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Environmental DNA metabarcoding reveals primary chemical contaminants in freshwater sediments from different land-use types

Yuwei Xie [a], Jizhong Wang [b], Jianghua Yang [a], John P. Giesy [a, c, d, e], Hongxia Yu [a], Xiaowei Zhang [a, *]

[a] State Key Laboratory of Pollution Control & Resource Reuse, School of the Environment, Nanjing University, Nanjing, 210023, China
[b] Laboratory for Nanomineralogy and Environmental Material, School of Resources and Environmental Engineering, Hefei University of Technology, Hefei, 230009, China
[c] Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[d] School of Biological Sciences, University of Hong Kong, Hong Kong Special Administrative Region
[e] Department of Biology, Hong Kong Baptist University, Kowloon, Hong Kong Special Administrative Region

HIGHLIGHTS

- Annelida and Arthropoda dominated the eukaryotic communities in sediment samples.
- Variations of the eukaryotic communities were associated with land-use types.
- Dissimilarity of community structures revealed the effects of primary contaminants.

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ABSTRACT

Land-use intensification threatens freshwater biodiversity. Freshwater eukaryotic communities are affected by multiple chemical contaminants with a land-use specific manner. However, biodiversities of eukaryotes and their associations with multiple chemical contaminants are largely unknown. This study characterized *in situ* eukaryotic communities in sediments exposed to mixtures of chemical contaminants and assessed relationships between various environmental variables and eukaryotic communities in sediments from the Nanfei River. Eukaryotic communities in the sediment samples were dominated by Annelida, Arthropoda, Rotifera, Ochrophyta, Chlorophyta and Ciliophora. Alpha-diversities (Shannon entropy) and structures of eukaryotic communities were significantly different between land-use types. According to the results of multiple statistical tests (PCoA, distLM, Mantel and network analysis), dissimilarity of eukaryotic community structures revealed the key effects of pyrethroid insecticides, manganese, zinc, lead, chromium and polycyclic aromatic hydrocarbons (PAHs) on eukaryotic communities in the sediment samples from the Nanfei River. Furthermore, taxa associated with land-use types were identified and several sensitive eukaryotic taxa to some of the primary contaminants were identified as potential indicators to monitor effects of the primary chemical contaminants. Overall, environmental DNA metabarcoding on *in situ* eukaryotic communities provided a powerful tool for biomonitoring and identifying primary contaminants and their complex effects on benthic eukaryotic communities in freshwater sediments.

1. Introduction

Freshwater ecosystems are threatened by land-use intensification through changes of hydrodynamics and discharging of land-use specific chemical contaminants during rapid industrialization and intensive agriculture (Saxena et al., 2015). Alpha-diversity and beta-diversity of ecosystems may be reduced by the land-use intensification (Gossner et al., 2016). Chemical contaminants in

* Corresponding author. State Key Laboratory of Pollution Control & Resource Reuse, School of the Environment, Nanjing University, 163 Xianlin Great Rd, Nanjing, 210023, China.
E-mail address: howard50003250@yahoo.com (X. Zhang).

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sediments vary according to land-use types, for instance, metals and PAHs mainly released from the industrial areas (Ordonez et al., 2003; Sindern et al., 2007); organophosphorus pesticides and pyrethroids insecticides mainly discharged from nonpoint sources in the agricultural regions. Chemical pollution can cause long-term systematic effects at all levels of biological organization from the molecule, individual (population) to community and ecosystem, and eventually lead to losses of biodiversity and ecological functions (Eggleton and Thomas, 2004; Fent, 2003; Lake et al., 2000). Ecogenomics has been previously used to demonstrate metals and land-use pressures on prokaryotic communities in freshwater sediments (Saxena et al., 2015; Xie et al., 2016). However, ecological responses of aquatic eukaryotic communities, particularly eukaryotic microbial communities, to multiple chemical contaminants and land-use types were largely unknown.

Eukaryotic communities are the major components of freshwater aquatic biomass, even in very contaminated rivers (Amaral Zettler et al., 2002). Algae, fungi, protozoa and metazoa compose the freshwater benthic ecosystems through both direct and indirect trophic interactions and interaction cascades (Peterson et al., 2003). Within these assemblages, periphyton and benthic macroinvertebrates have been used as indicators of the health of ecosystems. Ubiquitous protists are essential components of food webs. Protists graze on bacteria and link primary producers and decomposers with higher trophic levels (Bonkowski and Brandt, 2002; Clarholm, 1985; Rocke et al., 2015). Their high rate of metabolism facilitates fluxes of carbon and energy through ecosystems (Bitencourt et al., 2014). Due to the enormous diversity of eukaryotic communities, parallel biomonitoring on various eukaryotic assemblages is limited by the bottlenecks of morphology-based taxonomy (Thomsen and Willerslev, 2015). The recently developed environmental DNA metabarcoding provides a powerful tool for fine-scale biomonitoring (Thomsen and Willerslev, 2015). A comprehensive survey of the taxa using environmental DNA metabarcoding is quicker and cheaper than the morphology-based approach.

Many vital ecological components, including benthic eukaryotic microbial communities, have always been neglected in conventional sediment quality assessments (Faris et al., 2009; Sabater et al., 2007). A decline in eukaryotic microbial biodiversity and activity caused by multiple chemical contaminants could affect the geochemical cycling of elements in aquatic ecosystems (Yergeau et al., 2012). However, we are still at the very beginning of understanding eukaryotic microbial communities in rivers at fine scales. The ecological responses of eukaryotic microbial communities to mixtures of chemical contaminants under field conditions are largely unknown.

To bridge the gaps between multiple chemical contaminants, land-use types and eukaryotic communities in sediments from the highly human-impacted Nanfei River (Anhui province, China). The Nanfei River flows through industrial, agricultural and confluence regions (Fig. 1) with different profiles of chemical contaminants (Ren et al., 2015; Wu et al., 2014; Xie et al., 2016). The objective of this study was to determine if *in situ* eukaryotic communities can be used to identify primary chemical pollution in freshwater sediments from different land-use regions. Specific objectives of this study were to: 1) characterize the eukaryotic communities in the freshwater sediment samples of three land-use types (industrial, agricultural and confluence regions) from the Nanfei River; 2) examine relationships of the alpha-diversities and structures of eukaryotic communities with concentrations of chemical contaminants and 3) investigate the primary chemical pollution driving *in situ* eukaryotic communities. In this study, the eukaryotic communities were characterized using the environmental DNA metabarcoding approach, semi-quantitative 18S rDNA gene amplicon next generation sequencing.

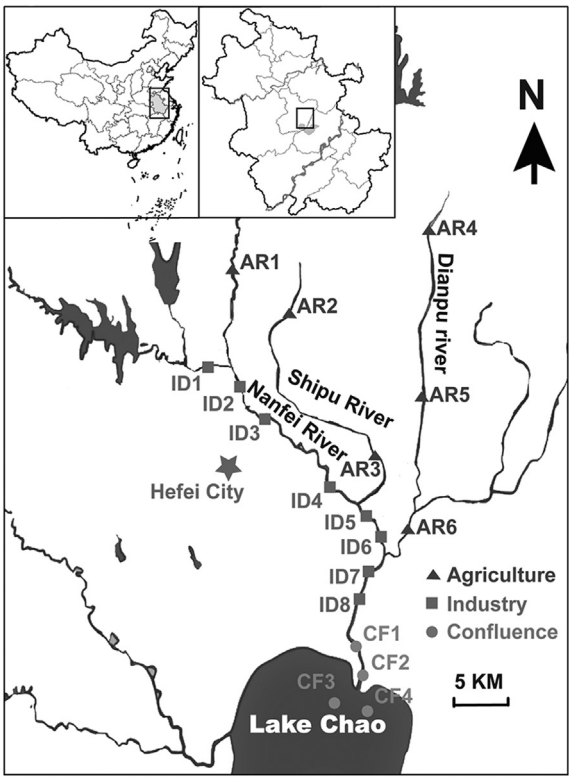

Fig. 1. Locations of sampling sites on the Nanfei River of Anhui province, China. Sampling sites were grouped into three types according to land-use characteristics and hydrology.

2. Materials and methods

2.1. Sampling sites

The Nanfei River is one of the largest rivers discharging into Lake Chao, China. It is about 70 km long with a catchment area of 1446 km^2. The Nanfei River and its tributary, the Shipu River, pass through an urbanized region with a population of more than 3 million, while another tributary of the Nanfei River, the Dianpu River, passes through an agricultural region with a population of approximately 0.9 million. The stream flow of Nanfei River is very slow (average 0.07 m/s). According to the hydrology and land-use characteristics, sampling sites were grouped into three land-use types (agriculture, industry or confluence) (GlobeLand30 web tools) (Han et al., 2015). In this study, the agricultural group contained six sites; the industrial group contained eight sites and the last confluence group contained four sites (Fig. 1, Supplementary Table S1).

2.2. Sediment collection and processing

Sediment samples were collected on September 25 & 26, 2012, along with the Nanfei, Shipu and Dianpu rivers. The temperature of water varied from 23.2 to 25.9 °C. Three grabs of sediment were collected at each site (approximately 2.5 m depth) using the Van Veen grab sampler. Surface sediments (top 5 cm) were sampled, pooled, and homogenized. About 40 mL aliquot of homogenized surface sediment was stored in a sterile 50 mL tube. Remaining, approximately 2 L of surface sediments from each site, were combined and stored in a plastic bag. Samples were delivered to the

laboratory in the dark on ice within the same day and frozen at −20 °C. Subsamples of sediments were freeze-dried and homogenized for measurement of environmental variables, including pH, grain size, organophosphorus insecticides (OPs), type I pyrethroid pesticides (TI-PRTs), type II pyrethroid pesticides (TII-PRTs), 16 USEPA priority PAHs, metals (Fe, Zn, Cd, Cr, Pb, Ni, Cu and Mn), total nitrogen (TN) and total phosphorus (TP). Analytical method and results of those environmental variables have been reported in the previously published papers (Supplementary Table S2) (Ren et al., 2015; Wu et al., 2014; Xie et al., 2016).

2.3. DNA isolation, PCR amplification and next generation sequencing

DNA was extracted from a 0.25 g aliquot of a homogenized sample with the MoBio Power Soil DNA Kit (MoBio Laboratories Inc., CA, USA). Extracted DNA was quantified and checked for purity at A260/280 nm in a Take3 microplate in the Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, VT, USA) before storage at −80 °C. Eukaryotic 18S rRNA genes were amplified by PCR from DNA using the V9 primers (Amaral-Zettler et al., 2009). A 20 μL reaction system was utilized for each PCR amplification with Platinum® Taq polymerase (Life Technologies, CA, USA). Amplifications were conducted in a SureCycler 8800 Thermal Cycler (Agilent Technologies, CA, USA) under the following conditions: initial denaturation at 94 °C for 2 min, 28 cycles at 94 °C for 15 s, 50 °C for 30 s and 68 °C for 30 s, and a final extension at 68 °C for 7 min. To minimize potential PCR bias, triplicate PCR reactions were performed for each sample. Ten ng of extracted DNA was used per 50 μL reaction mixture. PCR products were checked for size and specificity by electrophoresis on a 2% (w/v) agarose gel and were gel purified using the MinElute Gel Extraction Kit (Qiagen, CA, USA). Purified products were quantified by use of Qubit™ dsDNA HS Assay Kits (Invitrogen, CA, USA) and adjusted to 10 ng/μL in molecular grade water. Purified PCR products were pooled for subsequent sequencing. Sequencing adaptors were linked to purified DNA fragments with the Ion Xpress™ Plus Region Library Kit (Life Technologies, CA, USA). Before next generation sequencing, all DNA products were assessed for length and concentration using the Bioanalyzer 2100 (Agilent Technologies, CA, USA). Samples were adjusted to a final concentration of 100 pM. Sequencing templates were prepared with Ion OneTouch 2™ and sequenced in the Ion Proton sequencer (Life Technologies, CA, USA).

2.4. Bioinformatics

Raw reads were evaluated by use of the QIIME toolkit (Caporaso et al., 2010) and reads of low quality (mean quality < 20, scanning window = 50; contained ambiguous 'N'; sequence length: < 100 bp and > 180 bp) were discarded. Removal of chimeras and clustering of operational taxonomic units (OTUs) were conducted following the UPARSE pipeline (Edgar, 2013). Taxonomy annotation for each OTU was assigned by comparison with the Protist Ribosomal Reference database (Guillou et al., 2013). A small fraction of unexpected prokaryotic sequences was removed. To reduce biases resulting from differences in sequencing depth, the table of OTUs was rarefied at the least sequencing depth (23,514 sequences per sample). Alpha-diversity summarizes diversity of the eukaryotic community in each sample, whereas beta-diversity measures differences between samples. Observed OTUs (OTU richness) were measured at multiple sequencing depths. Shannon entropy, an alpha-diversity measurement of richness and evenness, was calculated on all twenty equal-depth rarefactions and then averaged. To determine whether eukaryotic community structures were different between land-use groups, beta-diversity was estimated by computing unweighted unifrac distances between samples.

2.5. Statistics

Statistical analyses were performed using R Statistical Language (https://cran.r-project.org/) and PRIMER7 with PERMANOVA+ add-on software (PRIMER-E Ltd, Ivybridge, UK). Environmental variables were firstly grouped or selected based on their chemical structure and concentration distribution, as the previously published paper (Xie et al., 2016). Environmental variables were natural logarithm transformed and then normalized. To identify the land-use type associated individual taxa, we conducted a univariate test for associations between groups and the eukaryotic classes using linear discriminant effect size (LEfSe) analyses (Segata et al., 2011). The nonparametric factorial Kruskal-Wallis (KW) test on ranks was conducted to detect significant differential features among the land-use types, and then, consistency was subsequently investigated using a set of pairwise tests among subgroups using the (unpaired) Wilcoxon rank-sum test. To determine whether overall eukaryotic community structures were different between land-use groups, unweighted unifrac distance matrices of the eukaryotic community were compared using permutational multivariate analysis of variance test (PERMANOVA). Statistical significance was set at $P < 0.05$, and the number of permutation test replicates was set at 9999. Relative contributions of contaminants and environmental variables in explaining differences in community structure were determined by forward selection distance-based linear modeling (distLM). The Mantel test was used to evaluate associations among community structures and environmental attributes of sediments (Diniz-Filho et al., 2013). Principal coordinate analysis (PCoA) was performed to visualize relationships between environmental variables and community structure. Due to the short length of the V9 region of 18s rDNA, for reliability, eukaryotic OTUs were collapsed at the class level. To get insight into an array of complex and previously poorly understood phenomena, network analysis was used for mapping and measuring of correlations (edges) between relative abundances of classes of eukaryotes and concentrations of contaminants (nodes) (Liu et al., 2015; Newman, 2006). An association network between eukaryotic classes was generated by SparCC (Friedman and Alm, 2012) with 100 bootstraps to assign P-values. The network was filtered to include only correlations with a correlation $\rho > 0.7$ and a 'two-tailed' P value < 0.01. Correlations between eukaryotic classes and environmental variables were confirmed to be robust if the Spearman's correlation coefficient ($|r_{spearman}|$) was > 0.6 and the adjusted FDR P value was statistically significant ($P_{FDR} < 0.05$). The network was displayed and analyzed with Cytoscape V3 (Shannon et al., 2003).

3. Results and discussion

3.1. Next generation sequencing data

Within 4,291,684 raw reads, the filtering process rejected 2,277,349 reads with low quality, and 669,004 reads with errors or mismatches in the barcode or primer sequences (Table S3). Rare 264,075 reads occurring only once or twice were abandoned. Another 174,633 reads (clustered as 1095 OTUs) un-annotated or misannotated against the reference database were discarded. Hence, a total of 906,623 eukaryotic reads were remained for further analyses after quality-checking. A total of 1,380 eukaryotic OTUs, representing 25 phyla, were discovered from those filtered reads. Sequencing depth of annotated reads in each sample varied from 23,514 to 126,738. Although rarefaction curves of observed

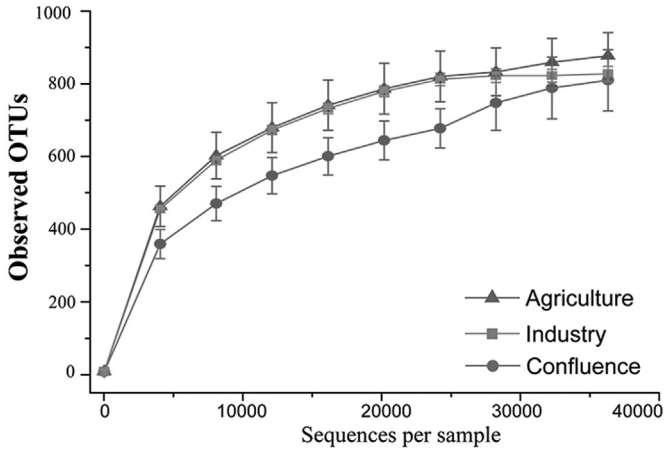

Fig. 2. Rarefaction curves of the observed numbers of eukaryotic OTUs.

OTUs were not saturated, most of the trends in the diversity of 18 samples were captured by rarefaction at 23,514 sequences per sample (Fig. 2). Low quality reads (accounting for about 53% of total raw reads) were due to PCR bias, poor quality of sequencing and short length (Bragg et al., 2013). About 4% of total sequenced reads (accounting for 44% of total clustered non-singleton OTUs) can't be assigned to known lineage due to lack of local reference database.

3.2. Compositions of eukaryotic communities in sediment from the Nanfei River

The eukaryotic Communities in sediment samples from the Nanfei River were dominated by metazoans (12.1–93.5%, mean 42.5%), and protozoa (3.6–54.5%, mean 29.1%), followed by algae (2.0–35.4%, mean 18.5%) and fungi (0.5–26.6%, mean 6.4%) (Fig. 3-A). The sum of these four assemblages accounted for about 98.7% of overall eukaryotes in the communities of sediment samples from the Nanfei River. Metazoans were dominated by Annelida (0.8–88.7%, mean 14.0%), Arthropoda (0.7–70.8%, mean 11.5%) and Rotifera (1.3–20%, mean 8.8%). Algae were dominated by Ochrophyta (1.2–22.9%, mean 8.6%), Chlorophyta (0.8–19.6%, mean 7.4%) and Dinophyta (0.1–9.5%, mean 2.6%). Ciliophora (2.6–43.7%, mean 21.6%), Cercozoa (0.3–9.7%, mean 3.0%) and Discoba (0.5–6.1%, mean 2.0%) dominated the protozoan assemblage (Fig. 3). Consistent with the results of previous studies, broad diversities of eukaryotes in the freshwater sediment were dominated by a small number of abundant taxa (Aguilera et al., 2007; Nolte et al., 2010; Simon et al., 2016; Slapeta et al., 2005).

3.3. Variations in the eukaryotic communities associated with land-use type

Environmental DNA metabarcoding revealed the land-use pressures on the eukaryotic communities in the sediment samples from the Nanfei River. Structures of eukaryotic communities significantly differed between the land-use types, as well as the bacterial communities in the sediments from the Nanfei River (PERMANOVA, $P < 0.001$) (Xie et al., 2016). The structures of eukaryotic communities displayed a significant separation among land-use types (Fig. 4-A). Additionally, alpha-diversity measures (Shannon entropy) were significantly different among land-use types (Fig. 4-B; KW tests, $P < 0.05$). However, Shannon entropy of bacterial communities in the sediments from the Nanfei River did not differ significantly between the land-use types, which might be due to the higher redundancy inherited from the great richness of bacterial communities (Torsvik et al., 2002; Xie et al., 2016). Five eukaryotic classes in the sediments associated with land-use type were identified (Fig. 4-C). Cercozoa novel clade 10–12 (Rhizaria) was enriched in the sediments of agriculture regions (Fig. 4-D); *Cryptomycotina* (Fungi) and *Chrysophyceae Synurophyceae* (Ochrophyta) were enriched in the sediments of industrial regions (Fig. 4-E and F); *Chromadorea* (Nematoda) and *Crustacea* (Arthropoda) were enriched in the sediments of confluence regions (Fig. 4-G and H).

Despite physical characteristics of the sediments, land-use pressures, mainly through chemical contaminants, may have effects on eukaryotic communities in the sediments from the Nanfei River. Chemical contaminants bound to sediments are a function of various agricultural, industrial and domestic activities of humans

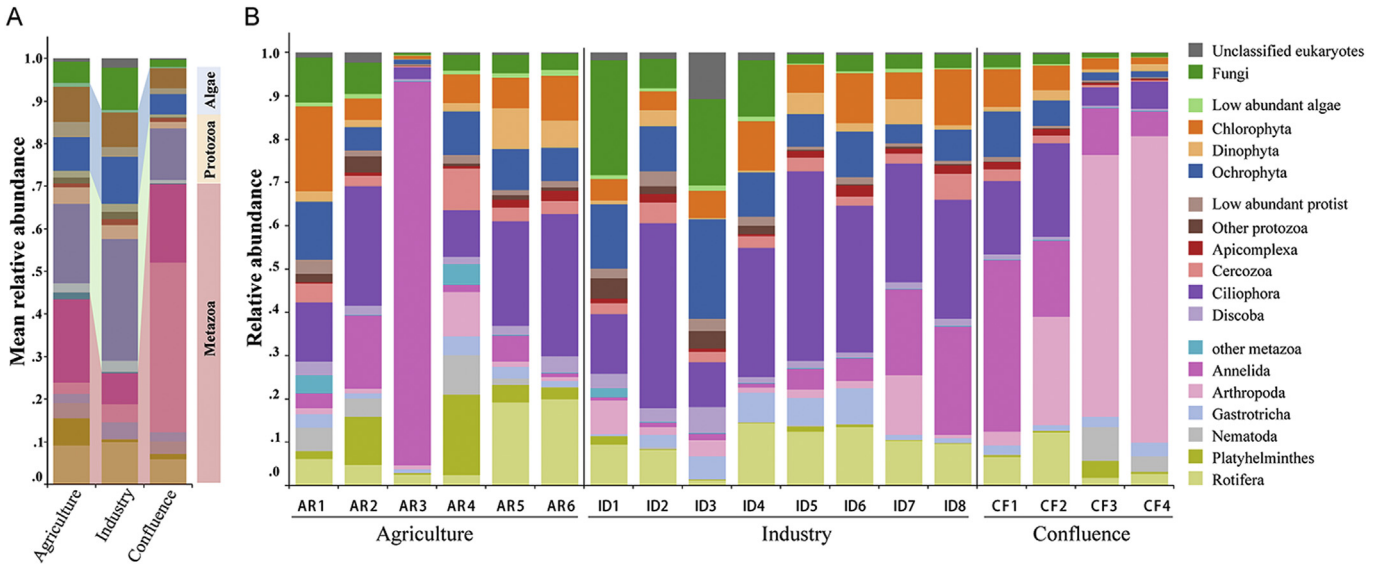

Fig. 3. Compositions of the eukaryotic communities in sediment samples from the Nanfei River. (A) Average relative abundances of eukaryotic taxa in each group. (B) Relative abundances of eukaryotic taxa in each sample. Low abundance phyla (<1%) were not presented. A significant fraction of denoised sequences could not be assigned to any taxa, indicating the extent of novel sequences in this study.

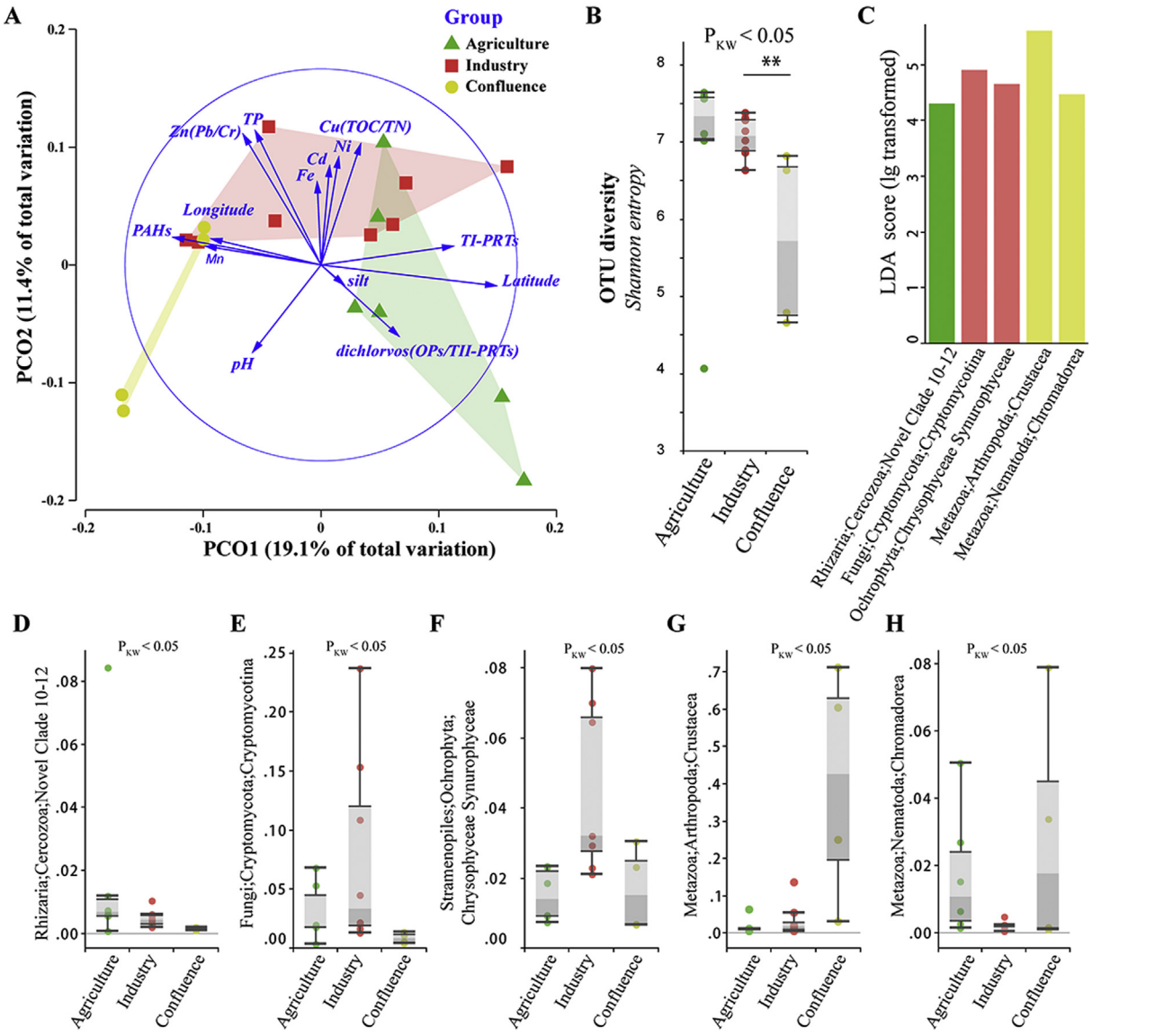

Fig. 4. Variations in the eukaryotic communities associated with land-use type. (A) Ordination of eukaryotic community structures (PCoA with unweighted Unifrac distance matrices). Blue vectors point to the direction of the increase for a given variable. Sediments with similar environmental variable profiles or eukaryotic communities are localized in similar positions in the diagram. (B) Alpha-diversity among different land-use groups. The Kruskal-Wallis test was performed, followed by post hoc Mann-Whitney U tests. P values < 0.01 (**). (C) LDA score of land-use type associated eukaryotic classes. (D) Relative abundances of land-use type associated eukaryotic classes among land-use groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

among the three land-use types. Metals in sediment are primarily discharged from traffic and industries and domestic activities such as wastewater treatment and can enter surface waters from point and non-point sources (Ordonez et al., 2003; Sindern et al., 2007). The primary sources of PAHs were emissions from transportation in urban-industrial areas of the Nanfei river basin. Pesticides mainly came from agricultural nonpoint sources. In most sediments from the regions classified as either industrial and/or confluence areas, concentrations of PAHs and metals (Cr, Cd, Cu, Pb and Zn) exceeded the threshold effects level (TEL), or even reached the probable effect level (PEL), where possible adverse biological effects would be expected (Long, 1992; Sun et al., 2013; Xie et al., 2016). Decreasing alpha-diversities from agricultural to industrial to confluence regions might be due to ecotoxicological effects caused by mixtures of toxic contaminants (Sun et al., 2013; Whale et al., 2003). Anthropogenic activities in different land-use areas might cause biodiversity loss of the eukaryotic communities in sediments through mixtures of chemical contaminants. The enrichment of *Synurophyceae* and *Cryptomycotina* in the sediments from the industrial region suggested that they were torrent to the effects of metals, while the enrichment of Novel Clade 10–12 (Cercozoa) in the sediments from the agricultural region potentially might be due to their sensitivities to PAHs and metals. Sediments classified as Confluence were enriched for *Chromadorea* and *Crustacea*, which was potentially due to their resistances to PAHs. However, no data on toxic potencies were available for these eukaryotic classes.

3.4. Variations in the structures of in situ eukaryotic communities associated with environmental variables

Observed differences in the structures of eukaryotic communities were consistent with the profiles of environmental variables among land-use types. Based on the results of the correlation analysis, 14 groups of environmental variabes were selected for

Table 1
DistLM results of eukaryotic community structures against fourteen selected predictor variables in the full analysis (9999 permutations). Bold = significantly correlated with community structure at a = 0.05. Prop. = Proportion of variation explained; Cumul. = Cumulative proportion of variation explained.

Environmental	Marginal tests			Forward selection sequential tests			
variable groups	Pseudo-F	P	Prop.	Pseudo-F	P	Prop.	Cumul.
Latitude	3.169	**<0.001**	0.165	3.169	**<0.001**	0.165	0.165
Zn(Pb/Cr)	1.882	**0.007**	0.105	1.760	**0.003**	0.088	0.253
Mn	2.068	**0.004**	0.114	1.612	**0.007**	0.077	0.330
Longitude	1.763	**0.012**	0.099	1.351	0.092	0.063	0.393
Silt	1.069	0.333	0.063	1.355	0.109	0.062	0.455
TI-PRTs	2.134	**0.003**	0.118	1.283	0.164	0.057	0.512
Cd	1.149	0.219	0.067	1.068	0.401	0.047	0.559
PAHs	2.280	**0.001**	0.125	1.103	0.368	0.048	0.607
Ni	1.332	0.103	0.077	1.007	0.473	0.044	0.651
Dichlorvos (OPs/TII-PRTs)	1.400	0.069	0.080	0.993	0.476	0.043	0.694
TP	1.767	**0.012**	0.099	1.387	0.212	0.057	0.752
Cu(TOC/TN)	1.466	0.052	0.084	1.422	0.226	0.055	0.807
pH	1.279	0.135	0.074	1.020	0.456	0.039	0.846
Fe	0.979	0.468	0.058	0.898	0.522	0.035	0.881

multiple association analyses (Table S4) (Xie et al., 2016). Strong, non-random associations between phylogenetic traits of the eukaryotic communities and groups measured environmental variables were also observed (Procrustes analysis, $M^2 = 275.1$, $P < 0.001$). About 48.2% of the variations in community dissimilarity were associated with the overall measured environmental variable distances (Mantel tests, $P_{PERM} < 0.001$). Based on the results of DistLM test, approximately 88% of the variations in community structure dissimilarity could be explained by 14 environmental variable groups altogether (Table 1). Latitude, longitude, Zn(Pb/Cr), Mn, TI-PRTs and PAHs significantly were correlated with the dissimilarity of community structures among sediments (Table 1; Marginal tests). Environmental variables, arranged in decreasing order of influence, were latitude, Zn(Pb/Cr), and Mn which together could explain 38.8% of community variations (Table 1; Forward selection sequential tests). Based on the results of Mantel tests (Table 2), the Geographic distance (latitude/longitude) was the most environmental variable associated with dissimilarity among structures of communities, followed by TI-PRTs, Mn, Zn(Pb/Cr), Cu(TOC/TN), TP, PAHs and dichlorvos (OPs/TII-PRTs). Ordination of community structures revealed greater concentrations of nutrient (TN, TP) and metals (Cd, Cr, Cu, Pb and Zn) in the sediments from industrial region, greater concentrations of PAHs and Mn in the sediments from confluence region and greater concentrations of OPs and TII-PRTs in the sediments from agricultural region (Fig. 4-A) (Xie et al., 2016). About 31.2% of variations among structures of communities were explained by the first two principal components (PCO1 and PCO2). Variations in pH, Mn, longitude, TI-PRTs, latitude, OPs and TII-PRTs were mostly loaded to PCoA1, whereas the other environmental variable groups were mainly loaded to PCoA2.

Chemical contaminants can influence eukaryotic communities in complex ways. Based on the consensus results of DistLM and Mantel tests, the dissimilarity of eukaryotic community structures in sediment samples from the Nanfei River was driven mostly by effects of TI-PRTs, Mn, Zn(Pb/Cr) and PAHs. Compared to the bacterial communities responding to the pressures of metals (Fe, Ni and Zn) and nutrients (TN, TP) (Xie et al., 2016), the eukaryotic communities were more sensitive to organic contaminants, for instance, PAHs and TI-PRTs. Zn and Mn have been classified as micronutrients, required by organisms that are cofactors for some enzymes (Andreini et al., 2008; Hansch and Mendel, 2009). However, some metals are toxic to freshwater ciliated protists (Madoni and Romeo, 2006), algae (Duong et al., 2010) and benthic macroinvertebrates (Faupel et al., 2012; Leung et al., 2016; Monteiro et al., 2014). Additionally, pesticides and PAHs can adversely affect the structures and functions of freshwater ecosystems and can contribute to local and regional losses of freshwater biodiversity and ecosystem services (Malaj et al., 2014). However, bioavailable organic contaminants can, under specific conditions, also be sources of carbon and energy for adapted eukaryotic microbial organisms (Subashchandrabose et al., 2013). Furthermore, since freshwater ecosystems are structured by both direct and indirect trophic interactions and interaction cascades (Peterson et al., 2003), chemical contaminants might influence the freshwater eukaryotic community through indirect effects (such as via food webs) as well (Almeda et al., 2014; Cerezo and Agusti, 2015; Enrique et al., 2007).

3.5. Association networks between eukaryotic classes and environmental variables

Network analysis highlighted the strong correlations of eukaryotic classes with Cd, PAHs, Zn, Pb, Cr and TP. Chemical contaminants (Cd, PAHs, Zn, Pb, Cr and TP) were significantly correlated with several eukaryotic classes and formed three clusters in the association network (Fig. 5). Cluster 1 was dominated by the classes from fungi and protozoa, which were significantly and negatively correlated with the concentrations of PAHs. Cluster 2 was dominated by the classes of algae and metazoan. The concentrations of TP, Zn, Pb and Cr were significantly and negatively

Table 2
Associations between eukaryotic community structures and each environmental variable of the sediments with Mantel test (9999 permutations). Geographic distances between every two sampling locations were calculated using the "Vincenty" formula (WGS-84) from the latitudes and longitudes. Dissimilarity matrices for the eukaryotic communities and environmental variables were constructed using unweighted unifrac and Euclidean distances, respectively. **Bold** = significantly correlated with community structure at a = 0.05.

Environmental variable	Rho	P_{PERM}
Geographic distance (Latitude/Longitude)	0.552	**<0.001**
TI-PRTs	0.481	**<0.001**
Mn	0.371	**0.002**
Zn (Pb/Cr)	0.315	**0.009**
Cu (TOC/TN)	0.276	**0.009**
TP	0.263	**0.005**
PAHs	0.245	**0.004**
Dichlorvos (OPs/TII-PRTs)	0.205	**0.043**
pH	0.098	0.159
Cd	0.073	0.259
Fe	0.029	0.371
Ni	−0.012	0.515
Silt	−0.114	0.834

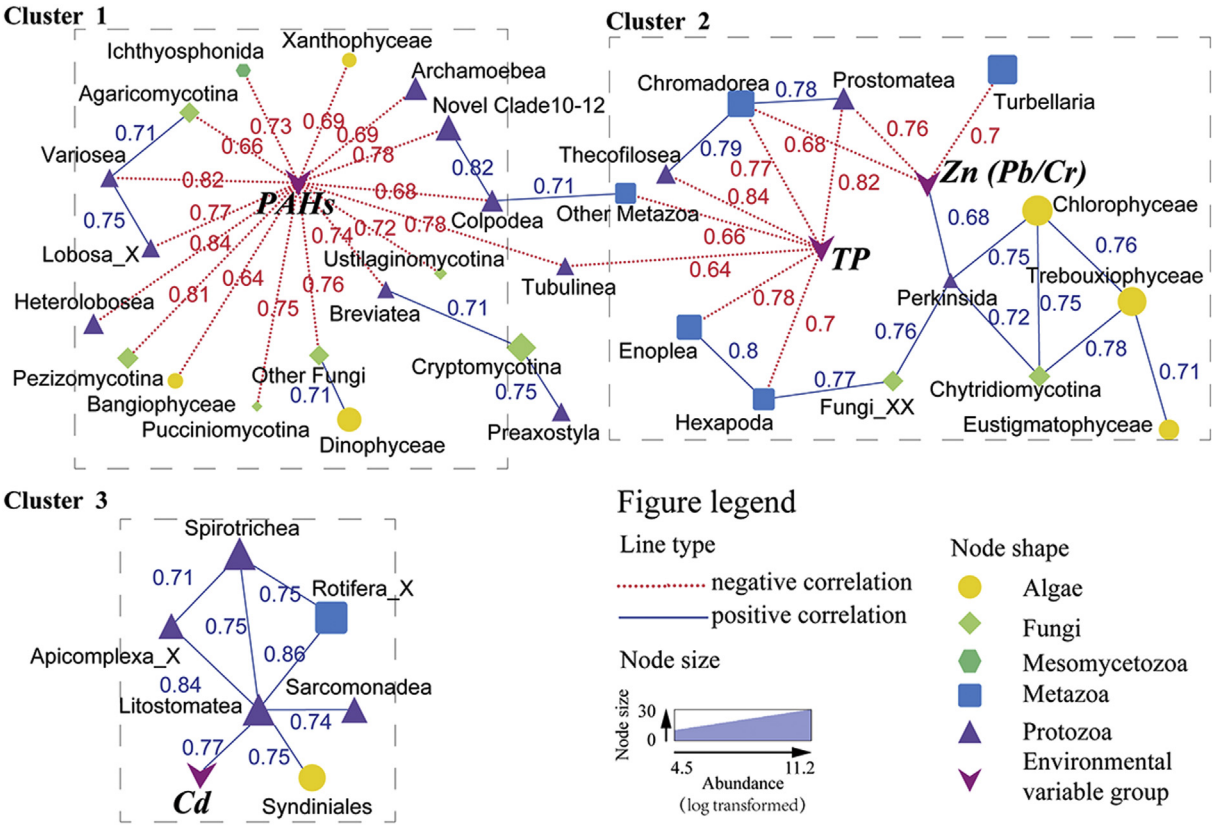

Fig. 5. Association networks between eukaryotic families and contaminants in sediment samples from the Nanfei River. Associations between eukaryotic classes were generated by SparCC with 100 bootstraps to assign P-values. The associations were filtered to include only correlations with a correlation $\rho > 0.7$ and a 'two-tailed' P value < 0.01. The correlations between eukaryotic classes and environmental variables were confirmed to be robust if the Spearman's correlation coefficient ($|r_{spearman}|$) was >0.60 and the adjusted FDR was statistically significant ($P_{FDR} < 0.05$). Correlation coefficients between two nodes were labeled, the positive coefficient in blue, while negative coefficient in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

correlated with the relative abundances of classes (*Tubulinea*, *Hexapoda*, *Enoplea*, *Thecofilosea*, *Chromadorea*, *Prostomatea*, and *Turbellaria*) from metazoan and protozoa, while *Perkinsida* was positively correlated with Zn (Pb/Cr). In cluster 3, the concentrations of Cd were positively correlated with the relative abundances of *Litostomatea*, which was potentially due to its tolerance to Cd.

The association network revealed ecological effects of chemical contaminants (PAHs, Zn, Pb, Cr and TP) on eukaryotic communities, which were consistent with the results of multivariate statistics (PCoA, distLM and Mantel tests). The concentrations of TI-PRTs were significantly associated with variations of eukaryotic community structures, while there were no eukaryotic classes which relative abundances were significantly correlated with the concentrations of TI-PRTs. Both abundant prokaryotic (Xie et al., 2016) and eukaryotic classes demonstrated effects of Zn, Pb, Cr and TP. However, compared with the bacterial-contaminant association network (Xie et al., 2016), eukaryotic classes were more sensitive to PAHs. Negative associations between eukaryotic classes and PAHs, Zn, Pb, Cr and TP suggested toxic effects of those contaminants to eukaryotic organisms. Protozoa were more sensitive to chemical contaminants (accounting for 42.3% of total negative correlations in the network) than other eukaryotic assemblages. Ubiquitous protozoans are susceptible to PAHs in freshwater ecosystems (Sibley et al., 2004). Overall, mixtures of PAHs, nutrients and heavy metals can cause systemic effects on freshwater ecosystems (Malaj et al., 2014; Rhind, 2009).

The environmental DNA metabarcoding and multivariate statistical approaches provide alternative evidence in identifying multiple drivers and their complex effects on freshwater ecosystems, rather than environmental parameter characterizing (Saxena et al., 2015). Since not all possible chemical residues were investigated, it is impossible to definitively state what the causative agents were. Here the relationships between environmental variables and eukaryotic communities were identified by correlation analysis, but not causal study. For instance, the correlations observed between structures of eukaryotic communities and PAHs might only be an indicator of TOC of sediments. It could also be a surrogate measure for oil and grease that are known to occur in more industrial and urban areas and also have adverse effects on benthic infauna. However, this analysis reveals effects of potential contaminant stressors on the overall structure of the eukaryote community. Other approaches, such as toxicity identification evaluation (TIE) experiment and sediment mesocosm studies, could be conducted to confirm the actual causative agents (Yi et al., 2015).

4. Conclusion

This study characterized *in situ* eukaryotic communities and assessed the relationships between environmental variables, land-use types and eukaryotic communities in the sediments from the Nanfei River. The eukaryotic communities were dominated by a small number of more abundant taxa. Variations in the eukaryotic communities were associated with land-use types. The dissimilarity of community structures revealed primary effects of TI-PRTs, Mn, Zn, Pb, Cr and PAHs on the eukaryotic communities. Land-use type and chemical contaminant associated eukaryotic classes were identified as potential indicators to monitor contaminants. The

environmental DNA metabarcoding approach applied in this study can be used to monitor the status and trends in biodiversities of eukaryotic communities as a function of space and time. Overall, *in situ* eukaryotic communities could provide a useful tool for sediment quality assessment. Furthermore, this study could provide an ecogenomics framework for the ecological risk assessment of riverine ecosystems nearby the Bohai Sea threatened by numerous chemical contaminants discharged from agricultural, industrial and domestic activities.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.12.117.

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Environmental DNA metabarcoding reveals primary chemical contaminants in freshwater sediments from different land-use types

Yuwei Xie[a], Jizhong Wang[b], Jianghua Yang[a], John P. Giesy[a,c,d,e], Hongxia Yu[a], Xiaowei Zhang[a*]

[a] State Key Laboratory of Pollution Control & Resource Reuse, School of the Environment, Nanjing University, Nanjing, 210023, China

[b] Laboratory for Nanomineralogy and Environmental Material, School of Resources and Environmental Engineering, Hefei University of Technology, Hefei, 230009, China

[c] Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[d] School of Biological Sciences, University of Hong Kong, Hong Kong, SAR, China

[e] Department of Biology, Hong Kong Baptist University, Kowloon, Hong Kong, SAR, China

* Corresponding author. State Key Laboratory of Pollution Control & Resource Reuse, School of the Environment, Nanjing University, 163 Xianlin Great Rd, Nanjing, 210023, China.

E-mail address: howard50003250@yahoo.com (XW Zhang)

Supporting information

Table S1. Minimum, maximum, median, means and SD values of chemicals measured in the study (Xie et al., 2016).

Variable	Minimum	Maximum	Median	Means	SD
TN (%)	0.06	0.55	0.21	0.22	0.12
TP (%)	0.06	0.66	0.38	0.39	0.19
TOC (%)	0.16	4.96	1.37	1.48	1.07
Cd*	0.02	6.47	0.46	0.81	1.42
Cr*	29.92	109.57	56.30	62.51	27.96
Pb*	8.44	77.56	25.26	34.78	21.64
Ni*	1.10	4.58	2.10	2.27	0.90
Cu*	9.89	122.62	32.04	43.15	31.61
Fe*	18244.84	48810.28	31902.89	31547.95	7788.12
Zn*	4.56	1175.27	205.20	393.23	390.17
Mn *	126.40	368.30	202.11	215.17	73.32
naphthalene #	194.79	36321.63	5660.32	10474.53	11710.54
acenaphtylene #	518.55	7280.16	2533.13	3215.66	2219.55
acenaphthene #	83.89	74424.28	1490.01	14273.89	20536.68
fluorene #	827.63	104462.70	9514.53	22830.72	28978.44
phenanthrene #	2989.38	173993.70	26366.82	47163.75	52307.06
anthracene #	432.62	55903.95	3777.19	15006.21	17858.86
fluoranthene #	8709.53	231412.40	42288.42	73447.05	72658.50
pyrene #	3101.82	170701.80	31515.77	54962.96	58737.24
benzo[a]anthracene #	6163.13	141781.00	30754.22	46888.67	42182.71
chrysene #	9882.39	146554.00	38543.78	50236.54	41028.09
benzo[b]fluoranthene #	1893.19	51016.95	12915.61	17767.94	15540.74
benzo[k]fluoranthene #	5143.66	81684.91	17501.07	24685.65	21010.32
benzo[a]pyrene #	6213.77	138447.80	24599.95	41004.63	36738.72
indeno[1,2,3-cd]pyrene #	12684.56	251734.50	48982.18	63091.90	62994.13
dibenzo[a,h]anthracene #	3645.64	83892.29	16855.00	22471.46	21948.00
benzo[g,h,i]perylene #	14985.86	304854.20	51709.13	72623.35	73392.90
methidathion #	0.50	12.87	0.92	3.15	4.36
dichlorvos #	44.68	618.20	151.32	188.42	141.31
dyfonate #	0.02	0.30	0.08	0.11	0.07
diazinon #	0.46	47.06	6.98	14.69	16.26
CFVM #	0.35	8.93	1.79	2.88	2.39
fenitrothion #	0.07	4.28	0.23	0.71	1.03
malathion #	0.10	2.10	0.37	0.59	0.60
parathion #	0.19	11.18	0.79	1.63	2.48
chlorfenvinphos #	0.02	3.76	0.12	0.38	0.84
ethion #	0.01	0.52	0.03	0.07	0.12
carbophenothion #	0.00	0.10	0.01	0.02	0.02

Variable	Minimum	Maximum	Median	Means	SD
phosalone #	0.05	50.17	0.87	4.00	11.28
tefluthrin #	0.01	3.26	0.15	0.66	0.93
bioallethrin #	0.70	6.35	2.00	2.69	1.70
prallethrin #	0.07	1.93	0.29	0.47	0.46
tetramethrin #	0.39	18.10	1.55	2.60	3.91
permethrin #	24.79	1639.65	262.73	356.62	378.58
cyfluthrin #	5.43	205.99	9.36	22.51	45.18
cypermethrin #	76.94	1135.08	239.16	305.56	282.47
fenvalerate #	39.46	2380.58	156.80	381.99	584.33
deltamethrin #	11.59	113.93	23.48	33.11	25.07
Cyhalothrin #	22.58	2580.55	81.80	230.87	573.53
esfenvalerate #	12.02	545.97	36.75	96.72	140.18
allethrin #	0.29	7.82	2.01	2.31	1.73

* (mg/kg dry wt)

\# (ug/kg TOC dry wt)

Table S2. Raw, denoised reads, and numbers of alpha diversity of each eukaryotic community.

Sample	Quality-checking			Alpha diversity			
	Filtered	clean	Annotated	OTU #	Faith's phylogenetic distance	Shannon	Pielou equitability
AR1	69984	53042	34881	910.5 ± 8.48	76.684 ± 0.619	7.585 ± 0.011	0.772 ± 0.001
AR2	97194	77961	69386	787.2 ± 9.31	68.888 ± 1.393	7.109 ± 0.01	0.739 ± 0.001
AR3	144303	130563	126738	478.2 ± 10.06	47.769 ± 1.422	4.066 ± 0.013	0.457 ± 0.002
AR4	105361	81922	68346	880.85 ± 13.33	70.973 ± 1.093	7.018 ± 0.013	0.717 ± 0.002
AR5	39650	29633	25043	916.3 ± 3.03	75.624 ± 0.618	7.562 ± 0.003	0.769 ± 0.001
AR6	99563	81071	56970	912.85 ± 10.33	74.365 ± 0.917	7.636 ± 0.013	0.776 ± 0.001
CF1	52047	43832	37897	801.85 ± 7.8	66.221 ± 0.815	6.819 ± 0.01	0.707 ± 0.001
CF2	37913	30686	27183	718.95 ± 3.46	62.404 ± 0.498	6.635 ± 0.007	0.699 ± 0.001
CF3	51300	44180	41308	590.45 ± 6.21	51.782 ± 0.817	4.79 ± 0.018	0.52 ± 0.002
CF4	145799	120186	113642	576.75 ± 9.61	52.123 ± 0.919	4.66 ± 0.016	0.508 ± 0.002
ID1	49103	39628	31590	802.6 ± 6.26	68.18 ± 0.719	6.891 ± 0.01	0.714 ± 0.001
ID2	67588	54127	39407	876.9 ± 7.71	73.175 ± 1.495	7.017 ± 0.01	0.718 ± 0.001
ID3	44627	34365	25366	804.85 ± 2.96	68.07 ± 0.34	6.863 ± 0.005	0.711 ± 0.001
ID4	88733	69450	52556	871.25 ± 9.39	73.522 ± 0.941	7.378 ± 0.013	0.755 ± 0.001
ID5	43079	27752	23514	791	65.525	7.143	0.742
ID6	91828	71020	54141	792.8 ± 8.5	66.036 ± 1.289	7.277 ± 0.011	0.756 ± 0.001
ID7	38190	30995	26700	742.3 ± 5.01	64.194 ± 0.409	6.635 ± 0.006	0.696 ± 0.001
ID8	79069	60843	51955	809.1 ± 9.93	68.292 ± 0.904	7.306 ± 0.017	0.756 ± 0.002

Table S3. Variables included in the sediment microbial community multivariate analyses chosen by identifying collinear groups. Environmental variables with correlations |r| > 0.75 were grouped together to account for co-linearity between predictor variables. Correlations (r) were calculated with Pearson correlation analysis. (Xie et al., 2016)

Group name	Variable used in analysis	Correlated variables (|r| > 0.75)
PAHs	SUM(PAHEPA16)	naphthalene, acenaphtylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene and benzo[g,h,i]perylene
Dichlorvos (OPs/TII-PRTs)	dichlorvos	TII-PRTs, total Ops, dichlorvos
TI-PRTs	TI-PRTs	
Cu (TOC/TN)	Cu	TN, TOC, Cu
Zn (Pb/Cr)	Zn	Zn, Pb, Cr
pH	pH	
Silt	Silt	
TP	TP	
Cd	Cd	
Ni	Ni	
Fe	Fe	
Mn	Mn	
Latitude	Latitude	
Longitude	Longitude	

Reference

Xie, Y., Wang, J., Wu, Y., Ren, C., Song, C., Yang, J., Yu, H., Giesy, J.P., Zhang, X., 2016. Using in situ bacterial communities to monitor contaminants in river sediments. Environ Pollut 212, 348-357.