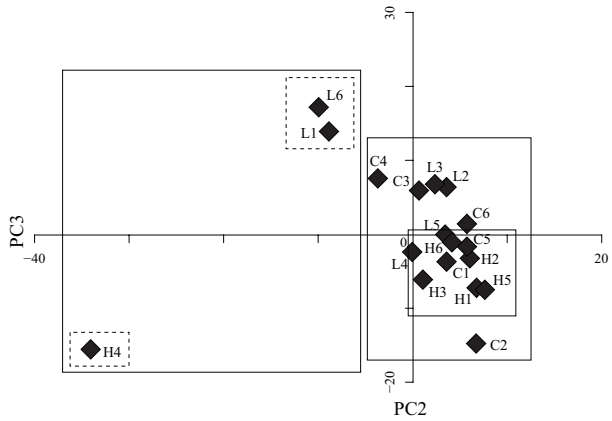

Figure 4. Samples plotted by principal component (PC)2 and 3 and grouped by principal component analysis cluster groups. Dashed rectangles show outlier groups (L1, L6, and H4). C = triclosan control; L = low triclosan treatment; H = high triclosan treatment.

Table 1. Summary of indicator taxa analysis performed on the three clusters (control/low, moderate, and high)[a]

Group cluster	Best match (BLAST)	Bit score	A[b]	B[c]	IV[d]	*p* value[e]	Phylum	Class[f]	Order[f]	Family[f]	Accession
Control/low											
	Sabatieria punctata	671	1	0.9	0.949	0.03	Nematoda	Chromadorea	Monhysterida	Comesomatidae	AY854236
	Sabatieria punctata	671	0.83	1	0.913	0.03	Nematoda	Chromadorea	Monhysterida	Comesomatidae	AY854236
	Sabatieria punctata	601	0.82	0.9	0.858	0.03	Nematoda	Chromadorea	Monhysterida	Comesomatidae	AY854236
	Sabatieria pulchra	668	1	0.8	0.894	0.03	Nematoda	Chromadorea	Monhysterida	Comesomatidae	JN968250
	Sabatieria pulchra	647	1	0.8	0.894	0.03	Nematoda	Chromadorea	Monhysterida	Comesomatidae	JN968250
	Metadesmolaimus sp.	668	1	0.8	0.894	0.03	Nematoda	Chromadorea	Monhysterida	Miraciidae	JN968218
	Stenhelia sp.	683	1	1	1	0.03	Arthropoda	Maxillopoda	Harpacticoida	Miraciidae	EU380291
Moderate											
	No indicator taxa found										
High											
	Paramphiascella fulvofasciata	673	1	0.6	0.775	0.03	Arthropoda	Maxillopoda	Harpacticoida	Miraciidae	EU380293
	Thaumatomastix sp.	686	1	1	1	0.03	Cercozoa[g]	Silicofilosea[g]	Thaumatomonadida	Thaumatomastigidae	GQ144681
	Nolandella sp.	478	1	0.8	0.894	0.03	Amoebozoa	Tubulinea	Euamoebida	Hartmannellidae	EU273456
	Uronychia multicirrus	664	1	1	1	0.03	Alveolata	Spirotrichea	Euplotida	Uronychiidae	HQ380025
	Gymnophrys sp.	679	1	0.8	0.894	0.03	Cercozoa[g]	Cercozoa_ucC	Cercozoa_ucO	Cercozoa_ucF	GU320582
Control/low and moderate											
	Paracyatholaimus intermedius	313	1	1	1	0.03	Nematoda	Chromadorea	Chromadorida	Cyatholaimidae	JN968220
	Paracyatholaimus intermedius	601	1	0.83	0.913	0.03	Nematoda	Chromadorea	Chromadorida	Cyatholaimidae	JN968220
	Praeacanthonchus sp.	538	1	0.92	0.957	0.03	Nematoda	Chromadorea	Chromadorida	Cyatholaimidae	JN968256
	Cyatholaimus sp	311	1	0.92	0.957	0.03	Nematoda	Chromadorea	Chromadorida	Cyatholaimidae	AY854213
	Sabatieria punctata	671	1	0.83	0.913	0.03	Nematoda	Chromadorea	Monhysterida	Comesomatidae	AY854236
	Sabatieria pulchra	596	0.8	0.92	0.899	0.03	Nematoda	Chromadorea	Monhysterida	Comesomatidae	JN968250
	Metachromadora remanei	666	1	0.92	0.957	0.03	Nematoda	Chromadorea	Desmodorida	Desmodoridae	AY854216
	Metachromadora remanei	666	1	0.83	0.913	0.03	Nematoda	Chromadorea	Desmodorida	Desmodoridae	AY854216
	Metadesmolaimus sp.	668	1	0.75	0.866	0.03	Nematoda	Chromadorea	Monohysterida	Xyalidae	JN968218
	Pycnophyes sp.	677	1	0.67	0.816	0.03	Kinorhyncha	Kinorhyncha_ucC	Homalorhagida	Pycnophyidae	AY859598
	Nutricola tantilla	631	1	0.92	0.957	0.03	Mollusca	Bivalvia	Veneroida	Veneridae	AM774569
	Nutricola tantilla	568	1	0.92	0.957	0.03	Mollusca	Bivalvia	Veneroida	Veneridae	AM774569
	Nutricola tantilla	659	1	0.75	0.866	0.03	Mollusca	Bivalvia	Veneroida	Veneridae	AM774569
	Nutricola tantilla	535	1	0.75	0.866	0.03	Mollusca	Bivalvia	Veneroida	Veneridae	AM774569
	Stenhelia sp.	683	1	0.92	0.957	0.03	Arthropoda	Maxillopoda	Harpacticoida	Miraciidae	EU380291
	Paulinella chromatophora 16S-like	686	0.83	1	0.913	0.03	Cercozoa[g]	Silicofilosea[g]	Euglyphida	Paulinellidae	X81811
	Massisteria marina	683	0.86	0.87	0.87	0.03	Cercozoa[g]	Cercozoa_ucC	Cercomonadida	Cercomonadidae	AF174374
	Allas sp.	684	1	0.75	0.866	0.03	Cercozoa[g]	Silicofilosea[g]	Thaumatomonadida	Thaumatomonadida_ucF	AY268040
Moderate and high											
	Paulinella chromatophora 16S-like	686	0.83	1	0.913	0.03	Cercozoa[g]	Silicofilosea[g]	Euglyphida	Paulinellidae	X81811
	Massisteria marina	683	0.88	0.86	0.87	0.03	Cercozoa[g]	Cercozoa_ucC	Cercomonadida	Cercomonadidae	AF174374

[a]The best match, bit scores, taxonomy and accession numbers are derived from the top hit from the National Center for Biotechnology Information nucleotide database. All presented operational taxonomic units (OTUs) received 100% similarity hits with MEGABLAST.
[b]A is the the conditional probability of the OTU as an indicator of the group cluster.
[c]B is the probability of finding the OTU in samples belonging to this cluster group.
[d]Indicator Value (IV) is an index with a maximum value of 1.00 occurring when a taxa is restricted to one group (or combination of groups) and present in all samples.
[e]Presented *p* values have been Sidak corrected.
[f]"uc" indicates an unclassified class (C), order (O), or family (F).
[g]Indicates unranked taxonomic groups.

metazoans were more susceptible to triclosan, with this being most evident in bivalves, nematodes, and kinorhynchs.

DISCUSSION

Molecular endpoints for characterizing ecological effects

Decisions regarding the ecological responses of aquatic organisms to environmental stressors are derived from the responses of only a fraction of representative biota [2]. Therefore, the ability to develop ecological endpoints that provide a comprehensive view of the targeted community has the potential to significantly enhance the way we examine the ecological ramifications of contaminants and other environmental stressors in sediment systems. The aim of the present study was to examine the viability of using molecular richness and composition data derived from high-throughput amplicon sequencing as an ecotoxicological endpoint. As we have clearly demonstrated, the molecular diversity data produced from pyrosequencing 18S rDNA encapsulates genes associated with a diverse range of eukaryl phyla. The diversity detected with the molecular approach surpassed that obtained through the traditional methods employed in a companion study that used the sample biological samples [9]; this is because many of the sequenced taxa (e.g., Labyrinthulida, Ciliophora, Gastrotricha, Bacillariophyta, Tubulinea, and Mucoromycotina; Figure 1) were either too small, cryptic, or taxonomically challenging to be included in traditional meiofaunal and macrofaunal analysis. With the exception of Nemertea, for which only 2 individuals were observed in 1 sample, all phyla traditionally enumerated from the same samples were detected by the molecular analyses [9]. The nondetection of this taxon is mostly likely because of the removal of all specimens prior to obtaining DNA samples or sampling error.

It is well established that nematodes are sensitive indictors for a range of environmental conditions [26,27]; however, their size and difficulties associated with identification often preclude them from benthic monitoring programs below the level of phylum [28]. In the present study, both the traditionally obtained and molecular data clearly showed that nematode survivorship was impaired in the high group, which contained samples only from the high triclosan treatment [9]. The information gained from the traditionally obtained data with respect to nematode responsiveness to triclosan was limited to the level of phylum, while class-, order-, and occasionally family- and genus-level taxonomic information was obtained from the molecular data, providing a much stronger basis for ecological interpretation. For example, we found clear reductions in the relative occurrences of a number of OTUs associated with monhysterid nematodes (including Comesomatidae and Xyalidae), even though these nonselective deposit feeders are considered to be relatively tolerant of pollutants and typically exploit anthropogenically disturbed environments [26]. This result is unsurprising given that the particulate and dissolved triclosan concentrations in the high group were 2 orders of magnitude greater than those reported in the most enriched naturally occurring sediments [13]. Furthermore, bacteria are a primary food source of monhysterids [29]. The antibacterial properties of triclosan likely reduced bacterial counts, which may have also significantly contributed to mortality.

Collectively, the traditionally collected meio- and macrobenthic data [9] and molecular data showed that the abundance and composition of crustaceans and mollusks were unaffected in the low triclosan treatment (control low and moderate groups), even though particulate (14 mg/kg) and interstitial concentrations ($\sim$10 μg/L) substantially exceeded those typically reported for aquatic environments [13]. In the highest dosed samples, both the traditional and molecular proponents suggest that the tolerance thresholds for a number of crustaceans and mollusks were indeed exceeded. This treatment had interstitial concentrations ($\sim$137 μg/L) essentially double that of the 96-h water-only median lethal concentrations for the amphipod *Ampelisca abdita* (73 μg/L) and the mysid *Americamysis bahia* (74 μg/L) [30], although still below that for the grass shrimp *Palaemonetes pugio* (305 μg/L) [31].

The primers used in the present study have been used extensively by a number of researchers to examine nematode composition [14,32]; however, for the most part, OTUs could only be confidently assigned to order, class, or phylum. This limitation is most likely an artifact of applying overly conservative taxonomic cutoffs, which was required to be applicable to all sequenced taxonomic groups, but also reflects the limitations associated with using a single primer set [33], and the limited number and composition (primarily European) of 18S rDNA sequences residing in GenBank [34]. While other genes—for example, cytochrome oxidase—are able to provide species-specific taxonomic resolution, multiple primer sets are required to capture a diverse range of taxonomic groups [35]. Because of the relatively low throughput of the Roche 454 FLX platform, extra sequencing runs would be required to accommodate additional primer sets without a loss in sequencing depth. However, this issue is ephemeral, with new platforms (e.g., Illumina and Ion Torrent) now able to produce higher throughput data with read lengths suitable for amplicon analysis. Although taxonomic assignment is important for aiding ecological and biological interpretation, it is emphasized that even without annotated taxonomic information, OTUs are unique entities (albeit artificially derived), and can still be used to characterize and discriminate biotic assemblages.

In contrast to a previous DNA diversity study in which we attempted to use OTU richness as a metric of contaminant-induced change [18], after reflection, we excluded richness from the present study because of its ambiguity when derived from bioinformatically created clusters of sequences (OTUs). This was clearly evident from the clustering of the reference sequence (*F. heteroclitus*) into more than 40 OTUs. While the bioinformatic approach undoubtedly contributed to the overinflation of OTUs with respect to the number of expected unique sequences, the relationship between and molecular richness and taxa richness is potential further obscured by intra- and interspecific variations in the 18S rRNA gene [36]. This suggests that taxa may not contribute evenly to OTU richness, and consequently the loss of some taxa may contribute only marginally to the overall pool of molecular diversity, while the loss of others may contribute considerably. Until robust and predictable relationships between molecular and true alpha richness are obtained, careful consideration should be given to the merit of OTU richness as a sensitive and suitable ecological endpoint.

Comparison of traditional and molecular based approaches

In the present experiment, there was a strong congruence in responses of number of taxa that could be examined using both traditional and molecular-based approaches (e.g., nematodes, crustaceans, kinorhyncha, and mollusks; Table 2). This is despite marked differences in the statistical attributes associated with the datasets—that is, the molecular data were present/absent—while the macro- and meiodata employed univariate and multivariate metrics derived from abundance measurements.

Table 2. Comparisons between the ecological data derived from molecular study and that obtained from traditional methods [9]

Endpoint	Molecular	Traditional	Ecological significance	Considerations
No. of taxa examined	1260 OTUs	Meiofauna = 11 taxa	Far more diverse range of organisms examined by the molecular approach.	Taxonomic assignment varied greatly within the molecular data and was limited by time and expertise in the traditional study. Molecular data includes potentially deceased, adhered and consumed organisms.
		Macrofauna = 24 taxa		Molecular data included numerous additional taxa fundamental to biogeochemical cycling, primary production and basal components of food webs.
Biotic richness	Not applicable due to poor relationship between OTU richness and 'true' richness.	Meio- and macrofaunal richness was lower in HT	Additional biota were recruited via flow-through	In order for richness to be viable molecular endpoint, bioinformatic software needs to be able to provide a more view of relationship between OTU richness and 'true' richness, i.e. each OTU should represent a unique sequence variant.
Diversity	Not applicable as the abundance could not be quantified.	No difference in meiofaunal diversity among manipulated treatments. Macrofaunal diversity was reduced in HT.	Declines in diversity are a potential indicator of contamination.	Quantifiable molecular data would greatly aid interpretation.
Composition	Three distinct clusters of samples: control/low group (6 × CT and 4 × LT) moderate group (2 × LT) and high group (5 × HT)	Meiofauna HT ≠ (CT = LT)	Regardless of approach, compositional changes were evident in the highest treatment.	In the molecular study, differences among treatments were driven by numerous OTUs contributing to small differences. Consequently, summarizing and interpreting the key differences can be difficult using traditional statistical techniques.
		Macrofauna HT ≠ (CT = LT)	The application of the test sediment significantly altered the composition of the residing biota.	
		FC ≠ CT		
Nematodes	Significant reductions in the occurrences of number of nematode OTUs occurred in the high group.	abundance: HT<(CT = LT)	Key components of estuarine sediments encompassing a diverse range of ecological niches. Composition can reflect environmental conditions.	Compositional information obtained from the molecular component was relatively comprehensive. Enumeration of nematodes below the level phylum requires specialized training.
Molluscs	OTUs associated with the family Veneridae with indicative of the control/low and moderate groups.	Macrofaunal bivalve abundance was lower in HT.	Changes in abundance can alter sediment geochemistry and the availability of food for fish.	Molecular data detected greater diversity than traditional data. The declines in abundance could be obtained only from the traditional data. Both sets of data provided relevant ecological endpoints.
Annelids	No clear pattern was observed in this phylum.	No observed differences in meiofaunal or macrofaunal polychaete abundances among manipulated treatments. Compositional changes in polychaetes were observed among some treatments.	Includes a range of well-established ecological indictors. Encompasses a diverse range of ecological niches. Some taxa are important bioirrigators, sediment processors and an important food source for fish.	Molecular data detected greater diversity than traditional data. The declines in abundance could only be obtained from the traditional data.
Arthropods	OTUs associated with the family Miraciidae were indicative of both the control/low, control/low/moderate and high groups.	Harpacticoids and their nauplii were less abundant in the HT.	Includes taxa that are critical for the transfer of energy (trophic), as well as taxa commonly used in ecotoxicological studies.	Different taxa were identified from two approaches.
		Amphipod taxa aided discrimination among HT and other spiked treatments.		The declines in abundance could be obtained only from the traditional data. Both data provided relevant ecological endpoints.

(*Continued*)

Table 2. (*Continued*)

Endpoint	Molecular	Traditional	Ecological significance	Considerations
Kinorhyncha	Completely absent in the HT.	Kinorhyncha abundance was lower in HT.	Potentially sensitive to environmental contamination.	Both approaches produced similar findings. More taxonomic specific information, i.e. the loss of OTUs associated with Pycnophyidae was obtained from the molecular data. This information could be used to further explore the sensitivity of taxa associated with this Phylum.
Microbiota (e.g., cercozoans and alveloates)	Indicative of taxa from the higher dosed treatments.	Not examined	Fundamental for the functioning of benthic environments, including the biodegradation of organic materials, sediment structure and primary production.	Emphasizes the fundamental ecological components that can easily be examined using the molecular approach. Additional studies are required to determine if these patterns are reproducible and applicable to other environmental stressors.

OTU = operational taxonomic unit; NR = Narrow River treatment, unmanipulated field-collected sediment at the commencement of the experiment; FC = field control, the unmanipulated NR treatment at the completion of the experiment; CT = control triclosan treatment; LT = low triclosan treatment; HT = high triclosan treatment.

Currently, there is considerable interest in developing molecular-based ecological techniques to support, validate, or even replace time-consuming and costly morphological studies [37]. For the current application, the true advantage lies not in the approach's potential as a surrogate for traditionally obtained observations but rather in obtaining ecological data from biota generally excluded from ecological assessments. For example, a number of the OTUs that aided discrimination between the control/low group and the higher dosed treatments were associated with taxa beyond the scope of routine meio- and macrofaunal studies. This includes taxa (e.g., alveolates and cercozoans) essential for maintaining and supporting sediment structure and sediment function, and facilitating the biodegradation of organic materials [38,39]. With respect to triclosan, changes in sediment structure, decomposition, and food availability can be expected to occur as a result of the chemical's antibacterial properties [40].

Although clearly present in the combined control/low and moderate groups, Kinorhyncha showed pronounced declines in the high-dose treatment, with the phylum being undetected by the molecular analysis and observed only twice in the enumerated meiofaunal samples [9]. Quantification of Kinorhyncha obtained by Ho et al. [9] was restricted to the taxonomic level of phylum. However, the molecular data provided more detailed taxonomic information indicating that a majority of these highly respondent OTUs were associated with the order Homalorhagida and its family Pycnophyidae. Re-examination of the findings by Chariton et al. [18] suggests that sensitivity to environmental contaminants is not restricted to Homalorhagida, with responses also evident in the family Echinoderidae, from the order Cyclorhagida. Rarely examined in routine monitoring programs, Kinorhyncha shows great promise as an indicator of environmental degradation [18,41,42].

The ecological interpretation of molecularly derived measurements of eukaryl composition has a number of caveats associated with it. First, the detection of an organism does not mean that the organism was alive at collection, and the latency between mortality and the degradation of DNA could potentially cofound interpretation [1]. As triclosan is a potent antibacterial agent, it is plausible that decomposition rates are attenuated with increasing triclosan concentration. While the experiments were designed to minimize the vertical migration of dead DNA from the triclosan-treated sediments to the DNA-free sediment placed above, the organisms detected by the analyses may include those that have been adhered to or consumed by another organism. Consequently, the presence of this DNA may not indicate that the organisms were in sufficient condition to vertically migrate through the applied sediment layers. Finally, the taxonomic assignment given to OTUs should be viewed conservatively, as assignment is highly dependent on the bioinformatic approach, the choice of primers, and the quality and relevance of sequences retained within online repositories [43,44]. While we have been cautious not to overinterpret the results, it is emphasized that multiple approaches are required to obtain fine-resolution taxonomic information for multiple phyla, with the validation of suitable markers for many taxa still unresolved.

The findings from the molecular analysis support those of Ho et al. [9] and clearly demonstrate that the application of sediment alone had a detrimental effect on the eukaryote composition measured (field control vs triclosan control). As anticipated, this artifact was more pronounced in molecular analyses (50% decline in richness), as a large proportion of the mortalities occurred in small, sessile microorganisms (diatoms and cercozoa) and fungi. In contrast, data derived from the present study clearly showed a pronounced increase in the biodiversity of the samples during the experimental period (original Narrow River sediment vs field control containing the same sediment 2 wk later). As previously noted, the filtered water used for the flow-through system is the only viable source of the extra DNA. In practical terms, this increase in diversity would have had little or no effect on either the traditionally obtained or molecular data, because the dispersal of the exogenous genes would be randomly spread across all treatments.

CONSIDERATIONS FOR FUTURE STUDIES

As demonstrated, combining DNA-derived ecological data with the environmental realism associated with microcosms has the potential to provide informative ecotoxicological data that encompass the responses of a diverse range of biota. Clearly, a number of aspects require refinement and consideration for

future studies. Although some limitations (e.g., sample size) are inherent in manipulative experiments [45], others (e.g., bioinformatic challenges) reflect some of the current limitations associated with obtaining robust ecological data from DNA [44] and are undoubtedly resolvable.

For future studies, we make the following suggestions and recommendations: First, as noted by Ho et al. [9], smothering during the application of the spiked sediment had a pronounced effect on biological composition. Modifications to the application procedure are currently being trialed to minimize this artifact. Second, to provide a more accurate view of the occurrences/absences of OTUs within and among treatments, it is suggested that multiple subsamples be sequenced from each sample. Third, chemical data should be obtained from each sample, enabling a greater range of statistical tools to be used to examine the relationships between the biota and contaminant. Fourth, multiple primers should be used to encapsulate a wider breadth of biota and to provide greater taxonomic resolution. Fifth, to reduce taxonomic uncertainty and to assist in developing predictive models, local databases should be created using validated, regionally relevant taxa. Lastly, bioinformatic pipelines need to be refined to address the ambiguity between OTUs and unique gene variants. Resolving this issue would not only provide a more accurate view of diversity, but also assist in identifying indicator taxa rather than artificially created OTUs, which vary in accordance with the choice of the bioinformatic pipeline and the parameters they contain.

The underlying assumption of this experiment was that biota would vertically migrate through the sediment to reach the oxic layer, whereby they would be sampled. With all but a few exceptions, eukaryotes are reliant on oxygen for respiration; however, atypical responses such as a preference for suboxic and even anoxic conditions have frequently been reported [46]. Some ciliates, nematodes, and oligochaetes inhabit low oxygen environments to facilitate their symbiotic relationships with sulfur bacteria [46], whereas others (e.g., nematodes from the genus *Sabatiera*, one of the indicators identified in the present study) appear to be resilient to extended periods of hypoxia [47]. Consequently, the omission of some taxa from the study's ecological endpoint may have not been associated with the toxicant but rather may reflect differences in anoxia tolerance and habitat preference.

CONCLUSIONS

The ability to obtain ecological data unconstrained by taxonomic expertise, size, and morphology provides an exciting prospect for ecologists. For ecotoxicologists, such ecological information is of considerable importance, as it is highly probable that many of the assumptions regarding the responses of biological communities to environmental stressors will require correction simply because conventional taxonomy has been so limited. As we have demonstrated, the most powerful attribute of such studies is that they allow a diverse range of organisms, including their varied life cycles, tolerances, and abiotic and biotic interactions, to be included in biodiversity assessments. The interpretation of such data is often complex, and for many taxa, there is no precedent to determine whether a response to a particular environmental condition is characteristic or not. We envision that, as the field matures and sufficient data become available, new robust and predictive ecological models will be developed to improve understanding of how total biodiversity and its various constituents respond to combinations of natural and anthropogenic stressors. While there are concerns, including those raised in the present study, regarding the tenuous links between OTUs and species, analytical biases (e.g., sequencing errors and PCR artifacts), and the inability to extract quantifiable information from complex mixtures of eukaryl genes, we believe that many of these issues are transient and are likely to be resolved by the refinements that single-molecule (PCR-free) sequencing and bioinformatics advancements promise. Despite these current limitations, we believe that the approach has considerable merit as an ecological line of evidence for ecotoxicological studies; however, we stress that it is purely one line of evidence, and where possible, the approach should be used to complement rather than replace traditional endpoints (e.g., toxicity testing and bioaccumulation studies).

SUPPLEMENTAL DATA

Table S1.
Figure S1. (70 MB DOC.)

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