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# Effect-Directed Analysis of Toxicants in Sediment with Combined Passive Dosing and in Vivo Toxicity Testing

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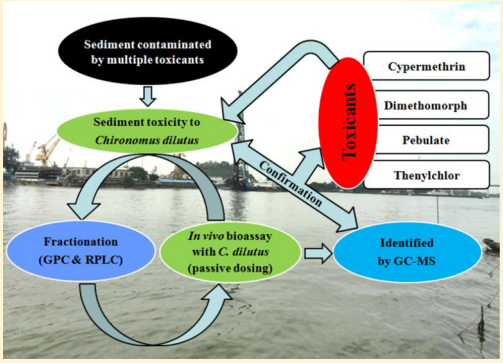
Supporting Information

**ABSTRACT:** Identifying key toxicants in sediment is a great challenge, particularly if nontarget toxicants are involved. To identify the contaminants responsible for sediment toxicity to *Chironomus dilutus* in Guangzhou reach of the Pearl River in South China, passive dosing and in vivo toxicity testing were incorporated into effect-directed analysis (EDA) to account for bioavailability. Fractionation of sediment extracts was performed with gel permeation chromatography and reverse phase liquid chromatography sequentially. Polydimethylsiloxane served as passive dosing matrix for midge bioassays. The fractions showing abnormal enzymatic response were subject to a nontarget analysis, which screened out 15 candidate toxicants. The concentrations of the screened contaminants (log-based organic carbon normalized) in sediments of 10 sites were compared to sediment toxicity (10 and 20 day mortality and 10 day enzymatic response) to *C. dilutus* using correlation analyses. The results suggested that oxidative stress induced by cypermethrin, dimethomorph, pebulate and thenylchlor may have in part caused the observed toxicity to *C. dilutus*. The present study shows that EDA procedures coupled with passive dosing and in vivo toxicity testing can be effective in identifying sediment-bound toxicants, which may pose high risk to benthic organisms but are not routinely monitored and/or regulated. The findings of the present study highlight the importance of incorporating environmentally relevant approaches in assessing sediment heavily impacted by a multitude of contaminants, which is often the case in many developing countries.




## INTRODUCTION

Sediment serves as a sink for a battery of hydrophobic contaminants in aquatic environment and deteriorated sediment quality due to multiple stressors has been reported worldwide, especially in rivers draining through large cities.[1,2] In the case of complex mixtures, the identification of toxicants causing ecotoxicological effects is critical for effectively selecting sediment management measures. Toxicity identification evaluation (TIE) and effect-directed analysis (EDA) are the two most widely used approaches for diagnosing causes of sediment toxicity.[3,4]

In the TIE work, researchers mainly focused on characterizing the contaminant classes causing toxicity, that is, organics, metals, and ammonia, then estimating the toxicity contribution of individual contaminants on the target lists (i.e., a prechosen list of chemicals to measure), and eventually identifying the toxicants causing adverse effects.[4,5] This approach, however, often fails to find the main toxicants when the toxicants are not present in the list of target analytes.[6] Alternatively, EDA techniques have been proposed to find organic toxicants by using analytical techniques to fractionate test samples for both chemical analyses and biological tests and confirm the identity of key toxicants in the toxic fractions without solely relying on the target lists.[3,6] As such, the EDA method is not limited to analyzing chemicals in specified target lists, but rather screens for contaminants of unknown identity under the guidance of the bioassay, providing a way to discover toxicants which are not monitored and/or regulated.[3,6−8]

Traditional EDA is mainly guided by cell-based in vitro bioassays, which are easy to achieve high-throughput analysis, however it lacks ecological relevance and ignores the

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bioavailability and toxicokinetic process of the contaminants.[8−10] The use of in vivo toxicity testing with benthic organisms is more environmentally realistic than cell-based assays for identifying sediment-bound toxicants. In addition, ignoring bioavailability may bias the estimation of toxicity of hydrophobic organic contaminants (HOCs) in sediment and provide false conclusions as to the suspected toxicants, which calls for the development of bioavailability-based EDA methods.[8−12] Passive dosing techniques with polydimethylsiloxane (PDMS) have been shown to successfully maintain constant concentrations of hydrophobic contaminants in water.[13−16] During the bioassays, PDMS serve as a partitioning delivery system to transfer chemical mixtures into water, acting as a surrogate for sediment organic carbon (OC).[17,18] Therefore, a EDA procedure combined with passive dosing and in vivo bioassays can take chemical bioavailability into account, improving the accuracy in diagnosing causes of sediment toxicity in aquatic system containing complex mixtures, such as urban rivers.

The Pearl River flows through Guangzhou, which is the largest city in South China. Various sediment-bound contaminants have been detected in Guangzhou reach of the Pearl River, including polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers, pesticides, and metals.[2,5,19] Current-use pesticides in sediment, particularly pyrethroid insecticides, were deemed as the principal causes of the mortality to benthic invertebrates in urban tributaries of the Pearl River based on TIE methods.[2,5] It was noted that urban sediments, such as those in Guangzhou reach of the Pearl River were quite complex with the presence of various pollutants many of which were of unknown identity. Similar to its tributaries, sediment-bound pyrethroids played a role in the toxicity to benthic invertebrates in Guangzhou reach of the Pearl River, yet their toxicity contribution was relatively small.[20] Meanwhile, the concentrations of other routinely monitored contaminants (e.g., metals) appeared to not contributing to sediment toxicity in this river.[2] These results suggested the need for exploration of nontarget contaminants, which would contribute to the observed adverse effects in benthic organisms.

The aims of the present study were to develop and validate an EDA method in combination with passive dosing and in vivo testing using *Chironomus dilutus* (abnormal enzymatic response as the endpoint) for diagnosing causes of sediment toxicity, particularly for identifying organic toxicants which are not targeted in common chemical analyses. The applicability of the method was validated by a thorough evaluation of sediment toxicity and identification of key toxicants in a complex aquatic system (using Guangzhou reach of the Pearl River as an example).

## MATERIALS AND METHODS

**Experimental Design.** An environmentally relevant EDA method for sediment-bound toxicants was developed and the stepwise procedures are shown in Figure 1. The experiments were separated into two parts: screening bioassays and EDA development. The screening bioassays evaluated sediment toxicity to *C. dilutus* collected from various sites along Guangzhou reach of the Pearl River. The EDA method was first developed with sediment from a representative site (P4), including sediment extraction, fractionation, bioassays and chemical screening, and the confirmation of potential toxicants was conducted with all sediments.

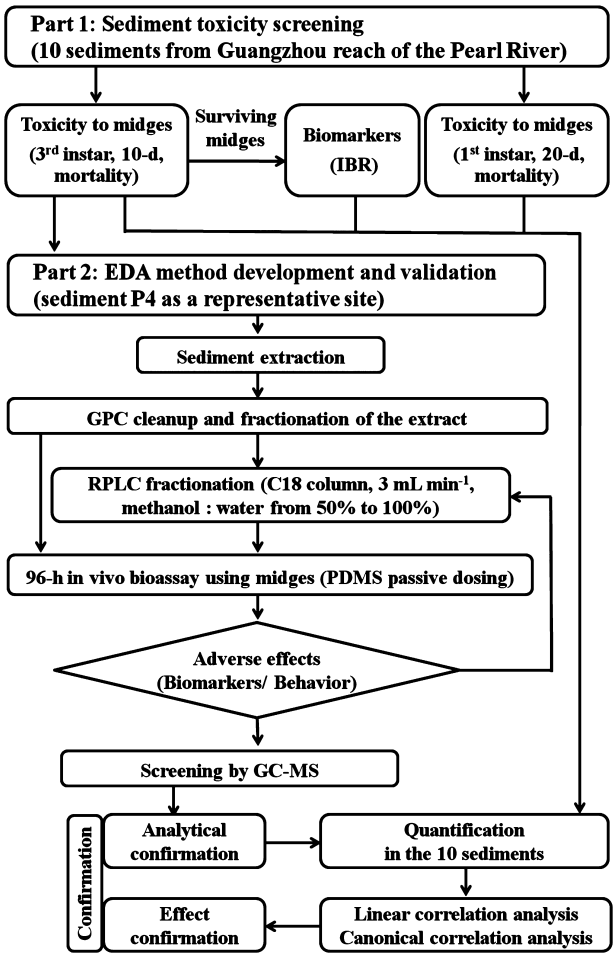


**Figure 1.** Visual schematic of effect-directed analysis in the Guangzhou reach of the Pearl River. GPC: gel permeation chromatography, RP-HPLC: reverse phase-high performance liquid chromatography, PDMS: polydimethylsiloxane, GC−MS: gas chromatography−mass spectrometry.

In the screening bioassays, 10 sediment samples were collected in Guangzhou reach of the Pearl River (Supporting Information (SI) Figure S1, and sediment toxicity was evaluated using first (20 day chronic testing) and third instar *C. dilutus* (10 day acute testing) as part of the screening bioassays (more information can be found in the SI and Cheng et al.[20]). In short, five of the 10 sediments exhibited acute lethality to the midges compared with the controls, and all of the sediments caused significant chronic toxicity to *C. dilutus* (SI Table S1). In addition, enzymatic activities in the surviving midges were significantly altered compared with the control, indicating ubiquitous sublethal toxicity in the study area. A previous study measured current-use pesticides (pyrethroids, organophosphates, and fipronil) and heavy metals in the sediments and evaluated their toxicity contributions.[20] Only pyrethroids (mostly cypermethrin) were correlated to sediment toxicity, which, however, can explain only a small portion of the observed toxicity.

To better explain the observed sediment toxicity, an EDA method using passive dosing and in vivo testing with C. *dilutus* was developed and used to diagnose other potential causes of toxicity besides the target analytes. The EDA procedure includes sediment extraction, fractionation, passive dosing, in vivo toxicity testing and nontarget chemical analysis and is


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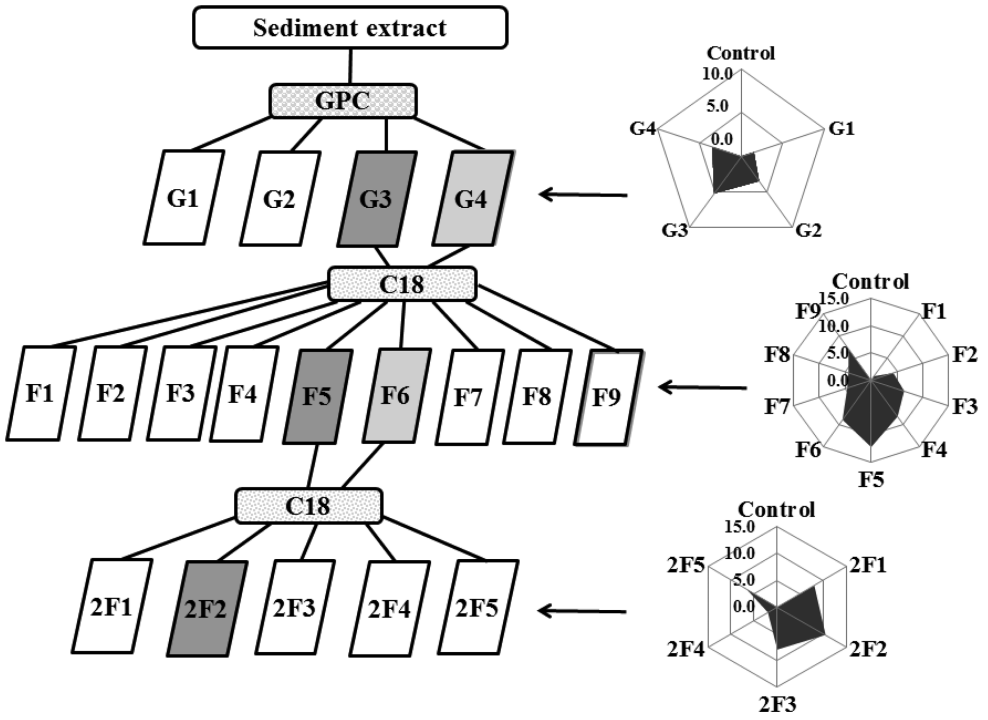


**Figure 2.** Integrated biomarker response (IBR) indices in *Chironomus dilutus* as exposed to the consecutive fraction series of site P4 sediment extract (via effect-directed analysis) in Guangzhou reach of the Pearl River.

complex and laborious. Thus, only the sediments from site P4, which exhibited moderate mortality (37 ± 4%) to the midges, was chosen to test the EDA procedure. This site is adjacent to Chebei Creek, which is a well-studied urban tributary of the Pearl River and polluted by a variety of contaminants including pyrethroids and fipronils.[5,12,19] After the candidate toxicants in sediment P4 were screened by the EDA procedure, their concentrations were determined in the remaining nine sediments and the toxicity contributions of these contaminants were assessed using linear correlation and canonical correlation analyses. Accordingly, key toxicants were identified in the sediments. Further information for each step of the EDA procedure is detailed below.

**Sediment Extraction and Fractionation.** To obtain sediment extracts for EDA analysis, ultrasound-assisted microwave extraction (UAME) was performed using a CW-2000 UAME extractor (Xintuo Company, Shanghai, China).[21] In brief, 40 g of freeze-dried sediment was extracted with 100 mL of a mixture of hexane and acetone (1:1, v/v). The extraction was carried out for 6 min with ultrasound and microwave power at 50 and 100 W, respectively. After decanting the extract, the extraction was repeated with an additional 50 mL of fresh extraction solution. The extracts were combined, filtered through a Whatman 0.45 $\mu$m filter, evaporated to near dryness, and solvent exchanged to 2 mL of dichloromethane.

The sediment extract was first fractionated using gel permeation chromatography (GPC) with a Biobeads S-X3 column (300 × 20 mm) and dichloromethane as the mobile phase at a flow rate of 5 mL min$^{-1}$.[22] Four fractions were collected at the time intervals of 0−4−8−18−27 min, and were solvent-exchanged to methanol for midge toxicity testing using the passive dosing method.

Two of the GPC fractions exhibited toxicity (G3 and G4) (Figure 2), which were combined, solvent exchanged to methanol and further fractionated for the second round of EDA analysis. Reverse phase liquid chromatography (RPLC; Lab-Tech Corporation, China) with a C18 semipreparative column (150 × 10 mm, 10 $\mu$m) was used for the fractionation and the mobile phase was consisted of methanol and water (with an initial composition of 50:50 and then increasing to 100% methanol at a flow rate of 3 mL min$^{-1}$).[23] A total of nine RPLC fractions were collected (F1−F9) over a period of 45 min at 5 min intervals.

On the basis of the toxicity results of these nine fractions, the third round of fractionations was carried out for the toxic F5 and F6 fractions (Figure 2). Again, the two fractions were combined and further separated using RPLC, resulting in five fractions that were collected every 2 min (2F1−2F5). The individual fractions were then solvent-exchanged to hexane and methanol for chemical analysis and toxicity testing, respectively. Passive dosing method was applied for all toxicity tests in the EDA procedure.

**Passive Dosing and Midge Toxicity Testing.** The PDMS films used for passive dosing procedures were made from a MDX4−4210 Bio-Medical grade elastomer kit (Dow Corning (China) Holding Company Limited, Shanghai, China), in accordance to the manufacturer's instruction. The method to prepare PDMS films[24] was modified from a previously developed method by Mayer and Holmstrup.[25] In brief, PDMS prepolymer and catalyst (10:1) were thoroughly mixed to cast into a film and cured at 23 °C for 72 h. The thickness of PDMS film was 0.25 mm (±0.1 mm) with a density of 1.11 g cm$^{-3}$. The impurities and oligomers in the cured films were removed using three sequential ultrasonic extractions with methanol and three rinses using Milli-Q water. The films were then cut into small pieces (2 × 4 cm) before use.

The fractions after GPC and RPLC separations were individually loaded onto the PDMS films. The loading of chemical mixture to the films was achieved by shaking


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methanol−water solutions of the extracts in beakers containing the films at 220 rpm for 48 h. Reconstituted water was gradually added to the solution to drive the contaminants into the PDMS film.[24,26] For dosing sediment extracts containing the mixtures with unknown identity into water, it was crucial to normalize the concentrations of contaminants in PDMS to sediment OC-based concentrations. Li et al.[27] suggested that the partitioning coefficient of a chemical between OC and PDMS was independent of its hydrophobicity and was a constant at approximately 2.1. Accordingly, the mass of PDMS used in the passive dosing was 2.1 times the mass of sediment OC and detailed calculations are presented in the SI. After loading, the PDMS films were placed into the reconstituted water and equilibrated for 48 h by stirring at 660 rpm to release the contaminants in PDMS into the aqueous phase. This passive dosing procedure has been validated with a series of polychlorinated biphenyls (PCBs) with log $K_{ow}$ values ranging from 5.35−7.42 and the results showed that the equilibrium time for all test PCBs was <36 h.[24] The water with the PDMS then served as the exposure media in the toxicity tests.

The 96 h water-only bioassays using PDMS films as dosing media were conducted in triplicate using third instar *C. dilutus* larvae and clean sand was added as a substrate to avoid cannibalization. Another PDMS film loaded with methanol was used as a solvent control in the 96 h toxicity tests. The toxicity of sediment extracts was significantly reduced due to the fractionation (no mortality occurred in any fractions of the EDA bioassays). Thus, change in burying behavior as well as abnormal enzymatic response in *C. dilutus* were used as test endpoints. Details regarding water quality and lighting regimes for the toxicity testing can be found in the SI.

**Measurements of Enzymatic Response.** The survived midges in the bioassays were sieved from the sediments, rinsed with reconstituted water, and stored at −20 °C before quantifying the enzymatic response in the midges using previously developed methods.[28] In brief, freeze-dried midges were homogenized in 50 mmol $L^{-1}$ chilled phosphate buffer saline (pH 7.4) and centrifuged for 15 min at 10 000 *g* and 4 °C. The supernatant was decanted to measure the activity of five enzymes, including two biotransformation enzymes (glutathione-S-transferase (GST) and carboxylesterase (CarE)), a specific enzyme indicating neurotoxicity (acetylcholinesterase (AChE)) and two biomarkers related to oxidization effects (calatase (CAT) and lipid peroxidation (LPO)). The activities of GST and CarE were spectrophotometrically quantified using 1-chloro-2,4-dinitrobenzene and $\alpha$-naphthyl acetate as the substrates, respectively. A kinetic method with acetylthiocholine iodide as the substrate was used to determine AChE activity. The CAT activity and LPO level were assessed with an ammonium molybdate method and a thiobarbituric acid reaction, respectively. After the enzymatic response was quantified, sequence of GST, CarE, AChE, CAT, and LPO was arranged so that the biomarkers with similar functions were adjacent on the star plot as suggested by Beliaeff and Burgeot.[29] The integrated biomarker response (IBR) index was calculated by summarizing the five enzymes into a single value and displayed on a star plot. The radius coordinates of the star plot represented the IBR indices and showed the stress levels to the midges at individual sites. A previous study showed the decrease of IBR values could serve as a good surrogate for the reduced emergence of the midges, which was related to midge toxicity on a population level.[28] Detailed calculations of IBR have been previously reported in the literature[28] and were performed in a similar manner herein.

**Chemical Screening and Confirmation.** Nontarget screening for candidate toxicants in the toxic fractions was performed using a semiquantification method for nearly 1000 analytes with a compound composer software (automated identification and quantification system) on a gas chromatograph−mass spectrometer (GC-MS) (Shimadzu QP-2010 plus, Shimadzu Corporation, Kyoto, Japan).[30] After semiquantitative screening, the screened toxicants were analytically confirmed using neat compounds as the calibration standards (SI and Tables S2 and S3).

Subsequently, the candidate toxicants in sediment P4, which were characterized using the EDA procedure, were quantified in the remaining nine sediment samples collected in Guangzhou reach of the Pearl River. The properties of the candidate toxicants, including toxicity data and log $K_{ow}$, were searched in the Toxicology Data Network from the U.S. National Library of Medicine and the USEPA's ECOTOX database. For effect confirmation, a linear correlation analysis evaluated the correlation between OC-based concentrations of candidate toxicants in all the 10 sediments to midge mortality (in acute and chronic tests) and the IBR indices in 10-d testing. Furthermore, a canonical correlation analysis was performed using the OC-based sediment concentrations of selected possible toxicants at all sites to individual enzymatic responses in *C. dilutus* to provide further evidence of causality.

**Statistical Analysis.** Data are reported as the mean ± standard deviation of at least three independent experiments. The statistically significant difference between the control and samples were assessed using a one-way analysis of variance (ANOVA) with a posthoc Dunnett's test and set at $p < 0.05$. The linear regression analysis was evaluated using SPSS 16.0 software and the canonical correlation analysis was analyzed using SAS 9.1.3 software.

## RESULTS AND DISCUSSION

**Development of EDA Methods with a Representative Sediment.** The toxicity of 10 sediments to *C. dilutus* was evaluated using acute and chronic toxicity tests and the results have been reported by Cheng et al.[20] In brief, all 10 sediment samples were chronically lethal to the midges with 20 day mortality greater than 65 ± 5%. While only half of the sites showed significant mortality to the midges after 10 day acute exposure, the IBR indices were 1 order of magnitude higher than the control (excluding site P2 due to complete mortality) (SI Table S1). The commonly analyzed contaminants failed to fully explain sediment toxicity in the midges.[20] Although pyrethroids in sediment were correlated to midge mortality, they only contributed to less than 0.56 acute toxic units (less than 50% mortality),[20] which strongly implied that traditional target analysis was not sufficient for identifying the key toxicants in these sediments.

As a complement of target analysis, EDA is particularly helpful for identifying the unknown toxicants that are not commonly evaluated or screened for, which was the case in this aquatic system. Because all 10 sediments were from the same river and shared similar sources and the screening procedure in EDA is time-intensive and costly, candidate toxicants were characterized only in one sediment (P4) which was considered to represent the entire river system.

Three consecutive fractionations using GPC and RPLC were conducted under the guidance of in vivo toxicity testing (Figure


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**Table 1. Concentrations of the 15 Identified Compounds Using Effect-Directed Analysis ($C_s$) for P4 Site Sediment in All 10 Sediments Sampled from the Guangzhou Reach of the Pearl River**

| | $C_s$ (ng g$^{-1}$ dry weight)[a] | | | | | | | | | | coefficient of determination ($r^2$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | mortality | | IBR[b] |
| sediment | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 | 10-d[c] | 20-d[d] | 10-d[e] |
| azoxystrobin | 0.66 | 0.77 | 0.62 | 5.75 | 0.54 | 1.12 | 0.50 | 0.58 | 2.70 | 0.51 | 0.04 | 0.02 | 0.01 |
| chlorfenapyr | 5.56 | 3.03 | 2.56 | 2.66 | 0.71 | 4.91 | 0.91 | 1.16 | 0.42 | 0.42 | 0.05 | 0.02 | 0.04 |
| chlorpyrifos | 2.22 | 7.78 | 1.64 | 1.42 | 1.19 | 6.54 | 1.26 | 0.34 | 1.23 | 0.61 | 0.28 | 0.21 | 0.03 |
| cinmethylin | 29.0 | 23.3 | 4.20 | 23.3 | 35.7 | 4.29 | 15.2 | 12.4 | 10.2 | 39.6 | 0.01 | 0.15 | 0.01 |
| dibenzofuran | 11.9 | 20.1 | 16.1 | 12.3 | 14.8 | 37.5 | 11.7 | 12.2 | 7.41 | 13.4 | 0.01 | 0.08 | 0.01 |
| **dimethomorph** | 1.37 | 8.06 | 6.07 | 5.30 | 3.61 | 2.24 | 2.59 | 0.80 | 3.39 | 0.97 | 0.26 | **0.44**[f] | 0.19 |
| hexaconazole | 1.06 | 1.52 | 9.30 | 2.12 | 3.87 | 2.09 | 1.18 | 1.98 | 1.57 | 2.28 | 0.01 | 0.01 | 0.01 |
| imibenconazole | 0.36 | 0.35 | 3.85 | 11.2 | 2.25 | 0.93 | 1.02 | 1.53 | 4.76 | 2.25 | 0.14 | 0.01 | 0.01 |
| **pebulate** | 2.84 | 5.06 | 4.86 | 3.14 | 4.13 | 14.8 | 1.35 | 5.89 | 0.98 | 19.6 | 0.02 | 0.26 | **0.38** |
| propham | 2.29 | 10.5 | 3.83 | 7.71 | 8.24 | 8.61 | 1.59 | 0.87 | 0.74 | 0.85 | 0.25 | 0.06 | 0.02 |
| propiconazole | 18.5 | 15.1 | 9.74 | 3.57 | 5.33 | 15.3 | 2.45 | 1.19 | 3.01 | 4.17 | 0.07 | 0.01 | 0.02 |
| pyrazoxyfen | 7.26 | 5.24 | 11.4 | 16.7 | 8.29 | 2.21 | 7.14 | 3.02 | 11.1 | 10.7 | 0.04 | 0.11 | 0.08 |
| **thenylchlor** | 1.94 | 1.54 | 1.28 | 1.86 | 0.90 | 0.85 | 1.41 | 2.46 | 1.72 | 1.12 | 0.03 | **0.33** | 0.11 |
| tetramethrin | 1.43 | 3.79 | 2.13 | 1.66 | 3.58 | 2.91 | 1.59 | 2.60 | 2.16 | 2.09 | 0.04 | 0.04 | 0.04 |
| **triadimenol** | 6.60 | 88.8 | 199 | 30.8 | 37.8 | 96.5 | 47.6 | 25.6 | 56.7 | 144 | 0.02 | **0.36** | 0.01 |
| mortality in probit (3rd, 10-d) | 3.87 | 8.00 | 5.18 | 4.67 | 4.75 | 4.05 | 4.82 | 4.39 | 3.72 | 3.72 | | | |
| mortality in probit (1st, 20-d) | 4.48 | 8.00 | 8.00 | 6.18 | 6.18 | 6.04 | 6.13 | 5.39 | 5.52 | 5.44 | | | |
| IBR[b] (3rd, 10-d) | 3.59 | | 3.97 | 4.17 | 3.06 | 2.49 | 6.50 | 3.07 | 3.06 | 2.08 | | | |

[a]The reporting limit for individual compounds in sediment was 0.16 ng g$^{-1}$ dry weight. [b]IBR: integrated biomarker response. [c]Determination of coefficient ($r^2$) of correlation analysis between the percentage of mortality in probit form measured by 10-d acute tests (SI Table S1) using *Chironomus dilutus* and log values of the organic carbon normalized sediment concentrations. [d]The $r^2$ of correlation analysis between the percentage of mortality in probit form measured by 20-d chronic tests with *C. dilutus* (SI Table S1) and log values of the organic carbon normalized sediment concentrations. [e]The $r^2$ of correlation analysis between the IBR indices measured by 10-d acute tests of *C. dilutus*(SI Table S1) and log values of the organic carbon normalized sediment concentrations. [f]Statistically significant values of $r^2$ and the corresponding compound were highlighted in bold ($p < 0.05$).

2). Chemicals were separated based on their molecular sizes when using GPC, and some macromolecule matrix interferences and elemental sulfur were removed as well. The RPLC separated the analytes by polarity. To imitate sediment OC, the contaminants in the fractions were introduced into water using passive dosing with PDMS. The toxicity of individual fractionations was assessed with 96 h in vivo aquatic toxicity testing using *C. dilutus*, which had a time frame of the standard water-only toxicity testing and proportionally reflected the observed sediment toxicity.[31] Although sediment P4 showed moderate mortality (37 ± 4%) in 10 day sediment toxicity testing with the midges after 10 day, no midge died in the process of aquatic toxicity testing in EDA analysis. This result has been previously reported in the literature, as the fractionation procedure in the EDA method would cause adverse effects in organism to be reduced and eventually lost.[7] Alternatively, abnormal enzymatic response in the midges (using IBR as an index) were more sensitive than the mortality and thus were selected as the endpoint in the EDA analysis.

Exposure of *C. dilutus* to the GPC fractions G3 and G4 significantly changed their enzymatic response, yet only slight effects were noted in the midges exposed to the G1 and G2 fractions. Moreover, the inability to bury into the sand substrate during aquatic bioassay was observed for the midges exposed to fraction G3 (Figure 2 and SI Table S4). The reduced capability of burying behavior confirmed that the midges were adversely impacted. These results demonstrated that the toxicants to the midges were mainly in the G3 and G4 fractions. Hence, the second round of fractionation was conducted for the combined fraction of G3 and G4 and this sample was separated to nine fractions using RPLC with a C18 column. Exposure of *C. dilutus* to the second set of individual fractions yielded two fractions exhibiting relatively high IBR indices (F5 and F6), and it should be noted that midges were again observed to not be able to bury into the substrate in the F5 fraction (Figure 2 and SI Table S5). Based on these results, the F5 and F6 fractions were combined for further fractionation by RPLC to yield five subfractions of 2 min time intervals, which were again subjected to midge bioassays and the highest IBR index was detected in the 2F2 fraction (Figure 2 and SI Table S6).

Bioavailability strongly influences sediment toxicity assessment due to site-specific sediment characteristics[32] and the same is true for the case of EDA for sediment samples.[7−9] Ignoring bioavailability of the contaminants may bias the judgment of the key contributors to sediment toxicity.[11] The PDMS films acted as surrogates for sediment OC to deliver the contaminants into water by partitioning for aquatic bioassays in the EDA method.[17] Individual fractions were loaded to PDMS films, and the partitioning of chemicals in the PDMS−water system imitated chemical desorption process in the sediment−water system. This EDA method takes bioavailability into consideration in aquatic bioassays,[18] increasing the accuracy in identifying the key toxicants in sediment.

**Characterizing Candidate Toxicants in Toxic Fractions.** Instead of performing a nontarget chemical analysis for the whole sediment extract, GC-MS screening was performed for the most active fraction 2F2. In doing so, interfering chemicals were reduced and the accuracy in identifying the key toxicants was improved. In the meantime, the "less/non-toxic" fractions (2F1, 2F3, 2F4, and 2F5) were also chemically



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analyzed to eliminate the main contaminants present, which did not induce significant effects. In total, 15 chemicals were found to be possibly responsible for the noted toxicity to the midges in fraction 2F2 (Table 1). Most of these 15 chemicals characterized by the EDA procedure were not included in the list of target analytes which were commonly monitored and thus were believed to account for some of the unknown toxicity of site sediments (a common problem in traditional risk assessments). Unfortunately, no toxicity data for *C. dilutus* were available for the majority of these chemicals, thus the toxicity data to *Daphnia magna* are shown instead (SI Table S7).

As shown in SI Table S7, the 15 candidate toxicants had log $K_{ow}$ values ranging from 2.50 to 4.96. This was surprising as previous studies found pyrethroids were one of the main toxicants causing acute lethality to the midges in Guangzhou urban waterways and they had log $K_{ow}$ values greater than 6.[2,5,12,19] In the present study, however, the fractions containing chemicals with high hydrophobicity (such as the fractions that would contain pyrethroids) exhibited little or no toxicity to the midges. The discrepancy between this EDA investigation and previous studies may be partially explained by the underestimation of the toxicity contribution of relatively highly hydrophobic contaminants using the EDA procedure, which is further discussed below.

Cheng et al.[20] found a moderate contribution of pyrethroids to midge toxicity in Guangzhou reach of the Pearl River (probit = 5.11 toxic unit +3.58, $r^2 = 0.52$, $p < 0.05$, acute toxic units of 0.03−0.56 for all sediments), yet this relatively small contribution was possibly compromised during the EDA fractionation procedure. The toxicity contribution from hydrophobic contaminants may also be reduced upon redosing chemical mixtures into water. A previous study suggested that the choices of endpoints and dosing mechanisms in EDA may compromise the characterization of toxicants.[33] Brack et al.[8] also showed that the dosing strategies for bioassays strongly affected prioritization of toxicants in EDA assessments. The advantage of passive dosing is that it is able to maintain water concentrations of hydrophobic contaminants by gradually releasing chemicals from PDMS into water, thus compensating for the chemical loss due to evaporation, degradation, beaker sorption, and organism uptake.[18] Although passive dosing was preferable in incorporating bioavailability into EDA, its application for highly hydrophobic contaminants was still challenging. Passive dosing kinetics in bioassays are expected to be slower for highly hydrophobic compounds (such as pyrethroids) than moderately hydrophobic ones (such as the compounds identified here).[34] Consequently, local depletion of the compounds with high log $K_{ow}$ in water may occur, resulting in underestimation of their toxicity to *C. dilutus* and possible removal from the list of expected toxicants.

**Validating Potential Toxicants in Conjunction with All Site Sediments.** The 15 candidate toxicants were quantified in all 10 sediments using calibration standards prepared by neat compounds, and their concentrations ranged from 0.36 to 199 ng $g^{-1}$ dry weight (Table 1). Effect confirmation was achieved by correlation analyses between sediment toxicity and contaminant concentrations across sediment samples.

First, linear correlation analyses were performed by comparing OC-normalized sediment chemical concentrations (log-based) and the mortality (in probit form) in 10 day and 20 day tests and the IBR indices of *C. dilutus* in 10 day tests (Table 1). These results suggested that the concentrations dimethomorph, pebulate, thenylchlor and triadimenol were significantly correlated to the sediment toxicity to *C. dilutus* ($r^2 > 0.32$, $p < 0.01$).

Subsequently, canonical correlation analysis was conducted to confirm the link between enzymatic responses and OC-normalized sediment concentrations of the selected contaminants. Apart from the four contaminants screened out in the EDA assessment, a pyrethroid, cypermethrin, which contributed the most to sediment toxicity in the study area,[2,5,12,19] was also included in the canonical correlation analysis. One set of variables ($X_1$−$X_5$) represented enzymatic responses including GST, CarE, AChE, CAT, and LPO, and the other set of variables ($Y_1$−$Y_5$) was the OC-normalized sediment concentrations (log-based) of cypermethrin, dimethomorph, pebulate, thenylchlor and triadimenol, respectively. Four pairs of canonical correlations were obtained in the analysis, but only the first pair (Corr ($U_1$, $V_1$)) was significantly correlated, with a canonical coefficient ($\rho_1$) of 0.99 ($p < 0.01$). The original variables ($X_i$ and $Y_i$) bearing canonical loading values greater than 0.50 indicated a meaningful interpretation between $U_1$ and $V_1$.[28,35] As shown in Table 2, the canonical variables of biological effects ($X_i$) were high only for CAT (0.63) and LPO (0.56), and the variables representing chemical concentration ($Y_i$) were strong for cypermethrin (canonical loading: − 0.57), dimethomorph (−0.67), pebulate (−0.82), and thenylchlor (−0.73). On the contrary, the contribution from triadimenol and other enzymatic responses (GST, CarE and AChE) were minimal. Hence, exposure to cypermethrin, dimethomorph, pebulate and thenylchlor in the sediments from Guangzhou reach of the Pearl River was the most likely associated with the oxidative stress (CAT and LPO), which in turn caused the observed toxicity in *C. dilutus*.

**Table 2. Canonical Correlation between the Two Sets of Variables [Biomarkers ($X_1$−$X_5$) Converted to the Arcsine of Their Square Roots and Log Values of the Organic Carbon-Normalized Sediment Concentrations of Contaminants ($Y_1$−$Y_5$)]**

| corr ($U_1$, $V_1$), $\rho_1 = 0.99$ ($p < 0.01$)[a] | | | | | |
|---|---|---|---|---|---|
| variable ($X$) | $a_1$[b] | loading[c] | variable ($Y$) | $b_1$[b] | loading[c] |
| GST[d] | 3.19 | 0.26 | cypermethrin | 0.09 | **−0.57** |
| CarE | −1.37 | −0.04 | dimethomorph | −0.52 | **−0.67** |
| AChE | −0.015 | −0.17 | pebulate | 0.32 | **−0.82** |
| CAT | −2.1 | **0.63** | thenylchlor | −0.44 | **−0.73** |
| LPO | 0.87 | **0.56** | triadimenol | 0.24 | −0.28 |

[a]Corr ($U_1$, $V_1$): first pair of canonical correlation ($U_1 = a_{1,1}X_1 + a_{1,2}X_2 + \ldots + a_{1,5}X_5$, $V_1 = b_{1,1}Y_1 + b_{1,2}Y_2 + \ldots + b_{1,5}Y_5$). $\rho 1$: coefficient of the first pair of canonical correlation. [b]$a_1$and $b_1$: standardized canonical coefficients. [c]Loading: canonical loadings values above 0.50 are given in bold. Values above 0.5 are believed to give a meaningful interpretation between the new pair of variables. [d]GST: glutathione *S*-transferase, CarE: carboxylesterase, AChE: acetylcholinesterase, CAT: catalase and LPO: lipid peroxidation.

While the toxicity of cypermethrin to benthic invertebrates has drawn increasing attention recently,[36] the other three toxicants have been largely overlooked as they are not regularly monitored. Dimethomorph is widely used as a fungicide to control plant phytophthora. This fungicide is toxic to aquatic macrophytes[37] and has shown acute toxicity to silkworms when being applied in or near mulberry field.[38] Pebulate is a widely used thiocarbamate herbicide.[39] Hunt et al.[40] reported that pebulate caused a decrease of Mg:Ca ratio in cattle. This



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chemical has also regarded to be an inhibitor of aldehyde dehydrogenase in mice and a possible cause of sensitizing agricultural workers to ethanol intoxication.[41] Moreover, pebulate has been found to inhibit fatty acid elongation, inducing toxicity to surface lipid biosynthesis of constituents of surface waxes and suberin.[42,43] Thenylchlor is also a herbicide but little toxicity information is available for this chemical.

Though these three pesticides showed some toxicity to other species, to our knowledge, this is the first study in which adverse effects to *C. dilutus* have been likely related to these pesticides (dimethomorph, pebulate, and thenylchlor). Further evaluations with these compounds with *C. dilutus* and other aquatic invertebrates would provide more evidence to support the conclusions herein. However, further confirmation of causal relationship for these aforementioned compounds is limited by small sample amount, limited availability of neat standards, appropriate dosing techniques, and an overall lack of information on concentration−response relationships and joint toxicity effects.[8,44]

Despite these difficulties, incorporating passive dosing and in vivo toxicity testing into EDA techniques provided characterization of key toxicants that were difficult to be characterized using other traditional means. This technique was able to establish a more environmentally realistic testing method for assessing sediment toxicity to benthic organisms with the consideration of bioavailability,[8−10] which in turn improved the characterization of emerging toxicants in sediment. The current work showed that EDA techniques could complement and strengthen traditional sediment toxicity assessment techniques by providing additional information to characterize and define key toxicants that are not commonly evaluated.

Overall, incorporating passive dosing and midge bioassays with enzymatic response as the chosen endpoint into the EDA approach allowed for three nontarget contaminants (dimethomorph, pebulate and thenylchlor) to be identified in addition to a previously detected pyrethroid (cypermethrin) as the key toxicants to *C. dilutus* in sediments from Guangzhou reach of the Pearl River. Effect confirmation with canonical correlation analysis showed that oxidative stress caused by these chemicals was the possible cause for the adverse effects in the midges. Passive dosing techniques were incorporated into the EDA procedure to take bioavailability into consideration. Although more studies are required to improve the efficiency of dosing chemicals with high hydrophobicity, the current technique is able to provide insight into chemicals that may be causing aquatic risk but being overlooked by traditional means.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b00540.

Detailed method descriptions, a figure showing sampling sites, and tables containing information on candidate toxicants and toxicity data of the midges exposed to individual fractions in the EDA procedures (PDF)

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### Notes

The authors declare no competing financial interest.

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