**Abstract:**

Comprehensive enrichment of contaminants in drinking water is an essential step for accurately determining exposure levels of contaminants and testing their biological effects. Traditional methods using a single absorbent for enriching contaminants in water might not be adequate for complicated matrices with different physical-chemical profiles. To examine this hypothesis, we used an integrated enrichment system that had three sequential stages: XAD-2 resin, poly (styrene–divinylbenzene) and activated charcoal to capture organic pollutants and disinfection by-products (DBPs) from drinking water in Shanghai. Un-adsorbed Organic Compounds in Eluates (UOCEs) named UOCEs-A, -B, and -C following each adsorption stage were determined by gas chromatography-mass spectrometry to evaluate adsorption efficiency of the enrichment system. Meanwhile, biological effects such as cytotoxicity, effects on reactive oxygen species (ROS) generation and glutathione (GSH) depletion were determined in human LO2 cells to identify potential adverse effects on exposure to low dose contaminants. We found that poly-styrene–divinylbenzene (PS-DVB) and activated charcoal (AC) could still partly collect UOCEs-A and -B that the upper adsorption column incompletely captured, and that potential carcinogens like 2-naphthamine were present in all eluates. UOCEs-A at (1-4000), UOCEs-B at (1000-4000), and UOCEs-C at (2400-4000) folds of the actual concentrations had significant cytotoxicity to LO2 cells. Additionally, ROS and GSH change in cells treated with UOCEs indicated the potential for long-term effects of exposure to some mixtures of contaminants such as DBPs at low doses. These results suggested that an enriching system with a single adsorbent would underestimate the exposure level of pollutants and the biological effects of organic pollutants from drinking water. Effective methods for pollutants’ enrichment and capture of drinking water should be given priority in future studies on accurate evaluation of biological effects exposed to mixed pollutants via drinking water.
<table>
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Keywords: Organic pollutants, enrichment system, XAD-2, disinfection by-products, DBPs, biological effects
Enrichment system is vital to the exposure and biological effects.
**Highlights**

1. Capacity of enriched pollutants from water by a single adsorbent is limited.
2. 69.5% of target chemicals are efficiently enriched by series of adsorption.
3. Unabsorbed organic compounds in eluate are cytotoxic and impact cell viability.
4. Low concentrations unabsorbed chemicals in eluate alter the ROS and GSH of cells.
5. Single sorbent underestimated exposures and effects of drinking-water pollutants.
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RE: No. CHEM93484 entitled “Single enrichment systems possibly underestimate both exposures and biological effects of organic pollutants from drinking water”

Dear Professor Zhang,

We very much appreciate the comments of the reviewers to help improve our manuscript (CHEM93484). We have carefully addressed each of the comments (see below) and made all the changes requested, and this is now reflected in our completely revised manuscript. We have accepted the reviewer’s suggestion to revise the title of the manuscript, which has been changed to: “Single enrichment systems possibly underestimate both exposures and biological effects of organic pollutants from drinking water”

We have kept the original reviewers’ order number and line numbers of the text in order that you can easily find them.

As suggested by the first reviewer, we have revised the Highlights, manuscript and clarified the issues. A native English-speaking author has helped edit the manuscript extensively.

For the third reviewer’s suggestion, we have corrected our errors in the text and revised Table 1 in order that readers can easily follow our methods.
We have discussed all the issues and responses with the co-authors. All the main changes are highlighted in blue, and the locations and revised contents are addressed in our relevant response connected to each comment. In addition, we have carefully checked the references that we cited.

We believe the changes made in response to the reviewers' constructive comments have greatly improved our study, which we anticipate will make an important contribution to the field.

Please note the edits we have made in the revised manuscript have changed the numbers of some pages, lines, figures, and references mentioned in the reviewers’ original comments.

Thank you very much for your assistance. We look forward to hearing from you soon.

Yours sincerely,

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COMMENTS FROM EDITORS AND REVIEWERS

Reviewer #1:

This is an important work in that it identified a suite of contaminants that could pass through SPE if a single type of resin were used. The authors also backed up the findings with real river water. Overall, I think this is a good paper with important findings and I would recommend publishing it in Chemosphere after some revision. Below are my comments.

-Response: Thank you very much for your comments and encouragement. We have carefully revised the manuscript and clarified issues that you addressed in the review. Now the quality of the manuscript has been greatly improved.

1. Highlight point 1, it probably is common knowledge that no method is capable of 'completely enriching' organic pollutants from water, as no method claims 100% recovery. I would revise this point.

-Response: We have modified the first highlight to “Capacity of enriched pollutants from water by a single adsorbent is limited. Please see Highlight column.

2. English needs improvement, e.g. 'low concentrations unabsorbed chemicals in...', 'drinking-water'

-Response: Done. A native English-speaking author has helped edit the manuscript extensively.

3. Title probably requires attention as its current state may lead to misunderstandings. Per my understanding, despite the fact that single enrichment systems do not capture all organic pollutants, the resulted negative biological mechanism(s) may remain identical and the only difference between effects may simply stem from the doses. Likewise, an underestimation of exposures may simply come from an underestimation of the doses. Therefore, in essence, both the underestimation of exposures and biological effects of these organic pollutants are probably all a result of an underestimation of the exact doses.
-Response: Thank you very much for your question and comments. We have modified the title of this manuscript to avoid misunderstanding.

The core point of the present study is to show that a single enrich system may omit some pollutants in water, which may affect subsequent investigations on pollutants exposure and the biological effects induced by enriched pollutants.

If the pollutants are enriched with a single adsorbent, some pollutants in water may not be completely and effectively adsorbed and captured. Therefore, these pollutants are not included in the subsequent analytical results of the pollutant spectrum, which have been lost during the process of enrichment. Obviously, potential adverse effects from missing pollutants would be omitted and neglected in the analysis on pollutant exposure and health effects. Confirmation of the pollutants should be the focus of prevention and control and so develop the formulation of effective public health policies. Thus, it is not only the issue for doses of compounds used in the bioassay that is important, but also the composition of the pollutants.

4. I am not sure what 'at (1-4000)' means here, as they were never mentioned before. MW in Dalton? As I read on, I learned that these numbers stand for concentration folds. Please specify.

-Response: Sorry, our description was confusing. You are right. 1-4000 means that the concentrations of contaminants are equal to folds in the real world rather than the molecular weight. We have modified this sentence.

5.Line 75, 'Complete and effective enrichment' should probably be modified, as no method/methods 'completely' recover the pollutants in water.

-Response: Done. We have modified this sentence. Please see Page 3 in Line 74~75.

6.'integrated enrichment system' sounds very vague, integrated how? This keyword probably demands attention.

-Response: Done. We have modified with series enriching systems and given a concise explanation.
7. Line 108 to 110, I do not understand 'these methods do not meet requirements for enriching sufficient amounts of chemicals from the same batch of water samples to conduct multiple biological effect assays'. To me, having enough concentrated chemicals for multiple biological assays simply entails having more water samples to start with.

-Response: What this paragraph illustrates is that the ideal enrichment system is to concentrate and capture contaminants in the water as much as possible, so as to facilitate the analysis and the pollutants spectrum and the confirmation of biological effects. The adsorption capacity of enriched materials is related to its properties. The enrichment ability of the enrichment materials to different types of pollutants in water is different, which is demonstrated by their selective differences. Although increasing the water sample size can make up for the shortage of insufficient adsorption, this way not only increases the sampling quantity, but also increases the processing time. Water samples treated over a long time will increase various reactions such as REDOX reactions among chemical substances in water and alter the original water quality profiles, which may change the results of biological effect analysis.

This sentence has been modified to “Organics in water were sufficient and effective enrichments that are helpful to confirm the exposure levels and composition of contaminants and increase accuracy of biological effects. Time-consuming enrichment processes may affect the stability of chemical substances in water and lead to enriched chemicals that are different from collected water samples, which reduces the accuracy of the analysis of biological effects. Please see Page 6 in Line 108~117.

8. Line 118, may not 'be' adequate to...

-Response: Done.

9. Line 138, please revise 'in drinking water and to objective evaluation drinking water safety.'.

-Response: Done.

10. Line 163, section 2.2, the description of the method is not clear why a synthetic water sample would be used when a real water sample was also collected.
To prove the limitations of a single enrichment system on pollutants’ adsorption, we designed a succinct experiment to test the hypothesis. Pollutants in a real water sample were determined by GC-MS to identify compounds and their concentrations. Then a synthetic water sample that was make up based on the result of detected pollutants and their concentrations was employed to examine the capacity of pollutants’ enrichment by different absorbents. The composition and concentrations of this synthetic water sample are clear, which was using to stimulate water sample in the real world and provide a good case for verifying our hypothesis. Our results demonstrated that a single enrichment system with XAD-2 resin omits some pollutants during the treatment of water samples, in comparison to an integrated enrichment system that had three sequential stages-XAD-2 resin, poly (styrene–divinylbenzene) and activated charcoal. Please see Page 8-9 in Line 171~178.

11. Line 166, 20 ug/40L = 0.5 ppb, super low, I wonder what the methods' detection limits were.

-Response: Twenty micrograms of each target compound were spiked into the 40 L of Milli-Q water to create the synthetic water sample. Here, 20 μg/40L = 0.5 ppb. the concentration of 0.5ppb refers to the spiked level of targeted compounds rather than the concentration of compounds in the sample for GC-MS detection. As described in the section of “Concentrating the Adsorbent Extracts “in the supplementary information, the extracted compounds were reconstituted with methanol and then concentrated to one millilitre. Therefore, the sample was first concentrated from 40L to 1 mL (around 20 ppm) followed by detection. In this study, the method’s detection limit was 1-200 ng/L for DPBs.

We have modified this sentence, please see Page 9 in Line 194~196.

12. Line 191 to 193, since the authors adapted from the current USEPA method, it is probably of interest to the readers how. I would suggest taking the appropriate parts in the SI and integrate in here. This is also because I can imagine that the concentration of the contaminants in the eluates were very low, having passed through one or more SPE.

-Response: Thank you very much for your suggestions. We have taken some contents from the SI and modified this part in the Methods section. Please see Page 10 in Line 208~217.
Briefly, we detected the compositions and concentrations of various pollutants in water according to their categories. and then we integrated all identified and detected pollutants from the water. In this way we clarified the compositions and concentrations of pollutants in the water.

13. line 194, I do not understand 'The total ion chromatogram of the 93 target compounds is 5 ng/μL'.

-Response: We apologise that this mistake produced ambiguity. Actually, we wanted to say ‘The total ion chromatogram of the 93 target compounds is obtained. To avoid misunderstanding and ambiguity, we have deleted this sentence.

14. Section 2.6, it was my understanding that the authors took the adsorbed organics, diluted that in methanol, making UOCEs-A, B, and C. This implies that the actual UOCEs share identical chemical characteristics with the adsorbed organics, is that correct? I think this may not be true. Some elaboration may be desired here.

-Response: Methanol here is just the solvent. Methanol should be dried naturally and then dissolved in dimethyl sulfoxide to appropriate concentrations for biological effect tests.
Reviewer #3:
In this work, the authors used an integrated enrichment system that employed three sequential adsorbents to capture organic pollutants and disinfection by-products (DBPs) from drinking water. The adsorption efficiency of the enrichment system was tested, and the biological effects of unabsorbed organic compounds in eluate (UOCEs) were determined. Enrichment of trace-level pollutants is critical for following chemical and biological analyses. This work may add information to sample enrichment after some revision.

-Response: Thank you very much for your comments and encouragement.

1. Line 67, please define "PS-DVB and AC" before using the abbreviation.

-Response: Done.

2. Line 69, it is unclear by "UOCEs-A at (1-4000), UOCEs-B (1000-4000), and UOCEs-C (2400-4000) times the actual concentrations had significant cytotoxicity".

-Response: Done. We have modified this sentence to improve understanding.

3. Lines 107-110, some recent studies have also focused on improving the enrichment prior to chemical and biological analyses (J. Environ. Sci. 2017, 58, 83-92; ES&T 2018, 52, 10552-10561). It may worth mentioning them to strengthen the introduction part.

-Response: Thank you. We have cited new related literature as per your suggestions.

4. Lines 122-128, please briefly explain why the three adsorbents can complement with each other in trapping different contaminants from drinking water.

-Response: Thank you. We have added a brief explanation on the working principle of the modified enrichment system.
5. Line 124 and elsewhere, "absorption" or "absorbed" may be changed to "adsorption" or "adsorbed".
   -Response: Done.

6. Line 167, to prepare the synthetic water sample, natural organic matter, which may significantly affect the adsorption of target compounds, should be added.
   -Response: Thank you very much for reminding us of this crucial issue. The synthetic water sample was prepared with high purity chemicals to avoid interference from natural organic matter in water. Please see Page 8-9 in Line 171~178.

7. Line 194, what was meant by "The total ion chromatogram of the 93 target compounds is 5 ng/µL"? 
   -Response: This is the nomenclature for chemicals analysis. It means that 93 compounds detected in water samples of the real world acted as targets for enrichment and detection. 93 compounds will be combined into a mixture of synthetic chemicals. To avoid misunderstanding and ambiguity, we have modified this sentence.

8. It would be better if the "Results" part and "Discussion" part could be combined, and some contents could be more concise. Also, "Conclusion" should be provided for this work.
   -Response: Thank you. We have modified the results and discussions, now it is more concise. In addition, we have added a conclusion for this work.

9. Lines 320 and 323, "MOPs" should be "OMPs".
   -Response: Thank you. We have corrected this error.

10. In addition to toxicity evaluation of UOCES-A, B, C, it might be more helpful if the toxicity of adsorbed organic compounds by XAD, PS-DVB and AC, respectively, and the toxicity of adsorbed organic compounds by sequential XAD, PS-DVB and AC could be evaluated and compared.
   -Response: The core hypothesis is that a single enriched system to treat organics in water might omit some pollutants. Because every kind of adsorbent has its own limits for enriching pollutants, when some...
pollutants with strong toxic effects cannot be effectively enriched by the specific adsorbent such as XAD-2 resin in this study. Not only were they omitted in the detection of pollutants’ composition and concentrations, but also were not considered for their potential biological effects. UOCEs-A, UOCEs-B, and UOCEs-C represent un-adsorbed organic compounds in eluates from every step. Therefore, if UOCEs are toxic, that will prove our hypothesis correct.

The method that you suggested is also a way to verify our hypothesis; both ways worked. Our method is advantageous as the explanation of the result, owing to the simple chemical composition.

11. Table 1, it might be misleading that from Table 1, the recoveries of PS-DVB and AC for many organic compounds are not detectable or very low.

-Response: Thank you. The results indicated that 82 of the 118 target compounds were completely enriched by the XAD-2 resin, so the fact that these 82 target compounds in PS-DVB and AC were not detected were in line with the expected results. We have modified Table 1 to clearly demonstrate what we have done.
Single enrichment systems possibly underestimate both exposures and biological effects of organic pollutants from drinking water

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Abbreviations

AC, activated charcoal; ANOVA, analysis of variance; CCK-8, Cell Counting Kit-8; DBPs, disinfection by-products; DFTPP, Decafluorotriphenylphosphine; DMEM, Dulbecco's Modified Eagle Media; ER, enrichment recovery; FBS, fetal bovine serum; GC-MS, gas chromatography-mass spectrometer; GSH, glutathione; HAAs, haloacetic acids; HALs, haloacetaldehydes; HKs, haloketones; IARC, International Agency for Research on Cancer; OMPs, organic micropollutants; P/S, penicillin–streptomycin; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; PS-DVB, poly-styrene–divinylbenzene; ROS, reactive oxygen species; SI, supporting information; THMs, trihalomethanes; Tris-HCl, tris-(hydroxymethyl)aminomethane; UOCEs, unadsorbed organic compounds in eluates; U.S. EPA, the United States of America Environmental Protection Agency.
Abstract (284 words)

Comprehensive enrichment of contaminants in drinking water is an essential step for accurately determining exposure levels of contaminants and testing their biological effects. Traditional methods using a single absorbent for enriching contaminants in water might not be adequate for complicated matrices with different physical-chemical profiles. To examine this hypothesis, we used an integrated enrichment system that had three sequential stages-XAD-2 resin, poly (styrene–divinylbenzene) and activated charcoal to capture organic pollutants and disinfection by-products (DBPs) from drinking water in Shanghai. Un-adsorbed Organic Compounds in Eluates (UOCEs) named UOCEs-A, -B, and-C following each adsorption stage were determined by gas chromatography-mass spectrometry to evaluate adsorption efficiency of the enrichment system. Meanwhile, biological effects such as cytotoxicity, effects on reactive oxygen species (ROS) generation and glutathione (GSH) depletion were determined in human LO2 cells to identify potential adverse effects on exposure to low dose contaminants. We found that poly-styrene–divinylbenzene (PS-DVB) and activated charcoal (AC) could still partly collect UOCEs-A and-B that the upper adsorption column incompletely captured, and that potential carcinogens like 2-naphthamine were present in all eluates. UOCEs-A at (1-4000), UOCEs-B at (1000-4000), and UOCEs-C at (2400-4000) folds of the actual concentrations had significant cytotoxicity to LO2 cells. Additionally, ROS and GSH change in cells treated with UOCEs indicated the potential for long-term effects of exposure to some mixtures of contaminants such as DBPs at low doses. These results suggested that an enriching system with a single adsorbent would underestimate the exposure level of pollutants and the biological effects of organic pollutants from drinking water. Effective methods for pollutants’ enrichment and capture of drinking water should be given priority in future studies on accurate evaluation of biological effects exposed to mixed pollutants via drinking water.
Keywords: Organic pollutants, enrichment system, XAD-2 resin, disinfection by-products, DBPs, biological effects
1. **Introduction**

Safe drinking water is necessary for human health and well-being. However, more than 663 million people worldwide still use unimproved drinking water sources (UNICEF and World Health Organization Geneva, 2015). Micropollutants in aquatic environments, including surface water (De Baat et al., 2020), groundwater (Li et al., 2020), and wastewater (Thomaidi et al., 2015), have been widely reported over the last few decades (Schwarzenbach et al., 2006). These micropollutants are released directly or indirectly into the aquatic environment by urban, industrial, agricultural, and other anthropogenic activities, or enter as transformation products formed during incomplete degradation by effluent treatment (Knopp et al., 2016). The presence of various hazardous organic compounds in drinking water may cause long-term health effects, including gastrointestinal and urinary tract cancers (Reungoat et al., 2010). However, current drinking-water treatment plants are not specifically designed to eliminate micropollutants from aquatic environments (Haddad et al., 2019). Thus, many of the organic micropollutants (OMPs) are not removed and remain in drinking water. Some emerging OMPs have already been detected in drinking water (Yang et al., 2017). Additionally, some micropollutants may react with disinfectants to form disinfection by-products (DBPs) during the water treatment process. Epidemiological studies have found an association between exposure to DBPs and bladder cancer (Diana et al., 2019). In view of the ubiquity of a high number of potentially toxic OMPs in drinking water and the largely unknown long-term effects of these pollutants on human health, OMPs including DBPs in drinking water have aroused considerable public concern (Li et al., 2018).

Exposure assessment and adverse effect identification are crucial starting points for health risk assessment of chemicals. Because most organic contaminants in drinking water are volatile (Rajasärkkä et al., 2016) or at least semi-volatile (Wu et al., 2013) and present at sub-
microgram to microgram per liter, it is necessary to concentrate and enrich trace organic pollutants in water before analyzing possible biological effects. Liquid-liquid extraction and solid-phase extraction are classic methods for enriching volatile and semi-volatile organic constituents in drinking water (How et al., 2021), but these methods are unsuitable for enriching chemicals from water to conduct biological effect assays. This is because liquid-liquid extraction and solid-phase extraction are commonly used for sample extraction and purification for determination of targeted chemicals in water rather than for testing multiple biological assays. Moreover, time-consuming enrichment processes may affect the stability of chemical substances in water and lead to enriched chemicals that are different from collected water samples, which reduces the accuracy of the analysis of biological effects. In recent years, a series of novel methods with multiple liquid-liquid extractions (LLEs) have been developed to improve extraction efficiencies of polar halogenated DBPs (Han et al., 2017; 2018), which increased our understanding of enrichment and extractions of pollutants.

Traditional methods for enriching OMPs in various water sources mainly rely on single absorbents such as Amberlite XAD resins to extract large scale water samples for biological assay (Xiao et al., 2012). The Amberlite XAD-2 resin has been widely used as a low polarity adsorbent for non-polar and low polarity organic pollutants enrichment to detect mutagens in water (Egea et al., 2021). However, owing to diverse components in water sources and the complexity of intrinsic physicochemical properties of these chemicals, the use of a single absorbent that focuses on specific chemical characteristics of pollutants for enriching waters may not be adequate to capture the broader suite of chemicals and low concentrations of these chemicals likely to be present in the complex mixtures in various water sources (Stalter et al., 2016).
Any single adsorbent would have its own specific limitation, so we designed an integrated strategy using three different adsorbents, arranged in series, to trap a larger portion of contaminants from drinking water to observe and evaluate adsorption efficiency of the enrichment systems. After passing through an XAD resin adsorption, the eluate moved through a poly (styrene–divinylbenzene) (PS-DVB) co-polymer adsorbent with a high sorption capacity for medium polarity of organic contaminants (Mirnaghi et al., 2012) and highly polar compounds such as phenols (Moret et al., 2005). A third adsorbent stage following the PS-DVB adsorbent used an activated charcoal (AC) adsorbent to capture chemicals that had not been removed by the first two stages (Li et al., 2010). Therefore, the overall objective of this study was to determine whether a single adsorbent system efficiently captured most contaminants in drinking water. To pursue and verify this hypothesis, we determined the unabsorbed organic compounds in eluate (UOCEs) following each stage by gas chromatography-mass spectrometer (GC-MS) and measured the cytotoxicity, reactive oxygen species (ROS) generation, and glutathione (GSH) depletion in human LO2 cells induced by eluates from different stages of our sequential adsorbent procedure. Our study demonstrated that an enriching system with a single adsorbent would underestimate the exposure levels and the effects of some contaminants in drinking water; this may help develop novel integrated enrichment systems to comprehensively understand biological effects induced by contaminants in drinking water and to objectively evaluate drinking-water safety.

2. Materials and Methods

2.1. Agents

The chemicals were purchased from Sigma (St Louis, MO), Dr. Ehrenstorfer (Augsburg, Germany) Riedel-de Haen (Seelze, Germany), and Toronto Research Chemicals (Toronto,
Canada), respectively, unless specified otherwise, and listed in supporting information (SI). All pesticide residue grade solvents (including methanol, acetone, dichloromethane, n-hexane, ethanol, acetonitrile, ethyl acetate, and n-propanol) were obtained from Dikama (USA).

Dulbecco's Modified Eagle Media (DMEM), 0.25% Trypsin-EDTA, 5-and-6-Chloromethyl-2 and Penicillin–streptomycin (P/S) were purchased from ThermoFisher (Waltham, MA, USA), Fetal bovine serum (FBS) from Mediatech, Inc. (Manassas, VA, USA). Cell Counting Kit-8 (CCK-8) and total glutathione (GSH) quantification kit from Dojindo (Tokyo, Japan), and tris-(hydroxymethyl)aminomethane (pH7.6) was from Beyotime Biotechnology (Shanghai, China).

The Amberlite® XAD-2 resin (20-60 mesh, CAS No. 9060-05-3), StratoSphere™ PS-DVB resin (100-200 mesh, CAS No. 9003-70-7), and AC (20-40 mesh, CAS No.7440-44-0) were obtained from Sigma (St. Louis. MO, USA). A Milli-Q Integral Water Purification System for Ultrapure Water (MilliporeSigma, Burlington, MA, USA) supplied laboratory reagent water for all experiments.

All glassware was washed with acetone and water, soaked overnight in concentrated sulfuric acid containing 5% $\text{K}_2\text{Cr}_2\text{O}_7$ solution, and baked at 200 °C for two hours after washing with ultra-pure water. Care was taken to use plastic products to the smallest extent possible to avoid contamination.

### 2.2. Synthetic Water Sample and Real Water Sample

To prove the limitations of a single enrichment system on pollutants’ adsorption, we designed a succinct experiment to test the hypothesis. Pollutants in a real water sample were determined by GC-MS to identify compounds and their concentrations. Then a synthetic water sample that was made up based on the results of detected pollutants and their concentrations with high purity chemicals to avoid
interference from natural organic matter in water, which was employed to exam the capacity of pollutants’ enrichment by different adsorbents. The known composition and concentrations of this synthetic water sample was used to simulate water samples in the real world and provide a good case for verifying our hypothesis.

In this study, 118 volatile and semi-volatile chemicals were selected as targeted compounds according to their high detection frequency in drinking water that used the Huangpu River as a water source (Chen et al., 2008). Twenty micrograms of each target compound were spiked into the 40 L of Milli-Q water to create the synthetic water sample. The suite of compounds was analyzed as described in Supplementary File SI (Table S1). The comparison of 118 target compounds with existing regulatory values and standards for water quality throughout the world is also given in table S2 of SI.

Forty liters of the drinking water of Shanghai was sampled into clean amber glass bottles. Before sample collection, each acid-treated bottle was thoroughly pre-rinsed with Milli-Q water at the laboratory and then rinsed with sample water prior to sample collection. Water samples were immediately shipped to the laboratory at 4°C in ice and stored at -80°C for analysis. Analyses were all conducted within 4 days of freezing (O'Toole et al., 2009; Kim et al., 2020).

2.3. Sample Enrichment Procedure

Briefly, appropriate amounts of XAD-2 resin, PS-DVB resin, and activated charcoal were first conditioned and then transferred to home-made glass cartridges. Twenty micrograms of each target compound were spiked into the 40 L of Milli-Q water to create the synthetic water sample. And then this water sample was spiked with 20 µg of each of the 118 target compounds was loaded onto the XAD-2 resin. After adjustment of the eluate to pH between 2.0 to 3.0, the
eluate from the XAD-2 resin cartridge flowed into two PS-DVB cartridges arranged in series. The eluate from the second PS-DVB cartridge was treated to adjust pH to 5.0 and then passed on to the activated charcoal cartridge. A variety of eluting solvent mixtures were used for removing extracted target compounds from the solid absorbents. Each adsorbent extract was evaporated to dryness at room temperature and then reconstituted with methanol. The detailed process of sample pretreatment is presented in SI.

2.4. GC-MS Analysis

We adapted the United States of America Environmental Protection Agency (U.S. EPA) 525.2 method (EPA525.2 method, Revision 2.0) to determine the targeted analytes except for DBPs (see SI). Briefly, the adsorbent extracts were analyzed using a Finnigan Trace GC ultra/Trace DSQ gas chromatograph equipped with mass spectrometry (Thermo Fisher Scientific, Waltham, MA). A capillary GC column DB-5MS (30 m×0.25 mm×0.25 µm) (Agilent, USA) was programmed for heating as follows: starting from 40 °C kept 1 min to 130 °C at a rate of 30 °C/min and kept 3 min, from 130 °C to 180 °C at a rate of 12 °C/min, from 180 °C to 240 °C at a rate of 7 °C/min, and from 240 °C to 320 °C at a rate of 12 °C/min and kept 5 min at 320 °C. High purity helium (99.999%) was used as the carrier gas. Splitless injection of samples (2 µl) into the GC-MS was performed at 280 °C. The electron ionization conditions were as follows: ion energy, 70 eV, ion source temperature 280 °C, and m/z=50-600 full scan for qualitative analysis and SIM mode for quantitative analysis. All of the characteristic ions and quantitative ions for target analytes are presented in Table S1. DBPs were determined according to U.S. EPA recommended methods (Pan et al., 2017).
2.5. Enrichment Capacity

For the assessment of the enrichment capacity of packed adsorbent columns, the enrichment recovery (ERs including ER\textsubscript{XAD-2}, ER\textsubscript{PS-DVB} and ER\textsubscript{AC}) was calculated by formula 1, respectively. \(C_a\) is the extracted concentration of target compounds in the adsorbent phase and \(C_o\) is the initial concentration of compounds in the synthetic water sample.

\[
ER = \frac{C_a}{C_o} \times 100\% \quad (1)
\]

As reference points, ER values were ranked as follows: (i) if ER was greater than 80\%, the extraction capacity was ranked good; (ii) if ER ranged between 60\% and 80\%, the extraction capacity was ranked medium; (iii) if ER was less than 40\%, the extraction capacity was ranked poor.

2.6. Unadsorbed Organic Compounds in Eluates (UOCEs)

According to these ER values, we obtained the concentration (\(C_b\)) of each un-adsorbed organic compound in the eluate of adsorbents by formulas 2-4, respectively.

\[
C_b \text{ (in the eluate of XAD-2)} = C_o \times (100 - ER_{XAD-2}) \quad (2)
\]

\[
C_b \text{ (in the eluate of PS-DVB)} = C_o \times (100 - ER_{XAD-2} - ER_{PS-DVB}) \quad (3)
\]

\[
C_b \text{ (in the eluate of AC)} = C_o \times (100 - ER_{XAD-2} - ER_{PS-DVB} - ER_{AC}) \quad (4)
\]

Furthermore, based on \(C_b\), we created three mixtures in methanol named UOCEs-A, -B and -C respectively (Table S3). These samples were stored at -20 °C. Prior to biological effect tests, the methanol in these samples were first volatilized to dryness by natural means and then the solutes were re-dissolved in dimethyl sulfoxide to appropriate concentrations for biological effect tests.
2.7. Water Samples Analysis

The 40 L aliquots of drinking water were added five surrogate standard chemicals and then flowed through the pre-conditioned XAD-2, PS-DVB, and AC sorbents column in series. The OMPs from the samples were extracted and then desorbed by different eluting solvents. For OMPs adsorbed by the XAD-2 resin, the 40 mL of methanol-acetone (7:3, V/V), 30 mL of acetone-hexane (1:1, V/V) and 45 mL of dichloromethane were sequentially passed through the cartridge and the eluates were collected in a clean glass vial; for OMPs adsorbed by PS-DVB, 60 mL of methanol-ethyl acetate (1:1, V/V), 20 mL of hexane and 20 mL of dichloromethane flowed through the cartridge and the eluates were collected in turn; for OMPs adsorbed by AC adsorbent, 40 mL of acetone-hexane (1:1, V/V) and 20 mL of dichloromethane were passed through the cartridge and the eluates were collected separately. All elution solvents were equilibrated on the cartridges for 15 minutes and then solvent flowed through the columns at a rate of 3-5 mL/min. The eluates were concentrated to 1.0 mL under a nitrogen flow and 2 µL of aliquot was injected for the GC-MS analysis. The qualitative analysis was performed by both the retention times and characteristic ions. The quantitative analysis was carried out by use of internal calibration curves.

2.8. Quality Control

Quality controls for all target compounds were confirmed by equipment calibration checks, blank control experiments and quality control charts during sample analysis. First, a 2 µL aliquot of decafluorotriphenylphosphine (DFTPP) (2.5 ng/µL) and 2 µL aliquot of p,p’-DDT was injected into GC-MS before any samples were analyzed and also intermittently throughout sample analysis to evaluate the equipment operations. If the DFTPP mass spectrum did not meet all ion abundance criteria, the MS was returned. If degradation of p,p’-DDT exceeded
20%, maintenance was performed on the GC injection port or other parts of the system. The results of calibration check of DFTPP and p,p’-DDT is given in the Supplementary Information Table S4 and S5. Secondly, continuous monitoring of 2.0 ng/µL aliquot of target compounds for twenty days was applied to create a quality control chart (data not shown). Thirdly, laboratory reagent blanks and fortified method blanks were performed daily to ensure that the GC-MS system was free of contamination.

As for DBPs analysis, a field blank (distilled water) was set for each sampling campaign. For every batch of 10 samples, both a solvent blank and a procedural blank (both for aqueous and solid samples) were added to ensure that the samples and the analysis process were free of contamination. No quantifiable analytes were detected in the blanks.

2.9. Cell Cultures

Human embryo liver LO2 cells, purchased from the Cell bank of the Chinese Academy of Sciences, were cultured in DMEM with 10% FBS and 1% P/S at 37 °C in an incubator with 5% CO₂ in air (Yang et al., 2018).

2.10. Cytotoxicity

LO2 cells (1×10⁴ cell per well) were plated in 96-well plates, incubated for 24 h and treated with UOCEs-A, -B and -C at 1 to 4000 times actual concentrations in eluates for 24 h. Methanol (1%, V/V) served as the vehicle control and CdCl₂ (25 μmol/L) as the positive control. Following, every well was added CCK-8 solution (10 μL) and their optical density values were determined at 450 nm after incubating for 30 min. Cell viability was expressed as percent relative to the vehicle control. Experiments were performed in five replicates.
2.11. Intracellular ROS and Total GSH Levels

ROS were determined by a microplate reader using CM-H$_2$DCFDA (Li et al., 2008), at 488 nm excitation and 525 nm emission. Total GSH levels were determined following the protocol of the Total GSH Quantification Kit through the reaction between 5, 5'-dithiobis (2-nitrobenzoic acid) and GSH. The levels were expressed as a percentage relative to the vehicle control. The experiment was performed in four and three replicates, respectively.

Statistical Analysis. The results between and within groups were analyzed by analysis of variance (ANOVA) by IBM SPSS Statistics Version 22. If the variance was homogeneous, a Student-Newman-Keuls test was used to do multiple comparison; otherwise, a Games-Howell tests was used. A $p$-value<0.05 was defined as statistically significant. Figures were drawn by GraphPad Prism Version 7.00.

3. Results

3.1. Effect of Adsorbent.

In this study three adsorbents of XAD-2, PS-DVB, and AC were connected in series and used for extracting duplicate blank spiked samples and their ER values were then calculated. As shown in Table 1, it was found that 80 out of total 118 target analytes could be satisfactorily enriched by XAD-2 resin with an ER of greater than 60%. Moreover, 39 out of the 80 analytes were not extracted by PS-DVB or by AC. In total, thirty-one of the target analytes were poorly extracted by XAD-2. A total of 41 target analytes were only partly extracted with the PS-DVB and 28 by AC. (See Table S6.)

3.2. Effect of Eluting Solvent.
The choice of the eluting solvent was an important determinant of extraction efficiency. Organic solvents such as methanol, acetone, dichloromethane, and n-hexane have commonly been used to elute the XAD-2 resin (Lynch et al., 1975; Kumar et al., 2011). In the present study methanol-acetone (7:3, V/V) (Lan, 1994) acetone-hexane(1:1, V/V) (Kanno et al., 2010; Xu et al., 2010) and dichloromethane (Woudneh et al., 2006) were examined with regard to elution of trace organic compounds adsorbed from XAD-2. Based on previous studies, methanol-ethyl acetate (1:1, V/V), hexane, and dichloromethane were investigated as eluting solvents for PS-DVB (Pietrzynska et al., 2013) and acetone-hexane (1:1, V/V) was used for AC (Kitamura et al., 2004). In our study, 54 % of target analytes absorbed on XAD-2 resin were satisfactorily eluted by methanol-acetone (7:3, V/V) with more than 60% ER. Moreover, some of the remaining adsorbed compounds could be eluted by acetone-hexane (1:1, V/V) and dichloromethane. The compounds adsorbed on PS-DVB were completely eluted with methanol-ethyl acetate (1:1, V/V), whereas neither hexane nor dichloromethane provided complete extraction. Some compounds adsorbed on AC, like phthalate esters, were mainly eluted by acetone-hexane (1:1, V/V) and dichloromethane. Because of these behaviors, methanol-acetone (7:3, V/V), acetone-hexane (1:1, V/V), and dichloromethane were selected as elution solvents of XAD-2 resin; methanol-ethyl acetate (1:1, V/V) was used as elution solvent of PS-DVB; and acetone-hexane (1:1, V/V) and dichloromethane was used with AC.

3.3. Evaluation of Mass Overload

Mass overload is also an important consideration for evaluating the enrichment process (Cui et al., 2012). After reloading the effluents of AC column into the proposed enrichment system, three adsorbents extracts were re-analyzed sequentially. No mass overload occurred when the proposed enrichment system was used to extract six types of target analytes, i.e., pesticides, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), amines,
hormones and antibiotics. Although a few analytes of phthalate esters, 2,6-di-tert-butyl-4-methylphenol, as well as stearyl acetate, cholesterol, stigmasterol, and suqalene were detected in the reloading process, these results might result from the background of the enrichment system because a majority of these compounds were also present in the reagent blank (data not shown).

### 3.4. Analysis of Real Samples

The enrichment system described has been applied to real-world drinking water samples. The result demonstrated that 43 organic pollutants were identified from 14 classes of OMPs. Of these 8 pollutants are in the List of Priority Pollutants under the Clean Water Act of USA and 8 pollutants are in the Standards for Drinking Water Quality of China (GB 5749-2006). Eighteen of the identified OMPs were quantified and the results indicated that none of them were present at concentrations in excess of regulatory limits. However, several unregulated organic pollutants such as 2,6-di-tert-butyl-4-methylphenol, 4-cumylphenol, and N-phenyl-β-naphthylamide occurred at the concentrations of as high as 1 µg/L. The potential risk of these compounds to the human health should not be summarily dismissed. Because exposure persists throughout life, some contaminants even at low levels of exposure may have the possibility of causing adverse effects on specific human populations or at specific life stages (Greenlee et al., 2004). Moreover, the compounds occur as complex mixtures that could also influence toxicity. Clearly, the possible human health effects of long-term mixture exposure from these OMPs in drinking water remains a legitimate concern.

### 3.5. Cytotoxicity of UOCEs
To determine adsorbed efficiency and overload in eluates, LO2 cells were exposed for 24 h to UOCEs at concentrations equal to 1 to 4000 times the eluate concentrations. The concentration effects of UOCEs (followed by XAD-2, PS-DVB, and AC, respectively) on LO2 cells are shown in Figure 1. In comparison to the control, there was significant cytotoxicity (p<0.05), when LO2 cells were treated with UOCEs-A at 1-4000 times the actual concentrations in the eluate (Figure 1a), with UOCEs-B followed by XAD-2 and PS-DVB at 1000-4000 times (Figure 1b), and with UOCEs-C followed by three absorbents treatment at 2400-4000 times (Figure 1c), respectively. The cell viability of LO2 cells decreased with the increase in concentrations of the UOCEs, exhibiting concentration-dependent effects. The rank order of cytotoxicity in LO2 cell was A > B > C.

3.6. ROS Induced by UOCEs

To test ROS production induced by UOCEs in LO2 cell lines, we determined ROS using the fluorescent CM-H$_2$DCFDA that diffuses into cells and whose fluorescence intensity depends on the concentration of ROS (Lee et al., 2020). Generation of ROS induced by UOCEs resulted in changes with different patterns depending on time of exposure, as demonstrated by immunofluorescence assay (Figure 2A). Exposure to UOCEs-A for 1 h (Figure 2A), at organic mixtures 1 to 100 times actual concentrations in the eluate significantly increased the ROS levels of LO2 cells (p<0.05), while no significant differences were observed at 1000-3600 times actual concentrations in eluates compared to the vehicle control. In contrast, as the concentration increased to 4000 times of actual concentrations in the eluate, the ROS levels in LO2 cells decreased. ROS generation in LO2 cells induced by UOCEs-B and UOCEs-C (followed by PS-DVB, and AC, respectively) decreased with the concentration increase of
UOCEs-B (2000-4000 times) and UOCEs-C (100-4000 times), respectively (Figure 2B and Figure 2C)

After exposing the mixtures of UOCEs for 12 h, UOCEs-A at 1-10 times of the actual concentration in eluates caused a significant increase in ROS levels in LO2 cells (Figure 2A) similar with results from UOCEs-A exposure for 1 h. ROS levels were significantly decreased at more than 100 times of the actual concentration in the eluate (Figure 2B a); i.e. UOCEs-B at 100, 1000, 2000, 24000, and 4000 times of the actual concentration in the eluate. UOCEs-C at all concentrations caused significant decreases in ROS levels in LO2 cells relative to the vehicle control (Figure 2B and Figure 2C).

3.7. The Effect of UOCEs on Intracellular GSH Levels

To determine the effects of UOCEs on intracellular GSH levels, a non-enzyme antioxidant inhibitor was used to maintain intracellular redox homeostasis (Xie et al., 2020b). LO2 cell lines was exposed to UOCEs for 24 h with 1-4000 times actual concentrations of UOCEs. The concentration effects on LO2 cells with three UOCEs treatments demonstrated different patterns (Figure 3). As shown in Figure 3a, LO2 cells treated with UOCEs-A at 100-4000 times of the actual concentrations in eluate significantly increased intracellular GSH levels, while at 3600-4000 times of the actual concentrations in the eluate, intracellular GSH levels decreased significantly. Surprisingly, intracellular GSH levels first increased and then decreased with the increases of UOCEs-A exposure concentrations. The pattern of intracellular GSH was opposite to the changes in ROS in LO2 cells treated with UOCEs-A. In LO2 cells treated with UOCEs-B, intracellular GSH levels demonstrated a similar change with a significant increase at 100-3200 times of actual concentration of UOCEs-B in eluate and a significant decrease at 3600 times actual concentration UOCEs-B in eluate. However, the magnitude of intracellular GSH
changes were significantly reduced (Figure 3b). No significant difference was observed in intracellular GSH levels when LO2 cells were treated with UOCEs-C at any concentrations (Figure 3c).

4. Discussion

Enrichment of organic pollutants from water is essential for accurate assessment of biological effects and, especially, for identifying the biological effects of low-dose mixed exposure of pollutants. We tested and developed a method based on differential adsorption by in-line integration of various adsorbent materials to assess enrichment of trace amounts of organic contaminants with varying chemical characteristics in water. By determining chemicals that resisted adsorption by different columns, we were able to look at adsorbed and un-adsorbed organic compounds from various eluates with GS-MS and to test these materials for biological effects. With this approach, we confirmed that a single adsorbent did not completely enrich organic pollutants from water and that the escaping effluent components in the eluates showed some biological activity. These observations indicated that quantitative analysis and biological effect testing based on chemicals enrichment with a single adsorbent material may well underestimate the exposure level of organic pollutants in water and underestimate potential harmful effects.

4.1. Enrichment Capability and Overload

Characteristics of sorbents play a critical role in enriching pollutants from water samples (Wang et al., 2020). The enrichment capability of sorbents largely depends on specific surface area (Wang et al., 2017), pore size (Casado et al., 2019) and physicochemical characteristics
of target chemicals (Ng et al., 2018). Because organic pollutants in water have a diversity of polarity characteristics, a single sorbent is unlikely to provide complete enrichment of pollutants from water samples. We used three adsorbents - XAD-2, PS-DVB, and AC - and connected them in series to evaluate the biological effects of these effluents. The XAD-2 resin was used as the first adsorbent to retain non-polar (Marcillo et al., 2017) and poorly polar organics; the PS-DVB extracted polar organics of media solubility (Popov et al., 2021) and the AC extract a wide diversity of organics (Guillossou et al., 2020). We anticipated that the inclusion of PS-DVB and AC would collect organics that were incompletely extracted by XAD-2. Overall, the XAD-2 resin extracted 67.80% of target analytes with a good ER, 5.93% of target analytes with a median ER, and 26.27% of target analytes with a poor ER. The existing DBPs including THMs, HAAs (Wei et al., 2013; Liu et al., 2013), haloacetaldehydes (HALs), and haloketones (HKs) were efficiently extracted by XAD-2 resin in this study, an observation consistent with previous studies (Richardson et al., 2008) PS-DVB and AC supplemented the extraction of target analytes of 35.60% and 25.42%, respectively. Overall, 69.49% of target analytes were efficiently enriched by the series of adsorption of XAD-2 resin, PS-DVB, and AC, indicating that some pollutants were not readily adsorbed. Among the poorly retained compounds, 2-naphthalamine was classified by International Agency for Research on Cancer (IARC) as carcinogenic to humans, Group 1, and that others, N-nitrosodiethylamine, acrylamide and aldrin, are listed as probably carcinogenic to humans, Group 2A. Another group included 3-chloro-1,2-propanediol, naphthalene, tert-butyl-4-hydroxyanisole, and chlorothalonil that are listed as possibly carcinogenic to humans, Group 2B (Cancer, I. A. F. O., 2021) (see Table S7). As for these pollutants, therefore, other enrichment methods need to be developed to extract them efficiently.

4.2. Cytotoxicity of UOCES
A cytotoxicity test, a simple and effective method to detect the biological effects of chemicals and assess potency (Cheuk et al., 2017; Halle et al., 2017), has been widely employed to evaluate comprehensive effects caused by single or mixed exposure of pollutants, assess water quality and assure drinking water safety (Rosenmai et al., 2018). UOCEs had mild cytotoxicity to LO2 cells after the third-stage adsorption, indicating that a single adsorbent would not entirely enrich the pollutants from drinking water. Our mixed contaminants simulated the actual scenario in terms of components and concentrations. The observation that the UOCEs-A eluate demonstrated cytotoxicity at just 1 times of the actual concentration indicated that the complex organic mixture in drinking water has the potential for cytotoxicity after a relatively low degree of enrichment. Compared with UOCEs-A (in eluate after enriching of XAD-2), the concentrations causing cytotoxicity were higher in mimic components with UOCEs-B and UOCEs-C, indicating that even with double adsorption and triple adsorption some materials are not well retained. UOCEs-B and UOCEs-C eluates were still cytotoxic and would need to be examined to get any idea of the full toxic potency of the suite of chemicals in water.

Contaminants in drinking water escaped the thorough in-line adsorption with triple differential absorbents, but there were only minimal cytotoxic effects of UOCEs-C. Clearly, if a single absorbent were used to enrich a complex mixed components of drinking water, the toxic effect from drinking-water contaminants might be underestimated, and toxic effects from some escape components would be missed in reconstituting the adsorbed material. To avoid underestimating exposure levels and biological effects resulting from un-adsorbed pollutants, approaches to recover specific pollutants and a variety of adsorbing materials will need to be used to develop a more comprehensive picture of likely adverse responses to water-borne contaminants.
4.3. **Oxidative Stress Induced by UOCEs**

Oxidative stress and adaptive response are most important defense responses and play an important role in maintaining the normal physiological function and stability of the body (Ornatowski et al., 2020). Oxidative stress reaction were determined by testing ROS and GSH generation (Xie et al., 2020a), both of which can serve as early alarming indicators of exposure to bioactive pollutants (Escher et al., 2012). These assays have been used to identify the harmful effects of pollutants (Wang et al., 2013) such as pesticides (Odetti et al., 2020), phthalates (Al-Saleh et al., 2019), DBPs (Lundqvist et al., 2019), and PAHs (Yu et al., 2021) and to evaluate water quality (Peluso et al., 2021). Low level contaminants present in the UOCEs at low dose levels could lead to significant changes. Low concentrations stimulated ROS production of LO2 cells and high concentrations; however, paradoxically, the same exposures reduced ROS production LO2 cells. On the other hand, UOCEs at low concentrations increased the antioxidant GSH. Both of these endpoints had concentration- and time-dependent relationships, indicating that the decreases in ROS and increases in GSH, both of which could be viewed as beneficial responses, were consistent across the assays. These UOCEs-B and UOCEs-C effluents gave rise to changes in ROS and GSH in LO2 cells even though most pollutants had been adsorbed suggesting an even higher toxic potency for these compounds. These results indicated that the potential adverse effects on long-term continuous exposure to various low-dose contaminants from drinking water to public health should not be ignored and that current methods to assess toxicity from desorbed contaminants may miss some chemicals with the potential for adverse responses.

5. **Conclusions**

Our study employed a succinct and effective experimental design to examine an important issue and to verify a hypothesis that some chemicals may systematically escape concentrating
processes on adsorbent columns leading to misleading classification of adverse effects when looking at adsorbed compounds. Our results clearly demonstrated that adequate methods for enriching contaminants in water need to be carefully designed to capture pollutants and more accurately evaluate the possible biological effects of complex pollutants in drinking water. Any method intended for contaminant enrichment from water sources needs to be designed to capture as large a suite of pollutants in drinking water as possible in order to more accurately examine biological effects associated with the entire suite of whole pollutants present in any particular water source.

Acknowledgments

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Appendix A. Supplementary Material

Contains details on experimental methods, seven tables and four figures, about characterization, information and biological effects of target compounds (Table S1, S2, S6, S7 and Figure S1-S3), compounds of built UOCEs (Table S3) and some information of DFTPP and p,p'-DDT (Table S4 and S5)
References


preconcentration of Cd(II), Cu(II), Ni(II), and Pb(II) in water and food samples using Amberlite XAD-2 functionalized with 3-(2-nitrophenyl)-1H-1,2,4-triazole-5(4H)-thione and determination by inductively coupled plasma-atomic emission spectrometry. J. Agric. Food Chem. 59, 11352-11358. https://doi.org/10.1021/jf202409c


Wang, S., Zhang, H., Zheng, W., Wang, X., Andersen, M.E., Pi, J., He, G., Qu, W., 2013. Organic extract contaminants from drinking water activate Nrf2-mediated antioxidant response in a human cell line.


Figure legends

Table 1
Table 1. Enrichment recoveries of three adsorbents systems for 118 target analytes and 5 surrogates

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<thead>
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<th>Compounds</th>
<th>XAD-2</th>
<th>XAD-2+ PS-DVB*</th>
<th>XAD-2+ PS-DVB +AC*</th>
<th>Compounds</th>
<th>XAD-2</th>
<th>XAD-2+ PS-DVB*</th>
<th>XAD-2+ PS-DVB +AC**</th>
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### 5. Ethers

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<td>bis(2-chloroethyl)ether</td>
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<td>bis(2-chloroisopropyl)ether</td>
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<td>31.24</td>
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<td>4-cumylphenol</td>
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<td>151.96</td>
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### 7. Hormones and antibiotics

<table>
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<td>17α-ethylestradiol</td>
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### 8. Amines

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<th>PC 3</th>
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</tr>
<tr>
<td>Substance</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>N-phenyl-β-naphthlamide</td>
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<td>2-naphthamide</td>
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<tr>
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<td>hexchloropropanone</td>
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<tr>
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<td>100.00</td>
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</tr>
</tbody>
</table>
Note: *means one type of enrichment system by which the organic pollutants from drinking water would be captured via two sequential stages including XAD-2 and PS-DVB adsorption; **means another type of enrichment system by which the organic pollutants from drinking water would be captured via three sequential stages including XAD-2, PS-DVB and AC adsorption.
Single enrichment systems possibly underestimate both exposures and biological effects of organic pollutants from drinking water

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Abbreviations

AC, activated charcoal; ANOVA, analysis of variance; CCK-8, Cell Counting Kit-8; DBPs, disinfection by-products; DFTPP, Decafluorotriphenylphosphine; DMEM, Dulbecco's Modified Eagle Media; ER, enrichment recovery; FBS, fetal bovine serum; GC-MS, gas chromatography-mass spectrometer; GSH, glutathione; HAAs, haloacetic acids; HALs, haloacetaldehydes; HKs, haloketones; IARC, International Agency for Research on Cancer; OMPs, organic micropollutants; P/S, penicillin–streptomycin; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; PS-DVB, poly-styrene–divinylbenzene; ROS, reactive oxygen species; SI, supporting information; THMs, trihalomethanes; Tris-HCl, tris-(hydroxymethyl)aminomethane; UOCEs, unadsorbed organic compounds in eluates; U.S. EPA, the United States of America Environmental Protection Agency.
Abstract (284 words)

Comprehensive enrichment of contaminants in drinking water is an essential step for accurately determining exposure levels of contaminants and testing their biological effects. Traditional methods using a single absorbent for enriching contaminants in water might not be adequate for complicated matrices with different physical-chemical profiles. To examine this hypothesis, we used an integrated enrichment system that had three sequential stages-XAD-2 resin, poly (styrene–divinylbenzene) and activated charcoal to capture organic pollutants and disinfection by-products (DBPs) from drinking water in Shanghai. Un-adsorbed Organic Compounds in Eluates (UOCEs) named UOCEs-A, -B, and -C following each adsorption stage were determined by gas chromatography-mass spectrometry to evaluate adsorption efficiency of the enrichment system. Meanwhile, biological effects such as cytotoxicity, effects on reactive oxygen species (ROS) generation and glutathione (GSH) depletion were determined in human LO2 cells to identify potential adverse effects on exposure to low dose contaminants. We found that poly-styrene–divinylbenzene (PS-DVB) and activated charcoal (AC) could still partly collect UOCEs-A and-B that the upper adsorption column incompletely captured, and that potential carcinogens like 2-naphthamine were present in all eluates. UOCEs-A at (1-4000), UOCEs-B at (1000-4000), and UOCEs-C at (2400-4000) folds of the actual concentrations had significant cytotoxicity to LO2 cells. Additionally, ROS and GSH change in cells treated with UOCEs indicated the potential for long-term effects of exposure to some mixtures of contaminants such as DBPs at low doses. These results suggested that an enriching system with a single adsorbent would underestimate the exposure level of pollutants and the biological effects of organic pollutants from drinking water. Effective methods for pollutants’ enrichment and capture of drinking water should be given priority in future studies on accurate evaluation of biological effects exposed to mixed pollutants via drinking water.
Keywords: Organic pollutants, enrichment system, XAD-2 resin, disinfection by-products, DBPs, biological effects
1. **Introduction**

Safe drinking water is necessary for human health and well-being. However, more than 663 million people worldwide still used unimproved drinking water sources (UNICEF and World Health Organization Geneva., 2015). Micropollutants in aquatic environments, including surface water (De Baat et al., 2020), groundwater (Li et al., 2020), and wastewater (Thomaidi et al., 2015), have been widely reported over the last few decades (Schwarzenbach et al., 2006). These micropollutants are released directly or indirectly into the aquatic environment by urban, industrial, agricultural, and other anthropogenic activities, or enter as transformation products formed during incomplete degradation by effluent treatment (Knopp et al., 2016). The presence of various hazardous organic compounds in drinking water may cause long-term health effects, including gastrointestinal and urinary tract cancers (Reungoat et al., 2010). However, current drinking-water treatment plants are not specifically designed to eliminate micropollutants from aquatic environments (Haddad et al., 2019). Thus, many of the organic micropollutants (OMPs) are not removed and remain in drinking water. Some emerging OMPs have already been detected in drinking water (Yang et al., 2017). Additionally, some micropollutants may react with disinfectants to form disinfection by-products (DBPs) during the water treatment process. Epidemiological studies have found an association between exposure to DBPs and bladder cancer (Diana et al., 2019). In view of the ubiquity of a high number of potentially toxic OMPs in drinking water and the largely unknown long-term effects of these pollutants on human health, OMPs including DBPs in drinking water have aroused considerable public concern (Li et al., 2018).

Exposure assessment and adverse effect identification are crucial starting points for health risk assessment of chemicals. Because most organic contaminants in drinking water are volatile (Rajasärkkä et al., 2016) or at least semi-volatile (Wu et al., 2013) and present at sub-
microgram to microgram per liter, it is necessary to concentrate and enrich trace organic pollutants in water before analyzing possible biological effects. Liquid-liquid extraction and solid-phase extraction are classic methods for enriching volatile and semi-volatile organic constituents in drinking water (How et al., 2021), but these methods are unsuitable for enriching chemicals from water to conduct biological effect assays. This is because liquid-liquid extraction and solid-phase extraction are commonly used for sample extraction and purification for determination of targeted chemicals in water rather than for testing multiple biological assays. Moreover, time-consuming enrichment processions may affect the stability of chemical substances in water and lead to enriched chemicals that are different from collected water samples, which reduces the accuracy of the analysis of biological effects. In recent years, a series of novel methods with multiple liquid-liquid extractions (LLEs) have been developed to improve extraction efficiencies of polar halogenated DBPs (Han et al., 2017; 2018), which increased our understanding of enrichment and extractions of pollutants.

Traditional methods for enriching OMPs in various water sources mainly rely on single absorbents such as Amberlite XAD resins to extract large scale water samples for biological assay (Xiao et al., 2012). The Amberlite XAD-2 resin has been widely used as a low polarity adsorbent for non-polar and low polarity organic pollutants enrichment to detect mutagens in water (Egea et al., 2021). However, owing to diverse components in water sources and the complexity of intrinsic physicochemical properties of these chemicals, the use of a single absorbent that focuses on specific chemical characteristics of pollutants for enriching waters may not be adequate to capture the broader suite of chemicals and low concentrations of these chemicals likely to be present in the complex mixtures in various water sources (Stalter et al., 2016).
Any single adsorbent would have its own specific limitation, so we designed an integrated strategy using three different adsorbents, arranged in series, to trap a larger portion of contaminants from drinking water to observe and evaluate adsorption efficiency of the enrichment systems. After passing through an XAD resin adsorption, the eluate moved through a poly (styrene–divinylbenzene) (PS-DVB) co-polymer adsorbent with a high sorption capacity for medium polarity of organic contaminants (Mirnaghi et al., 2012) and highly polar compounds such as phenols (Moret et al., 2005). A third adsorbent stage following the PS-DVB adsorbent used an activated charcoal (AC) adsorbent to capture chemicals that had not been removed by the first two stages (Li et al., 2010). Therefore, the overall objective of this study was to determine whether a single adsorbent system efficiently captured most contaminants in drinking water. To pursue and verify this hypothesis, we determined the unabsorbed organic compounds in eluate (UOCEs) following each stage by gas chromatography–mass spectrometer (GC-MS) and measured the cytotoxicity, reactive oxygen species (ROS) generation, and glutathione (GSH) depletion in human LO2 cells induced by eluates from different stages of our sequential adsorbent procedure. Our study demonstrated that an enriching system with a single adsorbent would underestimate the exposure levels and the effects of some contaminants in drinking water; this may help develop novel integrated enrichment systems to comprehensively understand biological effects induced by contaminants in drinking water and to objectively evaluate drinking-water safety.

2. Materials and Methods

2.1. Agents

The chemicals were purchased from Sigma (St Louis, MO), Dr. Ehrenstorfer (Augsburg, Germany) Riedel-de Haen (Seelze, Germany), and Toronto Research Chemicals (Toronto,
Canada), respectively, unless specified otherwise, and listed in supporting information (SI). All pesticide residue grade solvents (including methanol, acetone, dichloromethane, n-hexane, ethanol, acetonitrile, ethyl acetate, and n-propanol) were obtained from Dikama (USA). Dulbecco’s Modified Eagle Media (DMEM), 0.25% Trypsin-EDTA, 5- and 6-Chloromethyl-2 and Penicillin–streptomycin (P/S) were purchased from ThermoFisher (Waltham, MA, USA), Fetal bovine serum (FBS) from Mediatech, Inc. (Manassas, VA, USA). Cell Counting Kit-8 (CCK-8) and total glutathione (GSH) quantification kit from Dojindo (Tokyo, Japan), and tris-(hydroxymethyl)aminomethane (pH7.6) was from Beyotime Biotechnology (Shanghai, China).

The Amberlite® XAD-2 resin (20-60 mesh, CAS No. 9060-05-3), StratoSphere™ PS-DVB resin (100-200 mesh, CAS No. 9003-70-7), and AC (20-40 mesh, CAS No.7440-44-0) were obtained from Sigma (St. Louis. MO, USA). A Milli-Q Integral Water Purification System for Ultrapure Water (MilliporeSigma, Burlington, MA, USA) supplied laboratory reagent water for all experiments.

All glassware was washed with acetone and water, soaked overnight in concentrated sulfuric acid containing 5% K₂Cr₂O₇ solution, and baked at 200 °C for two hours after washing with ultra-pure water. Care was taken to use plastic products to the smallest extent possible to avoid contamination.

2.2. Synthetic Water Sample and Real Water Sample

To prove the limitations of a single enrichment system on pollutants’ adsorption, we designed a succinct experiment to test the hypothesis. Pollutants in a real water sample were determined by GC-MS to identify compounds and their concentrations. Then a synthetic water sample that was made up based on the results of detected pollutants and their concentrations with high purity chemicals to avoid
interference from natural organic matter in water, which was employed to exam the capacity of pollutants’ enrichment by different adsorbents. The known composition and concentrations of this synthetic water sample was used to simulate water samples in the real world and provide a good case for verifying our hypothesis.

In this study, 118 volatile and semi-volatile chemicals were selected as targeted compounds according to their high detection frequency in drinking water that used the Huangpu River as a water source (Chen et al., 2008). Twenty micrograms of each target compound were spiked into the 40 L of Milli-Q water to create the synthetic water sample. The suite of compounds was analyzed as described in Supplementary File SI (Table S1). The comparison of 118 target compounds with existing regulatory values and standards for water quality throughout the world is also given in table S2 of SI.

Forty liters of the drinking water of Shanghai was sampled into clean amber glass bottles. Before sample collection, each acid-treated bottle was thoroughly pre-rinsed with Milli-Q water at the laboratory and then rinsed with sample water prior to sample collection. Water samples were immediately shipped to the laboratory at 4°C in ice and stored at -80°C for analysis. Analyses were all conducted within 4 days of freezing (O'Toole et al., 2009; Kim et al., 2020).

2.3. Sample Enrichment Procedure

Briefly, appropriate amounts of XAD-2 resin, PS-DVB resin, and activated charcoal were first conditioned and then transferred to home-made glass cartridges. Twenty micrograms of each target compound were spiked into the 40 L of Milli-Q water to create the synthetic water sample. And then this water sample was spiked with 20 µg of each of the 118 target compounds was loaded onto the XAD-2 resin. After adjustment of the eluate to pH between 2.0 to 3.0, the
eluate from the XAD-2 resin cartridge flowed into two PS-DVB cartridges arranged in series. The eluate from the second PS-DVB cartridge was treated to adjust pH to 5.0 and then passed on to the activated charcoal cartridge. A variety of eluting solvent mixtures were used for removing extracted target compounds from the solid absorbents. Each adsorbent extract was evaporated to dryness at room temperature and then reconstituted with methanol. The detailed process of sample pretreatment is presented in SI.

2.4. GC-MS Analysis

We adapted the United States of America Environmental Protection Agency (U.S. EPA) 525.2 method (EPA525.2 method, Revision 2.0) to determine the targeted analytes except for DBPs (see SI). Briefly, the adsorbent extracts were analyzed using a Finnigan Trace GC ultra/Trace DSQ gas chromatograph equipped with mass spectrometry (Thermo Fisher Scientific, Waltham, MA). A capillary GC column DB-5MS (30 m×0.25 mm×0.25 µm) (Agilent, USA) was programmed for heating as follows: starting from 40 °C kept 1 min to 130 °C at a rate of 30 °C/min and kept 3 min, from 130 °C to 180 °C at a rate of 12 °C/min, from 180 °C to 240 °C at a rate of 7 °C/min, and from 240 °C to 320 °C at a rate of 12 °C/min and kept 5 min at 320 °C. High purity helium (99.999%) was used as the carrier gas. Splitless injection of samples (2 µl) into the GC-MS was performed at 280 °C. The electron ionization conditions were as follows: ion energy, 70 eV, ion source temperature 280 °C, and m/z=50-600 full scan for qualitative analysis and SIM mode for quantitative analysis. All of the characteristic ions and quantitative ions for target analytes are presented in Table S1. DBPs were determined according to U.S. EPA recommended methods (Pan et al., 2017).
2.5. Enrichment Capacity

For the assessment of the enrichment capacity of packed adsorbent columns, the enrichment recovery (ERs including ER$_{XAD-2}$, ER$_{PS-DVB}$ and ER$_{AC}$) was calculated by formula 1, respectively. $C_a$ is the extracted concentration of target compounds in the adsorbent phase and $C_0$ is the initial concentration of compounds in the synthetic water sample.

\[ ER = \frac{C_a}{C_0} \times 100\% \]  \hspace{1cm} (1)

As reference points, ER values were ranked as follows: (i) if ER was greater than 80%, the extraction capacity was ranked good; (ii) if ER ranged between 60% and 80%, the extraction capacity was ranked medium; (iii) if ER was less than 40%, the extraction capacity was ranked poor.

2.6. Unadsorbed Organic Compounds in Eluates (UOCEs)

According to these ER values, we obtained the concentration ($C_b$) of each un-adsorbed organic compound in the eluate of adsorbents by formulas 2-4, respectively.

\[ C_b \text{ (in the eluate of XAD-2)} = C_0 \times (100 - ER_{XAD-2}) \]  \hspace{1cm} (2)

\[ C_b \text{ (in the eluate of PS-DVB)} = C_0 \times (100 - ER_{XAD-2} - ER_{PS-DVB}) \]  \hspace{1cm} (3)

\[ C_b \text{ (in the eluate of AC)} = C_0 \times (100 - ER_{XAD-2} - ER_{PS-DVB} - ER_{AC}) \]  \hspace{1cm} (4)

Furthermore, based on $C_b$, we created three mixtures in methanol named UOCEs-A, -B and -C respectively (Table S3). These samples were stored at -20 °C. Prior to biological effect tests, the methanol in these samples were first volatilized to dryness by natural means and then the solutes were re-dissolved in dimethyl sulfoxide to appropriate concentrations for biological effect tests.
2.7. Water Samples Analysis

The 40 L aliquots of drinking water were added five surrogate standard chemicals and then flowed through the pre-conditioned XAD-2, PS-DVB, and AC sorbents column in series. The OMPs from the samples were extracted and then desorbed by different eluting solvents. For OMPs adsorbed by the XAD-2 resin, the 40 mL of methanol-acetone (7:3, V/V), 30 mL of acetone-hexane (1:1, V/V) and 45 mL of dichloromethane were sequentially passed through the cartridge and the eluates were collected in a clean glass vial; for OMPs adsorbed by PS-DVB, 60 mL of methanol-ethyl acetate (1:1, V/V), 20 mL of hexane and 20 mL of dichloromethane flowed through the cartridge and the eluates were collected in turn; for OMPs adsorbed by AC adsorbent, 40 mL of acetone-hexane (1:1, V/V) and 20 mL of dichloromethane were passed through the cartridge and the eluates were collected separately. All elution solvents were equilibrated on the cartridges for 15 minutes and then solvent flowed through the columns at a rate of 3-5 mL/min. The eluates were concentrated to 1.0 mL under a nitrogen flow and 2 µL of aliquot was injected for the GC-MS analysis. The qualitative analysis was performed by both the retention times and characteristic ions. The quantitative analysis was carried out by use of internal calibration curves.

2.8. Quality Control

Quality controls for all target compounds were confirmed by equipment calibration checks, blank control experiments and quality control charts during sample analysis. First, a 2 µL aliquot of decafluorotriphenylphosphine (DFTPP) (2.5 ng/µL) and 2 µL aliquot of p,p’-DDT was injected into GC-MS before any samples were analyzed and also intermittently throughout sample analysis to evaluate the equipment operations. If the DFTPP mass spectrum did not
meet all ion abundance criteria, the MS was returned. If degradation of p,p’-DDT exceeded 20%, maintenance was performed on the GC injection port or other parts of the system. The results of calibration check of DFTPP and p,p’-DDT is given in the Supplementary Information Table S4 and S5. Secondly, continuous monitoring of 2.0 ng/µL aliquot of target compounds for twenty days was applied to create a quality control chart (data not shown). Thirdly, laboratory reagent blanks and fortified method blanks were performed daily to ensure that the GC-MS system was free of contamination.

As for DBPs analysis, a field blank (distilled water) was set for each sampling campaign. For every batch of 10 samples, both a solvent blank and a procedural blank (both for aqueous and solid samples) were added to ensure that the samples and the analysis process were free of contamination. No quantifiable analytes were detected in the blanks.

2.9. Cell Cultures

Human embryo liver LO2 cells, purchased from the Cell bank of the Chinese Academy of Sciences, were cultured in DMEM with 10% FBS and 1% P/S at 37 °C in an incubator with 5% CO2 in air (Yang et al., 2018).

2.10. Cytotoxicity

LO2 cells (1×10⁴ cell per well) were plated in 96-well plates, incubated for 24 h and treated with UOCEs-A, -B and -C at 1 to 4000 times actual concentrations in eluates for 24 h. Methanol (1%, V/V) served as the vehicle control and CdCl₂ (25 µmol/L) as the positive control. Following, every well was added CCK-8 solution (10 µL) and their optical density values were
determined at 450 nm after incubating for 30 min. Cell viability was expressed as percent relative to the vehicle control. Experiments were performed in five replicates.

2.11. Intracellular ROS and Total GSH Levels

ROS were determined by a microplate reader using CM-H$_2$DCFDA (Li et al., 2008), at 488 nm excitation and 525 nm emission. Total GSH levels were determined following the protocol of the Total GSH Quantification Kit through the reaction between 5, 5'-dithiobis (2-nitrobenzoic acid) and GSH. The levels were expressed as a percentage relative to the vehicle control. The experiment was performed in four and three replicates, respectively.

Statistical Analysis. The results between and within groups were analyzed by analysis of variance (ANOVA) by IBM SPSS Statistics Version 22. If the variance was homogeneous, a Student-Newman-Keuls test was used to do multiple comparison; otherwise, a Games-Howell tests was used. A $p$-value < 0.05 was defined as statistically significant. Figures were drawn by GraphPad Prism Version 7.00.

3. Results

3.1. Effect of Adsorbent.

In this study three adsorbents of XAD-2, PS-DVB, and AC were connected in series and used for extracting duplicate blank spiked samples and their ER values were then calculated. As shown in Table 1, it was found that 80 out of total 118 target analytes could be satisfactorily enriched by XAD-2 resin with an ER of greater than 60%. Moreover, 39 out of the 80 analytes were not extracted by PS-DVB or by AC. In total, thirty-one of the target analytes were poorly extracted by XAD-2. A total of 41 target analytes were only partly extracted with the PS-DVB and 28 by AC. (See Table S6.)
3.2. Effect of Eluting Solvent.

The choice of the eluting solvent was an important determinant of extraction efficiency. Organic solvents such as methanol, acetone, dichloromethane, and n-hexane have commonly been used to elute the XAD-2 resin (Lynch et al., 1975; Kumar et al., 2011). In the present study methanol-acetone (7:3, V/V) (Lan, 1994) acetone-hexane (1:1, V/V) (Kanno et al., 2010; Xu et al., 2010) and dichloromethane (Woudneh et al., 2006) were examined with regard to elution of trace organic compounds adsorbed from XAD-2. Based on previous studies, methanol-ethyl acetate (1:1, V/V), hexane, and dichloromethane were investigated as eluting solvents for PS-DVB (Pietrzynska et al., 2013) and acetone-hexane (1:1, V/V) was used for AC (Kitamura et al., 2004). In our study, 54% of target analytes absorbed on XAD-2 resin were satisfactorily eluted by methanol-acetone (7:3, V/V) with more than 60% ER. Moreover, some of the remaining adsorbed compounds could be eluted by acetone-hexane (1:1, V/V) and dichloromethane. The compounds adsorbed on PS-DVB were completely eluted with methanol-ethyl acetate (1:1, V/V), whereas neither hexane nor dichloromethane provided complete extraction. Some compounds adsorbed on AC, like phthalate esters, were mainly eluted by acetone-hexane (1:1, V/V) and dichloromethane. Because of these behaviors, methanol-acetone (7:3, V/V), acetone-hexane (1:1, V/V), and dichloromethane were selected as elution solvents of XAD-2 resin; methanol-ethyl acetate (1:1, V/V) was used as elution solvent of PS-DVB; and acetone-hexane (1:1, V/V) and dichloromethane was used with AC.

3.3. Evaluation of Mass Overload

Mass overload is also an important consideration for evaluating the enrichment process (Cui et al., 2012). After reloading the effluents of AC column into the proposed enrichment system, three adsorbents extracts were re-analyzed sequentially. No mass overload occurred when the proposed enrichment system was used to extract six types of target analytes, i.e., pesticides,
polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), amines, hormones and antibiotics. Although a few analytes of phthalate esters, 2,6-di-tert-butyl-4-methylphenol, as well as stearyl acetate, cholesterol, stigmasterol, and suqalene were detected in the reloading process, these results might result from the background of the enrichment system because a majority of these compounds were also present in the reagent blank (data not shown).

### 3.4. Analysis of Real Samples

The enrichment system described has been applied to real-world drinking water samples. The result demonstrated that 43 organic pollutants were identified from 14 classes of OMPs. Of these 8 pollutants are in the List of Priority Pollutants under the Clean Water Act of USA and 8 pollutants are in the Standards for Drinking Water Quality of China (GB 5749-2006). Eighteen of the identified OMPs were quantified and the results indicated that none of them were present at concentrations in excess of regulatory limits. However, several unregulated organic pollutants such as 2,6-di-tert-butyl-4-methylphenol, 4-cumylphenol, and N-phenyl-β-naphthylamide occurred at the concentrations of as high as 1 µg/L. The potential risk of these compounds to the human health should not be summarily dismissed. Because exposure persists throughout life, some contaminants even at low levels of exposure may have the possibility of causing adverse effects on specific human populations or at specific life stages (Greenlee et al., 2004). Moreover, the compounds occur as complex mixtures that could also influence toxicity. Clearly, the possible human health effects of long-term mixture exposure from these OMPs in drinking water remains a legitimate concern.

### 3.5. Cytotoxicity of UOCEs
To determine adsorbed efficiency and overload in eluates, LO2 cells were exposed for 24 h to UOCEs at concentrations equal to 1 to 4000 times the eluate concentrations. The concentration effects of UOCEs (followed by XAD-2, PS-DVB, and AC, respectively) on LO2 cells are shown in Figure 1. In comparison to the control, there was significant cytotoxicity (p<0.05), when LO2 cells were treated with UOCEs-A at 1-4000 times the actual concentrations in the eluate (Figure 1a), with UOCEs-B followed by XAD-2 and PS-DVB at 1000-4000 times (Figure 1b), and with UOCEs-C followed by three absorbents treatment at 2400-4000 times (Figure 1c), respectively. The cell viability of LO2 cells decreased with the increase in concentrations of the UOCEs, exhibiting concentration-dependent effects. The rank order of cytotoxicity in LO2 cell was A > B > C.

3.6. ROS Induced by UOCEs

To test ROS production induced by UOCEs in LO2 cell lines, we determined ROS using the fluorescent CM-H$_2$DCFDA that diffuses into cells and whose fluorescence intensity depends on the concentration of ROS (Lee et al., 2020). Generation of ROS induced by UOCEs resulted in changes with different patterns depending on time of exposure, as demonstrated by immunofluorescence assay (Figure 2A). Exposure to UOCEs-A for 1 h (Figure 2A), at organic mixtures 1 to 100 times actual concentrations in the eluate significantly increased the ROS levels of LO2 cells (p<0.05), while no significant differences were observed at 1000-3600 times actual concentrations in eluates compared to the vehicle control. In contrast, as the concentration increased to 4000 times of actual concentrations in the eluate, the ROS levels in LO2 cells decreased. ROS generation in LO2 cells induced by UOCEs-B and UOCEs-C (followed by PS-DVB, and AC, respectively) decreased with the concentration increase of
UOCEs-B (2000-4000 times) and UOCEs-C (100-4000 times), respectively (Figure 2B and Figure 2C).

After exposing the mixtures of UOCEs for 12 h, UOCEs-A at 1-10 times of the actual concentration in eluates caused a significant increase in ROS levels in LO2 cells (Figure 2A) similar with results from UOCEs-A exposure for 1 h. ROS levels were significantly decreased at more than 100 times of the actual concentration in the eluate (Figure 2B a); i.e. UOCEs-B at 100, 1000, 2000, 24000, and 4000 times of the actual concentration in the eluate. UOCEs-C at all concentrations caused significant decreases in ROS levels in LO2 cells relative to the vehicle control (Figure 2B and Figure 2C).

3.7. The Effect of UOCEs on Intracellular GSH Levels

To determine the effects of UOCEs on intracellular GSH levels, a non-enzyme antioxidant inhibitor was used to maintain intracellular redox homeostasis (Xie et al., 2020b). LO2 cell lines was exposed to UOCEs for 24 h with 1-4000 times actual concentrations of UOCEs. The concentration effects on LO2 cells with three UOCEs treatments demonstrated different patterns (Figure 3). As shown in Figure 3a, LO2 cells treated with UOCEs-A at 100-4000 times of the actual concentrations in eluate significantly increased intracellular GSH levels, while at 3600-4000 times of the actual concentrations in the eluate, intracellular GSH levels decreased significantly. Surprisingly, intracellular GSH levels first increased and then decreased with the increases of UOCEs-A exposure concentrations. The pattern of intracellular GSH was opposite to the changes in ROS in LO2 cells treated with UOCEs-A. In LO2 cells treated with UOCEs-B, intracellular GSH levels demonstrated a similar change with a significant increase at 100-3200 times of actual concentration of UOCEs-B in eluate and a significant decrease at 3600 times actual concentration UOCEs-B in eluate. However, the magnitude of intracellular GSH
changes were significantly reduced (Figure 3b). No significant difference was observed in intracellular GSH levels when LO2 cells were treated with UOCEs-C at any concentrations (Figure 3c).

4. Discussion

Enrichment of organic pollutants from water is essential for accurate assessment of biological effects and, especially, for identifying the biological effects of low-dose mixed exposure of pollutants. We tested and developed a method based on differential adsorption by in-line integration of various adsorbent materials to assess enrichment of trace amounts of organic contaminants with varying chemical characteristics in water. By determining chemicals that resisted adsorption by different columns, we were able to look at adsorbed and un-adsorbed organic compounds from various eluates with GS-MS and to test these materials for biological effects. With this approach, we confirmed that a single adsorbent did not completely enrich organic pollutants from water and that the escaping effluent components in the eluates showed some biological activity. These observations indicated that quantitative analysis and biological effect testing based on chemicals enrichment with a single adsorbent material may well underestimate the exposure level of organic pollutants in water and underestimate potential harmful effects.

4.1. Enrichment Capability and Overload

Characteristics of sorbents play a critical role in enriching pollutants from water samples (Wang et al., 2020). The enrichment capability of sorbents largely depends on specific surface area (Wang et al., 2017), pore size (Casado et al., 2019) and physicochemical characteristics
of target chemicals (Ng et al., 2018). Because organic pollutants in water have a diversity of polarity characteristics, a single sorbent is unlikely to provide complete enrichment of pollutants from water samples. We used three adsorbents - XAD-2, PS-DVB, and AC - and connected them in series to evaluate the biological effects of these effluents. The XAD-2 resin was used as the first adsorbent to retain non-polar (Marcillo et al., 2017) and poorly polar organics; the PS-DVB extracted polar organics of media solubility (Popov et al., 2021) and the AC extract a wide diversity of organics (Guillossou et al., 2020). We anticipated that the inclusion of PS-DVB and AC would collect organics that were incompletely extracted by XAD-2. Overall, the XAD-2 resin extracted 67.80% of target analytes with a good ER, 5.93% of target analytes with a median ER, and 26.27% of target analytes with a poor ER. The existing DBPs including THMs, HAAs (Wei et al., 2013; Liu et al., 2013), haloacetaldehydes (HALs), and haloketones (HKs) were efficiently extracted by XAD-2 resin in this study, an observation consistent with previous studies (Richardson et al., 2008) PS-DVB and AC supplemented the extraction of target analytes of 35.60% and 25.42%, respectively. Overall, 69.49% of target analytes were efficiently enriched by the series of adsorption of XAD-2 resin, PS-DVB, and AC, indicating that some pollutants were not readily adsorbed. Among the poorly retained compounds, 2-naphthamine was classified by International Agency for Research on Cancer (IARC) as carcinogenic to humans, Group 1, and that others, N-nitrosodiethylamine, acrylamide and aldrin, are listed as probably carcinogenic to humans, Group 2A. Another group included 3-chloro-1,2-propanediol, naphthalene, tert-butyl-4-hydroxyanisole, and chlorothalonil that are listed as possibly carcinogenic to humans, Group 2B (Cancer, I. A. F. O., 2021) (see Table S7). As for these pollutants, therefore, other enrichment methods need to be developed to extract them efficiently.

4.2. Cytotoxicity of UOCEs
A cytotoxicity test, a simple and effective method to detect the biological effects of chemicals and assess potency (Cheuk et al., 2017; Halle et al., 2017), has been widely employed to evaluate comprehensive effects caused by single or mixed exposure of pollutants, assess water quality and assure drinking water safety (Rosenmai et al., 2018). UOCEs had mild cytotoxicity to LO2 cells after the third-stage adsorption, indicating that a single adsorbent would not entirely enrich the pollutants from drinking water. Our mixed contaminants simulated the actual scenario in terms of components and concentrations. The observation that the UOCEs-A eluate demonstrated cytotoxicity at just 1 times the actual concentration indicated that the complex organic mixture in drinking water has the potential for cytotoxicity after a relatively low degree of enrichment. Compared with UOCEs-A (in eluate after enriching of XAD-2), the concentrations causing cytotoxicity were higher in mimic components with UOCEs-B and UOCEs-C, indicating that even with double adsorption and triple adsorption some materials are not well retained. UOCEs-B and UOCEs-C eluates were still cytotoxic and would need to be examined to get any idea of the full toxic potency of the suite of chemicals in water.

Contaminants in drinking water escaped the thorough in-line adsorption with triple differential absorbents, but there were only minimal cytotoxic effects of UOCEs-C. Clearly, if a single absorbent were used to enrich a complex mixed components of drinking water, the toxic effect from drinking-water contaminants might be underestimated, and toxic effects from some escape components would be missed in reconstituting the adsorbed material. To avoid underestimating exposure levels and biological effects resulting from un-adsorbed pollutants, approaches to recover specific pollutants and a variety of adsorbing materials will need to be used to develop a more comprehensive picture of likely adverse responses to water-borne contaminants.
4.3. Oxidative Stress Induced by UOCEs

Oxidative stress and adaptive response are most important defense responses and play an important role in maintaining the normal physiological function and stability of the body (Ornatowski et al., 2020). Oxidative stress reaction were determined by testing ROS and GSH generation (Xie et al., 2020a), both of which can serve as early alarming indicators of exposure to bioactive pollutants (Escher et al., 2012). These assays have been used to identify the harmful effects of pollutants (Wang et al., 2013) such as pesticides (Odetti et al., 2020), phthalates (Al-Saleh et al., 2019), DBPs (Lundqvist et al., 2019), and PAHs (Yu et al., 2021) and to evaluate water quality (Peluso et al., 2021). Low level contaminants present in the UOCEs at low dose levels could lead to significant changes. Low concentrations stimulated ROS production of LO2 cells and high concentrations; however, paradoxically, the same exposures reduced ROS production LO2 cells. On the other hand, UOCEs at low concentrations increased the antioxidant GSH. Both of these endpoints had concentration- and time-dependent relationships, indicating that the decreases in ROS and increases in GSH, both of which could be viewed as beneficial responses, were consistent across the assays. These UOCEs-B and UOCEs-C effluents gave rise to changes in ROS and GSH in LO2 cells even though most pollutants had been adsorbed suggesting an even higher toxic potency for these compounds. These results indicated that the potential adverse effects on long-term continuous exposure to various low-dose contaminants from drinking water to public health should not be ignored and that current methods to assess toxicity from desorbed contaminants may miss some chemicals with the potential for adverse responses.

5. Conclusions

Our study employed a succinct and effective experimental design to examine an important issue and to verify a hypothesis that some chemicals may systematically escape concentrating
processes on adsorbent columns leading to misleading classification of adverse effects when looking at adsorbed compounds. Our results clearly demonstrated that adequate methods for enriching contaminants in water need to be carefully designed to capture pollutants and more accurately evaluate the possible biological effects of complex pollutants in drinking water. Any method intended for contaminant enrichment from water sources needs to be designed to capture as large a suite of pollutants in drinking water as possible in order to more accurately examine biological effects associated with the entire suite of whole pollutants present in any particular water source.

Acknowledgments

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Appendix A. Supplementary Material

Contains details on experimental methods, seven tables and four figures, about characterization, information and biological effects of target compounds (Table S1, S2, S6, S7 and Figure S1-S3), compounds of built UOCEs (Table S3) and some information of DFTPP and p,p'-DDT (Table S4 and S5)


preconcentration of Cd(II), Cu(II), Ni(II), and Pb(II) in water and food samples using Amberlite XAD-2 functionalized with 3-(2-nitrophenyl)-1H-1,2,4-triazole-5(4H)-thione and determination by inductively coupled plasma-atomic emission spectrometry. J. Agric. Food Chem. 59, 11352-11358. https://doi.org/10.1021/jf202409c


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Figure legends.
Table 1. Enrichment recoveries of three adsorbents systems for 118 target analytes and 5 surrogates

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<th>Compounds</th>
<th>XAD-2</th>
<th>XAD-2+ PS-DVB*</th>
<th>XAD-2+ PS-DVB +AC*</th>
<th>Compounds</th>
<th>XAD-2</th>
<th>XAD-2+ PS-DVB*</th>
<th>XAD-2+ PS-DVB +AC**</th>
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<td>diethylstilbestrol</td>
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<td>Substance</td>
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<td>----------------------------------</td>
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<tr>
<td>acrylamide</td>
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<td><strong>9. DBPs</strong></td>
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<tr>
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<td>bromodichloroacetaldehyde</td>
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<tr>
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<tr>
<td>hexchloropropanone</td>
<td>95.90</td>
<td>100.00</td>
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Note: *means one type of enrichment system by which the organic pollutants from drinking water would be captured via two sequential stages including XAD-2 and PS-DVB adsorption; **means another type of enrichment system by which the organic pollutants from drinking water would be captured via three sequential stages including XAD-2, PS-DVB and AC adsorption.
Author Contributions Statement:

Conceptualization: WQ
Data curation: LY, YZ, LC, WL, HC
Formal analysis: LY, LC, YZ, WZ
Funding acquisition: WQ
Investigation: LY, YZ, WZ, ZJ, HC
Methodology: YZ, LY, LC, HC
Project administration: WQ
Resources: National Natural Science Foundation of China
Software: SPSS 20.0
Supervision: WQ, HL, YH, JC, MA
Validation: LY, YZ, WL, YH
Visualization: NA
Roles/Writing
Original draft: LY, YZ, WQ, JC, LC
Writing: LY, YZ, SJ, WQ, JC, MA
Review & editing: WQ, WL, JC, MA, WZ, YH

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Conflict of Interest Disclosure: The authors declare no competing financial interest.
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
Dec. 15, 2021

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RE: No. CHEM93484 entitled “Single enrichment systems possibly underestimate both exposures and biological effects of organic pollutants from drinking water”

Dear Professor Zhang,

We very much appreciate the comments of the reviewers to help improve our manuscript (CHEM93484). We have carefully addressed each of the comments (see below) and made all the changes requested, and this is now reflected in our completely revised manuscript. We have accepted the reviewer’s suggestion to revise the title of the manuscript, which has been changed to: “Single enrichment systems possibly underestimate both exposures and biological effects of organic pollutants from drinking water”

We have kept the original reviewers’ order number and line numbers of the text in order that you can easily find them.

As suggested by the first reviewer, we have revised the Highlights, manuscript and clarified the issues. A native English-speaking author has helped edit the manuscript extensively.

For the third reviewer’s suggestion, we have corrected our errors in the text and revised Table 1 in order that readers can easily follow our methods.
We have discussed all the issues and responses with the co-authors. All the main changes are highlighted in blue, and the locations and revised contents are addressed in our relevant response connected to each comment. In addition, we have carefully checked the references that we cited.

We believe the changes made in response to the reviewers' constructive comments have greatly improved our study, which we anticipate will make an important contribution to the field.

Please note the edits we have made in the revised manuscript have changed the numbers of some pages, lines, figures, and references mentioned in the reviewers’ original comments.

Thank you very much for your assistance. We look forward to hearing from you soon.

Yours sincerely,

Weidong Qu M.D. & Ph.D.
Professor in Environmental Health
Director of Centres for Water and Health
Department of Environmental Health
School of Public Health
Fudan University
Figure 1

LO2

Cell Viability (Fold of Control)

Vehicle

Fold of Real Conc. (lg)

24 h

- a
- b
- c

* * * * * * * * *
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Supplementary Material
2021-12-15-Supplementary Information-Weidong.docx
Single enrichment systems possibly underestimate both exposures and biological effects of organic pollutants from drinking water

Lan Yang, a, 1 Ying Zhou, a, b, 1 Li Chen, a, 1 Hanyi Chen, a Wenhao Liu, a Weiwei Zheng, a Melvin E. Andersen, c Yubing Zhang, d Yi Hu, a M. James C. Crabbe, c, f Weidong Qu a, *

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1 These authors contributed equally to this work.

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Figure Captions

Figure 1. Cytotoxicity of UOCEs in LO2 Cells. The a, b and c represent UOCEs after adsorption of XAD-2, PS-DVB and AC, respectively. Methanol (1%, V/V) served as the vehicle control and CdCl₂ (25 μmol/L) as the positive control; Data was expressed as Mean ± SD (n=5), the X-axis is displayed in logarithmic scale of the fold of real concentration. *, p<0.05 vs the vehicle control.

Figure 2. ROS Induced by UOCEs in LO2 Cells. The a, b and c represent UOCEs after adsorption of XAD-2, PS-DVB and AC, respectively. A. Effects of UOCEs on LO2 Cellular ROS (1h). B. Effects of UOCEs on LO2 Cellular ROS (12h). Methanol (1%, V/V) served as the vehicle control and CdCl₂ (25 μmol/L) as a positive control for ROS; Data was expressed as Mean ± SD (n=4), the X-axis is displayed in logarithmic scale of the fold of real concentration. *, p<0.05 vs the vehicle control.

Figure 3. The Effect of UOCEs on Intracellular GSH Levels in LO2 Cells. The a, b and c represent UOCEs after adsorption of XAD-2, PS-DVB and AC, respectively. Methanol (1%, V/V) served as the vehicle control and CdCl₂ (25 μmol/L) as a positive control for GSH; Data was expressed as Mean ± SD (n=3), the X-axis is displayed in logarithmic scale of the fold of real concentration. *, p<0.05 vs the vehicle control.