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Environmental Technology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tent20

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To cite this article: Xiaokun Liu , Xiaoyan Y. Ma , Ke Dong , Kai Zheng & Xiaochang Wang (2020) Investigating the origins of acute and long-term toxicity posed by municipal wastewater using fractionation, Environmental Technology, 41:18, 2350-2359, DOI: 10.1080/09593330.2019.1567602

To link to this article: https://doi.org/10.1080/09593330.2019.1567602

ENVIRONMENTAL TECHNOLOGY
2020, VOL. 41, NO. 18, 2350–2359
https://doi.org/10.1080/09593330.2019.1567602

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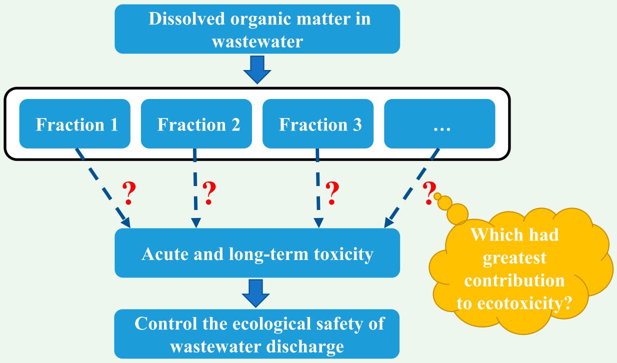
Investigating the origins of acute and long-term toxicity posed by municipal wastewater using fractionation

Xiaokun Liu[a], Xiaoyan Y. Ma[b], Ke Dong[b], Kai Zheng[b] and Xiaochang Wang[b]

[a]Xi'an Municipal Engineering Design & Research Institute Co., Ltd., Xi'an, People's Republic of China; [b]International Science & Technology Cooperation Center for Urban Alternative Water Resources Development; Key Lab of Northwest Water Resource, Environment and Ecology, MOE; Xi'an University of Architecture and Technology, School of environment and municipal engineering, Xi'an, People's Republic of China

ABSTRACT

It has been proven that the raw wastewater, secondary effluent and even reclaimed water may have toxic effects on aquatic organisms. In the present study, fractionation procedures combined with bioassays using luminescent bacteria were conducted to identify the fractions that contributed to the acute and long-term toxicity of municipal wastewater. Solid phase extraction was used to divide dissolved organic matter from the wastewater into three fractions, including non-polar, medium-polar and polar fraction. Among these fractions, although the acute toxicity of municipal wastewater was mainly caused by polar and medium-polar chemicals, the acute toxicity induced by the unit mass of the medium-polar fraction was the greatest. Using three kinds of resins, the organic substances in municipal wastewater were classified into six fractions, and the long-term toxicity of these fractions was further identified. The long-term toxicity of the hydrophobic neutrals, which were the primary toxic substances in raw wastewater, decreased after the conventional secondary biological treatment. Hydrophilic neutrals, which accounted for the majority of organic substances in the secondary effluent, were the main substances with long-term toxicity in the secondary effluent. The identification of fractions with acute and long-term toxicity in municipal wastewater is beneficial for further treatment to attenuate the ecotoxicity of wastewater before discharge into the aquatic environment.



ARTICLE HISTORY
Received 26 June 2018
Accepted 9 December 2018

KEYWORDS
Municipal wastewater; organic fractions; luminescent bacteria; acute toxicity; long-term toxicity

1. Introduction

The main goal of municipal wastewater treatment is to control general pollution indicators such as suspended solids, chemical oxygen demand (COD), nitrogen, and phosphorus, as well as to reduce potentially toxic substances. However, this process does not take decreases in ecotoxicity into account. After conventional secondary treatment, toxic pollutants are often not completely removed, part of which remain in the effluent [1]. New toxic substances may also be produced during the treatment processes [2]. Moreover, the treated municipal wastewater is either directly discharged or reclaimed after advanced treatment processes, which can release residual pollutants into the environment, where they have contact with aquatic organisms and humans.

Although the concentrations of pollutants in the secondary effluent are characterized by trace concentrations, their long-term persistence in aquatic ecological systems could result in chronic toxicity toward organisms. Moreover, chemicals with high

CONTACT Xiaoyan Y. Ma ✉ maxiaoyan@xauat.edu.cn International Science & Technology Cooperation Center for Urban Alternative Water Resources Development; Key Lab of Northwest Water Resource, Environment and Ecology, MOE; Xi'an University of Architecture and Technology, School of environment and municipal engineering, Xi'an 710055, People's Republic of China

toxicity can cause acute toxicity even at extremely low concentrations [3]. Some micropollutants can also accumulate in the bodies of aquatic organisms and then exert adverse effects on humans through transference via the food chain [4]. Leusch et al. applied 13 *in vitro* assays to evaluate the biological effects of potential pollutants in secondary effluents and reclaimed water, and found that the majority of the effluents had molecular and cellular effects, even when present at levels below chemical detections limits [5]. Hence, wastewater treatment designed to decrease conventional indices cannot guarantee the safety of the aquatic ecosystem and human health. Volker et al. attempted to extend anaerobic conditions in conventional activated sludge treatment processes to decrease endocrine disrupting effects by using seven *in vitro* bioassays. They found high anti-androgenic and minor dioxin-like and estrogenic effects in the treated effluent, despite the new reactor being able to effectively remove the observed effects [6]. Additionally, Tang et al. reported that the detected micropollutants explained less than 3% of the observed cytotoxicity detected by using luminescent bacteria [7]. Hence, the potential toxic substances that cause the ecotoxicity of municipal wastewater and their variation in treatment processes are unclear.

The United States Environmental Protection Agency (USEPA) listed 58 control indices to protect aquatic biological communities from the impact of acute or chronic toxicity. When compared with inorganic contaminants, the impact of organic substances from municipal wastewater on the environment has drawn widespread attention. Dissolved organic substances from municipal wastewater include natural organic matter (NOM), soluble microbial products (SMPs) and trace organic compounds (e.g. pesticides, pharmaceutical and personal care products, endocrine disrupting chemicals, disinfection by-products) [8]. Because of the heterogeneity of substances with diverse properties and varying origins in wastewater, it is difficult to quantify them separately. Furthermore, with the development of commerce and industry, nearly 10,000 new types of synthetic organic compounds are being generated every year, leading to an increased amount of unknown compounds entering into wastewater. Although more pollutants are being detected in wastewater because of the development and application of advanced instruments, all detected organic compounds constitute only a small part of the pollutants contained in wastewater [9]. Even if the composition and concentrations of pollutants in wastewater could be determined, their biological effects are difficult to deduce. Gradually, researchers have resorted to comprehensive toxicity evaluation to reduce or replace the determination of individual pollutants. Bioassays used to evaluate the toxicity of municipal wastewater need to exhibit toxicity quickly, be accurate and easy to operate. Of the toxicity assay using aquatic organisms, luminescent bacteria toxicity assay not only has these merits but also are well correlated with other *in vivo* bioassays [10]. Luminescent bacteria toxicity assays have been widely applied to manage wastewater discharge in many countries and regions [11]. The most commonly used luminescent bacterium is *Vibrio fischeri*. This organism is of marine water origin; therefore it is necessary to maintain a salty environment during toxicity assays that employ *V. fischeri*, which may change the property of substances in the testing sample [12]. In the present study, a freshwater bacterium, *Vibrio qinghaiensis* sp.-Q67 (Q67), which has similar luminescent property as *V. fischeri* without the need for a salty environment, was recommended for toxicity assay. In addition, luminescent bacterium is commonly used to conduct acute toxicity assay. In the present study, the long-term toxicity assay using Q67 was simultaneously conducted to reveal the ecotoxicity of municipal wastewater from another aspect.

The United States Environmental Protection Agency (USEPA) developed Toxicity Identification Evaluation (TIE) methods to identify the origins of toxicity for environmental samples [13–15]. However, the programme is cumbersome and lacks effectiveness, especially for atypical toxicants [16,17]. To track the toxicity of dissolved pollutants in environmental samples, new fractionation technologies were developed by simplifying the TIE programme. Lei and Aoyama applied reverse-phase high-performance liquid chromatography to isolate the organic pollutants in landfill leachates, then evaluated their toxicity using the Microtox test [18]. Their findings indicated that most toxicants were less polar organic compounds, and the concentrations of the compounds detected using gas chromatography/mass spectrometry (GC/MS) analysis were not high enough to be responsible for the observed toxicity in the original samples. Indeed, many toxicants are not successfully identified by GC/MS. On the other hand, Smital et al. applied large-volume solid-phase extraction (SPE) and silica gel fractionation to investigate the ecotoxicity of municipal wastewater, suggesting that the most significant contribution of toxic substances in the raw wastewater and secondary effluent of wastewater treatment plants (WWTP) was from the polar fractions [19]. There is currently a great deal of interest in identifying potential toxic substances from municipal wastewater. Therefore, the objectives of this study were to assess the acute and long-term toxicity of pollutants with different chemical characteristics using fractionation and luminescent bacteria toxicity test and then to identify the potential toxic fractions in municipal wastewater.

2. Materials and methods

2.1. Wastewater sampling

Wastewater samples including raw wastewater and secondary effluent were collected from a WWTP in Xi'an, China, with a treatment capacity of 1×10^5 m^3/day. The municipal wastewater was treated through a series of physical, chemical and biological units, including coarse and fine screens, a grid chamber, an oxidation ditch, a secondary settler, and a chlorination tank. Additionally, an anaerobic selection tank with a 0.5 h hydraulic retention time (HRT) was constructed prior to the oxidation ditch (HRT 18 h, sludge age 17 days) to fully mix the water with the return sludge (return ratio = 1.0). Triplicated sampling was conducted when the WWTP was operated under normal weather and operational conditions, after which the collected samples were transported to the laboratory and immediately filtered through a 0.45 μm mixed cellulose ester membrane (Φ 150 mm) mounted on a stainless-steel single filter holder. The filtrate of the first batch was then discarded. The membrane was boiled in milli-Q water before use. After filtering the water samples, the physicochemical analysis, fractionation and bioassays were performed within 24 h. Physicochemical analyses to determine the biochemical oxygen demand (BOD_5), COD, total phosphorus (TP), total nitrogen (TN), $NH_4^+ - N$ were conducted according to the standard methods [20]. Fractionation and bioassays were conducted described as below.

2.2. Sample solid-phase extraction and fractionation

To investigate the acute toxicity of different organic substances from the wastewater, 6 g Oasis HLB solid-phase cartridges (Waters Corporation, Milford, U.S.A), which had a high performance and could adsorb a broad polarity range of chemicals, were used to separate the organic substances into a non-polar fraction (A), medium-polar fraction (B) and polar fraction (C). The HLB solid-phase cartridge was packed with the macroporous copolymers polymerized with the monomer of lipophilic divinylbenzene and hydrophilic N-vinyl pyrrolidone. All operations were conducted on an SPE kit (Visiprep™ DL, SUPELCO, U.S.A).

The total organic substances in the wastewater were extracted as described below. The filtered water samples were passed through preconditioned HLB solid-phase cartridges at a flow rate of approximately 10 mL/min with the aid of a vacuum pump. After passing the water samples through the cartridge, 6 mL milli-Q water was passed through the cartridge to wash out the impurities. Vacuum pressure was then maintained at the maximum value for 15 min, after which the cartridge was centrifuged for 15 min at 4000 r/min to remove the residual water. The target chemicals were subsequently eluted with 20 mL methanol, after which the eluate was divided into two identical aliquots. A nitrogen evaporator (Sample Concentrator MD200, China) was then used to evaporate the aliquots at 40°C. One of the concentrated aliquots was subsequently redissolved in 0.5% dimethyl sulphoxide (DMSO) solution and transferred into 5 mL vials for toxicity assay. The other concentrated aliquot was further used for fractionation. The detailed operation procedure is shown in Figure 1. Briefly, the dried aliquots were redissolved in 2 mL n-hexane and then added into the silica gel column (500 mg/3 mL, Agela Technologies, China). The column was subsequently eluted with 20 mL n-hexane to obtain the non-polar fraction (Step 1 in Figure 1). Next, 2 mL dichloromethane was applied to dissolve the remaining residue, which was then added to the same silica gel column. The medium-polar fraction was obtained by eluting the column with 20 mL dichloromethane (Step 2 in Figure 1). The polar fraction was obtained by repeating the same procedure with methanol as the residue solvent and eluent (Step 3 in Figure 1). Each of the fractions was concentrated with a rotary evaporator, and then transferred into a 5 mL vial, further dried with a nitrogen evaporator, and redissolved in 0.5% DMSO for toxicity assay.

2.3. Fractionation of organic matter using resins

To investigate the long-term toxicity of organic substances in municipal wastewater, three kinds of resins, XAD-8 (SUPELITE DAX-8 macro porous resin), MSC (Dowex Marathon MSC strong acid cation exchange resin) and A-7 (Duolite A-7 weak base anion exchange resin), were used to isolate the organic matter into six fractions based on their polarity and acid-base properties, including hydrophobic acids (HoA), hydrophobic bases (HoB), hydrophobic neutrals (HoN), hydrophilic acids (HiA), hydrophilic bases (HiB) and hydrophilic neutrals (HiN).

Before fractionation, the municipal wastewater was concentrated using a reverse osmosis (RO) device (MSM-2008, Mosu Co. Shanghai, China) with a RO-1812/50G membrane module at a working pressure of 0.4–0.6 Mpa. The permeated solution was discharged, and the concentrate was recirculated to the sample reservoir to concentrate the sample continuously. The fractionation of organic substances in the concentrated wastewater was conducted as described by Wu et al. [21]. Milli-Q water was used as a blank control. The pH of all fractions was adjusted to be neutral using 6 mg/L NaOH or 6 mg/L

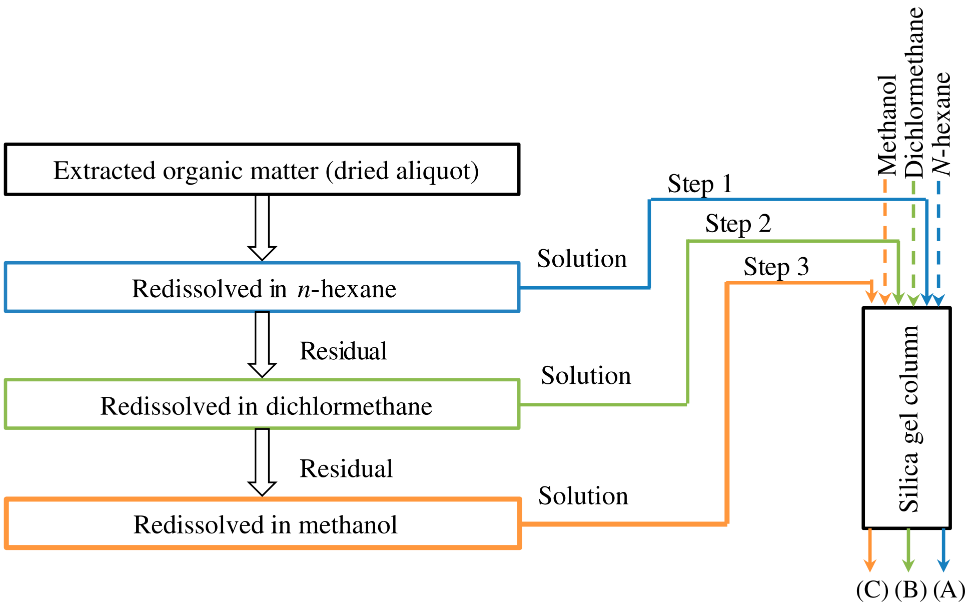


Figure 1. Diagram of organic substances fractionation using SPE. A: non-polar fraction; B: medium-polar fraction; C: polar fraction.

HCl, and all fractions were diluted to the volume of the unconcentrated wastewater samples using Milli-Q water before the long-term toxicity assay. The concentrations of all fractions were expressed as the total organic carbon (TOC) concentration, which was determined using a Shimadzu TOC-VC (Japan). The enrichment factor was evaluated by comparing the TOC concentration of the original sample with that of the concentrated solution. The UV absorbance at 254 nm (UV_{254}) was determined using an ultraviolet-visible spectrophotometer (UV-1650PC, Shimadzu, Japan).

2.4. Luminescent bacteria toxicity assay

The freshwater luminescent bacteria *V. qinghaiensis* sp. Q67, purchased from Beijing Hammatsu Photon Techniques Inc. China, was used for the acute and long-term toxicity assay following the optimized testing procedure in the authors' previous studies [22,23]. Based on the pretest of acute toxicity assay, the greatest enrichment factor was 20 times for raw wastewater, and 100 times for secondary effluent. The bioluminescence was detected using a Centrol IApc LB962 Microplate Luminometer (Berthold Technologies Company, Germany) with the unit of relative light unit (RLU). The acute and long-term inhibition values were calculated using Equations (1) and (2), respectively. To distinguish the results obtained from acute and long-term toxicity assays, the inhibition values obtained from the acute toxicity assays were defined as 'inhibition' (*I*) and the inhibition value obtained from the long-term toxicity assay was defined as 'integral inhibition' ($Inhibition_m$). An acute toxicity assay with 15 min of exposure and a long-term toxicity assay with 10 h of exposure were used to quantitatively evaluate the biological effects posed by the water sample. The effective concentration (EC_{50}) in the unit of enrichment factor (times) was obtained from the concentration (times)-inhibition curve. By definition, the higher the EC_{50} values, the lower the ecotoxic effects. For convenience of comparison, the toxicity impact index (TII_{50}) was introduced as Equation (3) [24]. Although TII_{50} is calculated in the same way as the toxicity unit (TU), they represent different concepts. TU is related to the amount of a known chemical, whereas TII_{50} is related to an amount of a mixture of unknown composition.

$$I(\%) = \frac{R_0 - R}{R_0} \times 100, \tag{1}$$

where *I* is the inhibition value for the acute toxicity assay (%), and *R* and R_0 are the RLU values of the parallel samples and the controls, respectively, after 15 min of exposure.

$$Inhibitionm(\%) = \left(1 - \frac{\sum_{i=0}^{m-1}(Lti + 1 + Lti)}{\sum_{i=0}^{m-1}(LCti + 1 + LCti)}\right) \times 100, \tag{2}$$

where $Inhibition_m$ is the integral inhibition value at time t_m (%) for the long-term toxicity assay, Lt_i is the average RLU of the parallel testing solutions at time t_i, and LCt_i is the average RLU of the controls at time t_i.

$$TII_{50}(\%) = \frac{1}{EC_{50}} \times 100. \tag{3}$$

3. Results and discussion

The conventional parameters of wastewater before and after the treatment processes are shown in Table 1. The pollutants in raw wastewater were effectively removed and the secondary effluent achieved the first level A requirements of discharge standard of pollutants for municipal wastewater treatment plants (GB 18918-2002).

3.1. Content of organic fractions obtained by SPE

The initial results showed that the luminous intensity of the bacteria could not be inhibited by the original unconcentrated municipal wastewater. RO is expected to be the best method for concentrating organic matter due to its 91–98% recovery under appropriate operations [25]. Nonetheless, the salts that could interfere with the acute toxicity assay towards luminescent bacteria were simultaneously concentrated during the RO process. Moreover, the RO concentrating were commonly combined with fractionation, using resins with different properties to separate dissolved organic substances into several groups. However, preliminary experiments showed that these fractions obtained by RO concentrating and resin fractionating did not acute toxicity on Q67. Seemingly, the method of combining RO with resin fractionating is not fit for investigating the acute toxicity of organic substances from municipal wastewater towards luminescent bacteria. On the other hand, SPE could eliminate the interference from inorganic matter and extract pollutants from wastewater.

To address the acute toxicity of organic substances from municipal wastewater, the organic substances were extracted and fractionated using SPE and eluents with different polarities to eliminate the inference of nutrients and part of the organic matter in the matrix. Table 2 shows the chemical properties and acute toxicity of all fractions in the raw wastewater and secondary effluent. For the contents expressed as TOC concentration, the polar fraction accounted for most of the total organic substances (63.73% of total TOC for raw wastewater and 80.37% of total TOC for secondary effluent). The second-highest constituent of the fractions was the medium-polar fraction (35.56% of total TOC for raw wastewater and 18.22% of total TOC for secondary effluent), while the non-polar fraction accounted for only a small part of the total organic matter in municipal wastewater. Furthermore, of the three fractions in the raw wastewater and secondary effluent, the polar fraction had a high degree of unsaturation because of its high absorbance of ultraviolet light at 254 nm (UV_{254}) (Table 2). After conventional biological treatment in the WWTP, the concentration of organic substances was distinctly reduced, especially that of the polar fraction. This is because polar fractions from wastewater such as surfactants, pharmaceuticals and phenols were readily degraded by microorganisms during the biological treatment processes [19,26]. Owing to the high concentrations of polar chemicals in the raw wastewater, the residual polar chemicals were still present at high levels in the secondary effluent, despite their high removal rate [27].

3.2. The contribution of organic fractions to acute toxicity

Of the three fractions from raw wastewater and secondary effluent, the acute toxicity of the polar fraction was the highest ($TII_{50} = 5.81\%$ for the raw wastewater and 1.09% for the secondary effluent), followed by that of the medium-polar fraction (Table 2). The non-polar fraction did not show apparent acute toxicity to Q67. This

Table 1. The conventional indicators of raw wastewater and secondary effluent and their removals in the WWTP.

Sample name	BOD_5 (mg/L)	COD (mg/L)	TP (mg/L)	TN (mg/L)	NH_4^+-N (mg/L)
Raw wastewater	293.4 ± 68.5	584.2 ± 179	6.31 ± 3.62	56.3 ± 9.2	36.2 ± 7.3
Secondary effluent	7.17 ± 1.9	26.4 ± 6.2	0.22 ± 0.11	12.1 ± 2.8	1.8 ± 1.9
Removal	97.56%	95.48%	96.51%	78.51%	95.03%

Table 2. Results of chemical analysis and acute toxicity assay for each fraction from raw wastewater and secondary effluent.

Wastewater	Categories	TOC (mg/L)	UV_{254} (1/cm)	EC_{50} (times)	TII_{50} (%)	TII_{50}/TOC ($\times10^{-2}$ L/mg)
Raw wastewater	Total organic substances	6.075 ± 0.725	0.0634 ± 0.058	12.74 ± 0.33	7.85	1.29
	A	0.050 ± 0.035	0.0004 ± 0.0002	–	–	–
	B	2.525 ± 0.177	0.0053 ± 0.0023	19.82 ± 0.19	5.05	2.00
	C	4.525 ± 1.114	0.0525 ± 0.0047	17.21 ± 0.70	5.81	1.28
Secondary effluent	Total organic substances	0.895 ± 0.170	0.0222 ± 0.0001	89.21 ± 6.16	1.12	1.25
	A	0.015 ± 0.011	0.0001 ± 0.0001	–	–	–
	B	0.195 ± 0.021	0.0002 ± 0.0001	135.3 ± 1.36	0.74	3.79
	C	0.860 ± 0.007	0.0160 ± 0.0018	92.12 ± 5.51	1.09	1.26

indicated that the polar and medium-polar fractions made dominant contributions to the acute toxicity of municipal wastewater. Hrubik also indicated that a polar fraction from raw wastewater caused cytotoxicity and increased the EROD activity based on fish liver cells (PLHC-1 cells), and increased mortality and developmental abnormalities in zebrafish embryos. In rat hepatoma cells H4IIE tests, polar fraction induced inhibition of cell growth and increased EROD activity, whereas human hepatoma cells HepG2 tests exerted low or no response to the exposure. Non-polar and medium-polar fractions were ineffective [28]. The acute toxicity of these fractions was closely related to their corresponding concentrations. The polar and medium-polar fractions probably contained several ecotoxic chemicals for household purposes such as pharmaceuticals, surfactants, disinfectant, alkylphenols, and pesticides [29,30]. The non-polar chemicals were commonly characterized by low solubility in water, which resulted in their extremely low concentrations in wastewater [31]. Therefore, its contribution to the acute toxicity of municipal wastewater is not evident. Smital et al. investigated the toxicity of polar, medium-polar and non-polar fractions of wastewater from a WWTP and found that the polar fraction had higher cytotoxicity, genotoxicity, and estrogenic potential than the medium-polar and non-polar fractions, which was in accordance with the results of the present study [19]. Moreover, because of the insufficient removal of contaminants in the wastewater treatment processes, the secondary effluent still exerted certain acute toxicity ($TII_{50} = 1.12\%$). Guo et al. who developed a screening strategy based on bioassay-directed fractionation to identify toxicants in wastewater from a WWTP, indicated that organic compounds extracted by SPE were the main toxic components and 6 pollutants, including imazalil, prometryn, propiconazole, tebuconazole, buprofezin and diazinon, were further confirmed and explained 48.79% of the observed toxicity to *Daphnia magna* [32].

To evaluate the acute toxicity of a unit mass of organic substances, the ratio of TII_{50} to TOC (TII_{50}/TOC) was used as an indicator to compare the contribution of unit mass fractions to the acute toxicity. Surprisingly, the acute toxicity per unit mass of the medium-polar organic matter was the highest (Table 2), though the content and acute toxicity of the polar fraction were dominant in the municipal wastewater. These findings suggested that the medium-polar chemicals in wastewater were more hazardous to aquatic organisms than the polar chemicals under the same concentration. Therefore, on the basis of decreasing polar organic matter, improving the removal of medium-polar chemicals in WWTPs should not be ignored.

3.3. Proportion of the resin fractionated organics in wastewaters

As stated in Section 3.1, the method of combining RO concentrating with resin fractionating may not be suitable for pretreatment prior to the acute toxicity assay using luminescent bacteria. However, long-term toxicity assays using aquatic organism are usually more sensitive than its short-term assays [33]. It has been proven that combining RO pretreatment with long-term toxicity assays using luminescent bacteria could eliminate the interferences caused by nutrients in the RO concentrated samples and improve the sensitivity of the analysis [23]. In order to identify the potential pollutants with long-term toxicity, the municipal wastewater was first concentrated by RO. Then, the organic matter was separated into six fractions using three kinds of resins. The TOC concentrations of the unconcentrated raw wastewater and secondary effluent were 42.96 and 6.20 mg/L, respectively. After RO concentrating, they were 292.63 and 174.88 mg/L, respectively. So, the greatest enrichment factors of the raw wastewater and secondary effluent were 6.85 and 28.20, respectively. In the resin fractionation process, 84.45% of the organic substances in the raw wastewater and 95.90% of the secondary effluent were recovered, respectively.

Figure 2 shows the proportions of TOC concentrations in the six fractions. For raw wastewater, the content of HiN was the highest, accounting for 45% of the total TOC concentration, followed by HoA. The other four were minor fractions of organic substances in the raw wastewater. For the secondary effluent, the content of HiN was equal to that of HoA, constituting 41% of the TOC concentration. This indicated that HiN and HoA were the main components of the organic substances in the municipal wastewater, even though the organic substances in the wastewater had decreased after the biological treatment. It has been demonstrated that HoA, followed by HiN, were the dominant fractions and the content of the soluble alkaline organic substances (HiB and HoB) was low in wastewater, which was consistent with the results of the present study [34,35]. When compared with SPE, concentration and fractionation using resin could adsorb not only micromolecular contaminants (e.g. trace organic compounds) but also macromolecular contaminants (e.g. NOM and SMPs). By using a three-dimensional excitation and emission matrix fluorescence spectroscopy, the organic matter in the six fractions was identified to be fulvic acid-like, SMP-like, protein-like, phenol-like, aromatic protein, and marine humic acids [35,36]. Additionally, the molecular weights of these fractions varied extensively, from several hundred to several thousand [37].

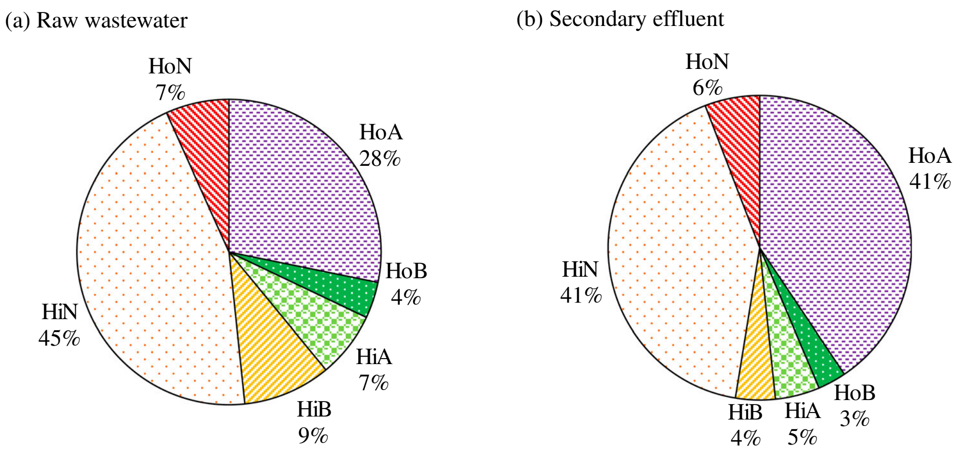


Figure 2. Percentage composition of six fractions from raw wastewater (a) and secondary effluent (b). HoA: hydrophobic acids; HoB: hydrophobic bases; HoN: hydrophobic neutrals; HiA: hydrophilic acids; HiB: hydrophilic bases; HiN: hydrophilic neutrals.

3.4. Contribution of organic fractions to long-term toxicity

Figure 3 shows the variations in the bioluminescence inhibition effects of the six fractions in the raw wastewater and secondary effluent in the long-term toxicity assay. For raw wastewater (Figure 3a), the integral inhibition of HoN increased slowly with exposure time. Until the bacteria entered the logarithmic growth phase (6–8 h), their integral inhibition increased rapidly, after which it decreased. These findings suggested that HoN could obstruct the bacterial reproduction, and the adverse effects tended to be weak along with the bacterial generation update. Similarly, HoA had the same tendency for integral inhibition as HoN that had the highest integral inhibition values of the six fractions. In contrast, the toxicity of HiN increased slowly after 8 h of exposure. The other three fractions had no observed inhibitory effect on Q67. Therefore, HoN followed by HoA and HiN, were the primary causes of long-term toxicity of the raw wastewater. The contribution of the other three fractions to the long-term toxicity of the raw wastewater was negligible.

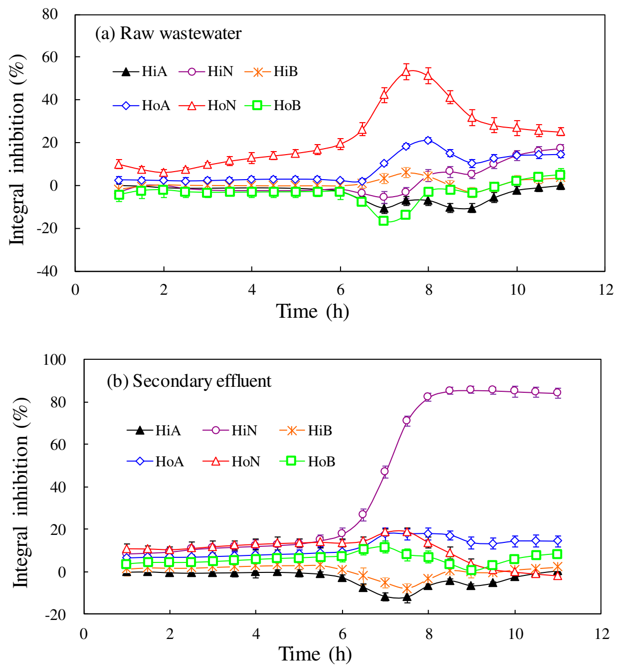


Figure 3. Variation in integral inhibition values of six fractions from raw wastewater (a) and secondary effluent (b) in the long-term toxicity assay. HoA: hydrophobic acids; HoB: hydrophobic bases; HoN: hydrophobic neutrals; HiA: hydrophilic acids; HiB: hydrophilic bases; HiN: hydrophilic neutrals.

Comparison of the long-term toxicity for the six fractions in the raw wastewater expressed as the integral inhibition with 10 h exposure (shown in Figure 3) to their contents (shown in Figure 2) revealed that HoN which only accounted for 7% of the total TOC concentration made the greatest contribution to the long-term toxicity of the raw wastewater. Fluorescent excitation–emission matrix (3DEEM) analysis revealed that the protein-like and humic-like substances were present in municipal raw wastewater [38]. In addition, this fraction of organic substances contained trace pollutants, which lead to reproduction toxicity at low concentration and were converted into chemicals with low toxicity through bacterial metabolism following the prolonged exposure time. Although HoA and HiN constituted 73% of the total TOC concentration in the raw wastewater, they only made a minimal contribution to the long-term toxicity of the raw wastewater. The contributions of HoB, HiB and HiA to the long-term toxicity of the raw wastewater were consistent with their contents. The organic substances in municipal wastewater, including dissolved organic matter and micropollutants, probably contribute to the toxicity of municipal wastewater

Table 3. The integral inhibition of six fractions with 10 h exposure in long-term toxicity assay using Q67.

Water sample	Integral inhibition (%) with 10 h exposure					
	HoA	HoB	HoN	HiA	HiB	HiN
Raw wastewater	14.07 ± 0.09	2.15 ± 2.07	26.83 ± 1.38	2.15 ± 2.08	2.43 ± 2.27	13.96 ± 1.03
Secondary effluent	14.41 ± 0.19	6.04 ± 1.09	−0.13 ± 0.59	2.45 ± 2.01	0.96 ± 0.85	85.06 ± 1.14

Notes: HoA: hydrophobic acids; HoB: hydrophobic bases; HoN: hydrophobic neutrals; HiA: hydrophilic acids; HiB: hydrophilic bases; HiN: hydrophilic neutrals.

[39]. Actually, dissolved organic matter in municipal wastewater exhibited contrasting effects towards aquatic organisms. It has been reported that dissolved organic matter can decrease metal uptake, leading to reduced bioavailability to exposed organisms and therefore low biotoxicity [40]. Additionally, dissolved organic matter can adsorb on cell membranes inducing toxic effects [40]. Moreover, dissolved organic matter can interact with micropollutants leading to the increased or decreased joint toxicity [41,42]. Hence, the different biological toxicity properties of organic fractions were present as a result of these combined actions.

Evaluation of the variation in toxicity of the six fractions in the secondary effluent with exposure time (Figure 3b) revealed that the integral inhibition of HiN increased sharply when the bacteria entered the logarithmic growth phase and remained stable after 8 h, while the integral inhibition of the other five fractions showed little change throughout the long-term toxicity assay. As shown in Table 3, the long-term toxicity of the secondary effluent was mainly caused by HiN, followed by HoA, while the contribution of the other four fractions was small. Associating the long-term toxicity of the six fractions with their contents in the secondary effluent, HiN, which constituted 41% of the TOC concentration in the secondary effluent, showed high long-term toxicity to Q67 ($Inhibition_{10h} = 85.06\%$). In contrast, HoA, which had the same content as that of the HiN fraction in the secondary effluent, had a minimal long-term toxic effect ($Inhibition_{10h} = 14.41\%$). The contents and long-term toxicity of the other four fractions were low enough to be ignored. Zhang et al. indicated that the HiN and HiA in reclaimed water showed high acute toxicity to luminescent bacteria [34]. To investigate the mutagenicity of organic substances from river and reservoir source water, it was indicated that for river water, HoA was mutagenic prior to chlorination and that chlorinated HoN, HiA, HoA and HiB had obvious mutagenic effects. Nonetheless, only HiA and HoA in the reservoir water were mutagenic after disinfection [43]. The fractions with different origins in wastewater probably have a different contribution to ecotoxicity.

Investigation of the variations in long-term toxicity after secondary biological treatment revealed that the long-term toxicity of HoN decreased in response to activated sludge treatment. The HoN fraction in secondary effluent consisted of soluble microbial products, protein-like or phenol-like, and simple aromatic proteins such as tyrosine, among which the soluble microbial products as the dominant composition was from the biodegradation of organic matter by microorganisms during the long retention time in the oxidation ditch [35]. The interaction between organic matter and microorganisms in the activated sludge treatment process might be similar to that with luminescent bacteria in the long-term toxicity assay. This was demonstrated by the reduction in integral inhibition of HoN in raw wastewater during the late stages of the exposure period (Figure 3a). In contrast to HoN, the long-term toxicity of HiN, which was mainly composed of marine humic acids and fulvic acid-like substances, increased significantly after the activated sludge treatment. It may be due to the formation of toxic hydrophilic neutral intermediates, which interfered with bacterial growth and reproduction [35].

4. Conclusions

With the increased consciousness of environmental protection, the purpose of wastewater treatment is not only to decrease the pollutant load but also to soften the negative effects of wastewater discharged into aquatic environments. To accomplish this, the identification of potential toxic substances in municipal wastewater is necessary. The results of this study showed that the acute toxicity of municipal wastewater was mainly caused by the polar and medium-polar fractions, which were obtained by solid-phase extraction and fractionation. Of the three fractions, the unit mass of the medium-polar organic substances caused the highest acute toxicity toward Q67. To evaluate the long-term toxicity of municipal wastewater, six fractions were obtained by using three kinds of resins. In raw wastewater, HoN made the greatest contribution to the long-term toxicity of wastewater, although its content was only 7% of the total TOC concentration in the wastewater. HoA and HiN, which constituted 73% of the total TOC concentration in the raw wastewater, made a minimal contribution to its long-term toxicity. However, the long-term toxicity of the secondary effluent was mainly caused by HiN. Based on the increase in long-term toxicity of HiN during the conventional secondary treatment process, further physical or chemical treatments, such as active carbon adsorption, are recommended to reduce the toxicity of HiN in the secondary effluent and preserve the ecological environment.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Natural Science Foundation of China [grant number 51508449] and [grant number 51778522], Scientific Research Program Funded by Shaanxi Provincial Education Department [grant number 18JK0468], and Foundation of Xi'an University of Architecture and Technology [ZR18025].

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