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Comparative Toxicity of Chlorinated Saline and Freshwater Wastewater Effluents to Marine Organisms

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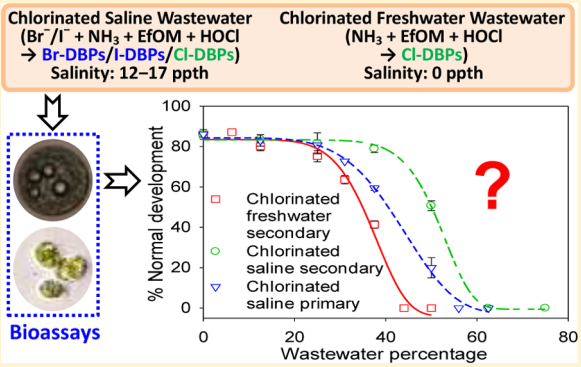
Supporting Information

ABSTRACT: Toilet flushing with seawater results in saline wastewater, which may contain approximately 33−50% seawater. Halogenated disinfection byproducts (DBPs), especially brominated and iodinated DBPs, have recently been found in chlorinated saline wastewater effluents. With the occurrence of brominated and iodinated DBPs, the adverse effects of chlorinated saline wastewater effluents to marine ecology have been uncertain. By evaluating the developmental effects in the marine polychaete *Platynereis dumerilii* directly exposed to chlorinated saline/freshwater wastewater effluents, we found surprisingly that chlorinated saline wastewater effluents were less toxic than a chlorinated freshwater wastewater effluent. This was also witnessed by the marine alga *Tetraselmis marina*. The toxicity of a chlorinated wastewater effluent to the marine species was dominated by its relatively low salinity compared to the salinity in seawater. The organic matter content in a chlorinated wastewater effluent might be partially responsible for the toxicity. The adverse effects of halogenated DBPs on the marine species were observed pronouncedly only in the "concentrated" chlorinated wastewater effluents. pH and ammonia content in a wastewater effluent caused no adverse effects on the marine species. The results suggest that using seawater to replace freshwater for toilet flushing might mitigate the "direct" acute detrimental effect of wastewater to the marine organisms.



INTRODUCTION

A prodigious population is centralized along or near coasts, which make up just 10% of the earth's land;[1] over 50% of the world's population lives within 60 km of the coast, and this number could reach 75% by the year 2020.[2] Several coastal cities and nations, mainly including Hong Kong, Avalon, Marshall Islands, and Kiribati, use seawater for toilet flushing, leading to the occurrence of dual water supply systems, which involve nonpotable and potable waters.[3,4] Hong Kong has been using a dual system since the 1950s and is the only city extensively using seawater for toilet flushing.[4] Approximately 80% of the population in Hong Kong is supplied with seawater, which has incontrovertibly reduced a considerable amount of freshwater demand in Hong Kong. To relieve the stress from higher rates of urbanization and the ever-increasing demand for freshwater, a stress that will only get more serious as the world population hits 8 billion by 2030,[5] more coastal cities may use seawater for toilet flushing. Problems induced by the practice, such as corrosion of pipelines and equipment, can now be solved or mitigated satisfactorily.[4]

The dual water supply system results in a saline wastewater because in a certain district, both used seawater (from toilet flushing) and used freshwater (from dishwashing, cloth-washing, showering, cleaning, and other domestic/commercial usage) are collected into the same sewerage system and conveyed to the same wastewater treatment plant.[4] The ratio of seawater to freshwater in the saline wastewater ranges from approximately 1:2 to 1:1, that is, 33%−50% of the saline wastewater is seawater. Therefore, such a practice leads to very high concentrations of Br^- and I^- from seawater into municipal saline wastewater treatment systems (20−32 mg/L and 30−60 μg/L respectively in Hong Kong's saline wastewater effluents).[6,7] Recently, Hong Kong has basically decided to use chlorination for disinfecting its 1.7×10^6 m^3/d of saline wastewater effluents.[8] It is well-known that chlorination aiming at microbial inactivation can produce a wide variety of unintended disinfection byproducts (DBPs).[9−23] In chlorination of saline wastewater effluents, Br^-/I^- can be oxidized to HOBr/HOI by chlorine or chloramines (from the reaction of chlorine with ammonia in the wastewater effluents); HOBr/HOI may further react with organic matter in the effluents to

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generate brominated and iodinated DBPs (Br-DBPs and I-DBPs).[6,7,24−27] Yang and colleagues[28] have examined the generation of four trihalomethanes (THMs) and nine haloacetic acids (HAAs) during chlorination of saline/freshwater wastewater effluents, indicating that the total THM and HAA concentrations as Br in the chlorinated saline wastewater effluents were significantly higher than those in the chlorinated freshwater wastewater effluents. Zhang's group[6,24−26] has demonstrated the generation of many new and unknown Br-/I-DBPs in chlorinated saline wastewater effluents, some of which have been identified, including 5-bromosalicylic acid, 2,6-dibromo-4-nitrophenol, 3,5-dibromo-4-hydroxybenzaldehyde, 3,5-dibromo-4-hydroxybenzoic acid, 2,4,6-tribromophenol, bromomaleic acid (Figure S1), 4-bromophenol, 2,5-dibromohydroquinone, 2,4,6-triiodophenol, 2,6-diiodo-4-nitrophenol, tetrabromopyrrole, tribromochloropyrrole, tribromoiodopyrrole, tribromopyrrole, and four iodinated 3,4,5-trihydroxybenzenesulfonic acids.

Evidence has proven that Br-DBPs and I-DBPs are generally considerably more toxic than corresponding chlorinated DBP (Cl-DBP) analogues in terms of mutagenicity, cytotoxicity, and genotoxicity.[29−31] It has been demonstrated recently that, for the newly identified and commonly known halogenated DBPs in chlorinated saline effluents, Br-DBPs and I-DBPs presented substantially higher developmental toxicity to a heterotrophic marine polychaete (*Platynereis dumerilii*) and growth inhibition to an autotrophic marine alga (*Tetraselmis marina*) than corresponding Cl-DBP analogues.[24,32]

After dechlorination to remove residual chlorine, the chlorinated saline wastewater effluents containing Br- and I-DBPs are discharged to the receiving water body, which in Hong Kong is coastal marine water, covering dozens of ecologically sensitive habitats and swimming beaches that are officially defined by the Hong Kong Environmental Protection Department.[33] The ultimate receiving water body for chlorinated wastewater effluents in most other cities should also be coastal marine water. However, the adverse effects of discharged saline wastewater effluents containing Br- and I-DBPs have not been questioned in an ecological context. Compared with chlorinated freshwater wastewater effluents, Br- and I-DBPs in chlorinated saline effluents may adversely affect marine species while the higher salinity may benefit marine species. Hence, there is no definite answer of whether chlorinated saline wastewater effluents are more or less toxic to the marine ecosystem than chlorinated freshwater wastewater effluents, which in turn determines whether the practice of using seawater for toilet flushing should be adopted and promoted.

As such, the primary aim of this work was to evaluate the comparative toxicity of chlorinated saline/freshwater wastewater effluents containing halogenated DBPs with the marine polychaete *P. dumerilii* and the marine alga *T. marina*. The two marine species were chosen because marine algae and polychaetes form the basis of the marine food web. Factors responsible for the toxicity were also investigated.

MATERIALS AND METHODS

Seawater and Chemicals. Seawater was drawn from a submerged intake in Hong Kong and had a salinity of approximately 35 parts-per-thousand (ppth); before use, it was filtered through a 0.22-μm pore size filter and sterilized by steam autoclaving for 20 min, and then it was cooled to room temperature and aerated with an air pump for 15 min. The seawater was used in maintaining the polychaete and the alga, and in preparing samples for toxicity tests. Ultrapure water was generated by a NANOpure system (Barnstead). A NaOCl stock solution was supplied by Allied Signal. It was diluted and standardized using the DPD titrimetric method.[34]

Wastewater Sampling and Characterization. Three wastewater effluent samples (undisinfected, 24 h composites, which can reflect time-weighted-average levels or temporal trends) were collected twice (once on 6 July 2010 and the other on 12 January 2011) from a freshwater secondary wastewater treatment plant, a saline secondary wastewater treatment plant, and a saline primary wastewater treatment plant. The saline and freshwater wastewater effluent samples used in the previous studies on halogenated DBPs[6,7,24−26,28] were also collected from these three wastewater treatment plants. The collected samples were delivered to the lab immediately and stored in the dark at 4 °C. The wastewater samples were brought back to ambient temperature prior to experiments. These samples were filtered through 0.45-μm membrane filters. Then, pH, dissolved organic carbon (DOC), bromide, and ammonia of each sample were measured with a pH meter, a total organic carbon analyzer (Shimadzu), an ion chromatograph (Dionex), and a flow injection analyzer (Lachat QuickChem), respectively.

Sample Preparation for the Developmental Toxicity Bioassay with the Marine Polychaete *P. dumerilii*. The wastewater effluent samples were disinfected by dosing 15 mg/L NaOCl as Cl_2. After a 30 min contact time, the chlorine residual in each sample was dechlorinated with 120% of the requisite stoichiometric amount of $Na_2S_2O_3$. For each chlorinated wastewater effluent sample, it was diluted with seawater to prepare a series of test samples that contained different percentages of wastewater (v/v). For comparison, the unchlorinated wastewater effluent samples were also prepared and diluted correspondingly. Then, the developmental toxicity of the seawater-diluted chlorinated/unchlorinated wastewater effluent samples was determined using the polychaete embryos aged 12 h postfertilization. The seawater and the seawater spiked with $Na_2S_2O_3$ served as controls. Images of embryos exposed in the wastewater effluent samples were recorded with an Olympus CKX 41 inverted microscope.

To reveal the effect of salinity, seawater was diluted with ultrapure water to prepare a set of samples with different salinities, which were measured with a salinity meter. Since the unchlorinated/chlorinated wastewater effluents had different salinities, to eliminate the effect of salinity and test other possible factors responsible for the toxicity, these wastewater effluents were adjusted to a salinity of 35 ppth by adding certain amounts of sea salt (from the Red Sea). Each salinity-adjusted wastewater effluent sample was diluted with seawater to prepare a series of test samples that contained different percentages of wastewater. One sample, which was ultrapure water adjusted to 35 ppth with sea salt, was used to check whether sea salt itself had an adverse effect. Then, the developmental toxicity of these samples was conducted using the polychaete embryos aged 12 h postfertilization.

To examine the effect of organics without/with DBPs in the unchlorinated/chlorinated wastewater effluents, these effluent samples were extracted and concentrated by following a widely used sample pretreatment procedure.[6,24−26] Briefly, 1 L of the water sample was adjusted to pH 0.5 with sulfuric acid, and 100 g of Na_2SO_4 was added to it. Then, the sample was extracted by liquid−liquid extraction with 100 mL of methyl *tert*-butyl ether

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(MtBE). After extraction, the upper organic phase was transferred to a rotary evaporator and concentrated to 3 mL. The 3 mL MtBE extract was transferred to a 40 mL bottle, followed by nitrogen sparging to remove MtBE. Then, 10 mL of the seawater was added to the dry organic matter without/with DBPs in the bottle, and the solution was ultrasonicated for 1 h to ensure complete dissolution, resulting in a 100-fold concentrated sample (from 1 L to 10 mL). It should be noted that some volatile DBPs might be lost in isolating the organic matter, but the contribution of volatile DBPs to the development toxicity of the whole DBP mixture has been demonstrated to be negligible.[35] To determine whether there were any toxicants introduced from the solvents in the extraction and concentration, a control sample was generated by repeating the same procedure with 1 L of ultrapure water. Finally, samples with different concentration factors were prepared by diluting the 100-fold concentrated sample with the seawater, and their developmental toxicity was determined using the polychaete embryos aged 12 h postfertilization.

Furthermore, samples were prepared to reveal the effects of chlorine dose, ammonia, and pH on the developmental toxicity, with details shown in the Supporting Information.

Developmental Toxicity Bioassay with *P. dumerilii*. Hutchinson and co-workers developed an in vivo assay with sensitive embryo-larval stages of *P. dumerilii*. They found this species to be ideally suited for evaluating the hazardous potential of contaminants in marine ecotoxicology.[36,37] In our study, this species was selected to represent the ecologically important group of marine invertebrates, many of which are broadly used in marine environmental quality programs.[38] In addition, *P. dumerilii* is widely distributed from the tropics to cold temperate latitudes in both hemispheres.[39]

Stock cultural conditions of *P. dumerilii* were maintained following a previous procedure.[36,39] By adopting and improving Hutchinson et al.'s bioassay method, we significantly lowered the relative standard deviation of it to <5%.[24] Then, impacts on embryo-larval development following exposure to chlorinated wastewater effluents can be evaluated reliably. The embryo exposure procedure was described previously,[24] with details given in the Supporting Information. By 24 h postfertilization (12 h aged embryos were exposed for 12 h), it is expected that normal embryos should achieve the first larval (trochophore) stage, with main features including a girdle of large ciliated cells in the equatorial region, four lipid macromeres, and free-swimming behavior. Conversely, underdeveloped individuals are absent of one or several of these characteristics.

For all the embryo exposure tests, duplicate samples were prepared and analyzed. All the data on the percent normal development present the means and the differences between the detected values and the means. Regression analysis was applied to each curve, which was used to calculate the EC_{50} value (the concentration that induced the percent normal development that was 50% of the control). The data were analyzed using SigmaPlot 12 (Systat Software Inc., San Jose, CA). A one-way analysis of variance (ANOVA) test was used to determine whether the prepared samples induced a significant level of abnormal development. A Holm-Sidak multiple comparison versus the control group analysis was conducted (power of performed test with alpha = 0.050:1.000).

Growth Inhibition Bioassay with *T. marina* for Unchlorinated and Chlorinated Wastewater Effluents. A marine culture of *T. marina* CCMP 898 was purchased from the National Center for Marine Alga and Microbiota (NCMA), USA. The microalgae were cultivated in the L1 medium (from NCMA)-enriched seawater,[32] with details shown in the SI. Eight *T. marina* cultures at the exponential growth phase were further concentrated and inoculated for experiments. Centrifugation (at 3000 rpm for 6 min) was conducted for each culture, and the subnatants of all cultures were collected and combined into a centrifugal tube. Then, the lugol's solution was added to the concentrated culture for fixation. The algal cell density of the culture was determined via cell counting with the aid of a microscope (Olympus). Six hundred mL of each wastewater effluent (collected on January 12, 2011) was filtered with a 0.45-μm membrane, and evenly divided into two aliquots, one of which was disinfected with 15 mg/L NaOCl as Cl_2 for 30 min, and the chlorine residual was dechlorinated with 120% of the requisite stoichiometric amount of $Na_2S_2O_3$. Each chlorinated or unchlorinated wastewater effluent was diluted with the medium-enriched seawater to prepare a series of 40 mL test samples (at wastewater percentages from 1% to 100%) in 100 mL Erlenmeyer flasks. The medium-enriched seawaters with and without the addition of $Na_2S_2O_3$ served as controls. Then the concentrated algal culture was spiked to each test or control sample to reach an initial algal density of 5×10^4 cells/mL. All the test or control samples were enclosed with flexible film with holes and cultivated in the cultivation chamber for 6 d. Repositioning of the samples was conducted for 4 times during the 14-h light-time every day to minimize the spatial difference in light intensity. The average specific growth rate of the alga in each test sample during the 6 d was evaluated through chlorophyll *a* concentration measurement according to Standard Method 10200 H,[34] and the inhibition percentage on algal growth in each test sample was then calculated.[32,40] The details are provided in the Supporting Information.

As shown later in Results and Discussion, the low salinity in a wastewater effluent (compared with the seawater salinity) was found to be the dominant factor causing algal growth inhibition. Accordingly, the salinity tolerance of the alga was tested (Supporting Information). To reveal the effect of salinity on the fine structure of the alga, the morphological changes of *T. marina* exposed to different percentages of the freshwater secondary effluent were studied. Briefly, four 10 mL test samples were prepared in Petri dishes, including the medium-enriched seawater (as control) and the freshwater secondary effluent that was diluted with the medium-enriched seawater to the wastewater percentages of 100% (undiluted), 85% and 70%. The algal culture was spiked to each sample at an initial density of 1×10^4 cells/mL. After well mixed, 200 μL of each test sample was transferred into a well of a 96-well plate (Techno Plastic Products AG), and then observed under a microscope (Olympus CKX41) for 48 h.

To eliminate the effect of salinity, the growth inhibition bioassay with *T. marina* was also conducted for each wastewater effluent whose salinity was initially adjusted to 35 ppth with sea salt.

Moreover, to investigate other possible factors responsible for the algal growth inhibition, the effects of ammonia, pH, and organic matter without/with DBPs from the unchlorinated/chlorinated saline primary effluent on algal growth were tested (Supporting Information).

RESULTS AND DISCUSSION

Developmental Toxicity of the Chlorinated and Unchlorinated Wastewater Effluents to the Marine Polychaete *P. dumerilii*. Three effluents, including a saline

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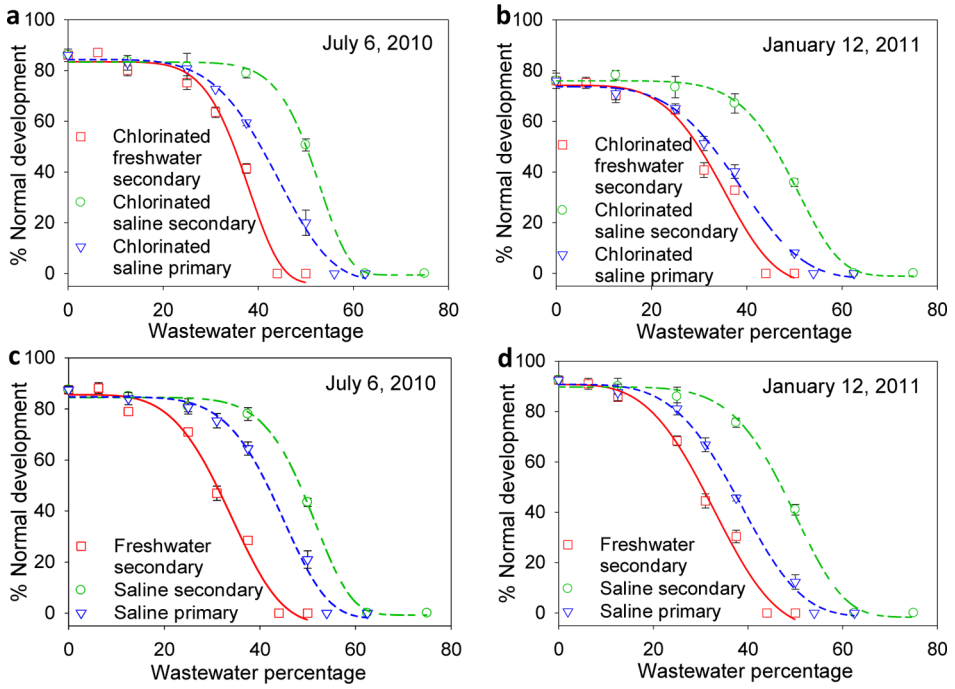

Figure 1. Developmental toxicity of the three chlorinated and unchlorinated wastewater effluents that were diluted with seawater to different wastewater percentages. The three unchlorinated wastewater effluents were collected on July 6, 2010 and January 12, 2011. The three chlorinated wastewater effluents were prepared by chlorinating the three unchlorinated wastewater effluents by dosing 15 mg/L of NaOCl as Cl_2 for a 30 min contact time, followed by dechlorinating with $Na_2S_2O_3$. Percentages of normal development of control samples for charts a–d were 86%, 76%, 88%, and 93%, respectively.

primary effluent, a saline secondary effluent and a freshwater secondary effluent, were chosen for this study, as they represent the most typical effluents from wastewater treatment plants. The chemical characteristics of the three effluents collected in the summer and winter seasons are given in Table S1. Irrelevant to season, the three effluents are characterized by their own features. For instance, a saline effluent contains high levels of salinity and bromide while a freshwater effluent contains nearly no salinity or bromide; a primary effluent contains substantially higher levels of DOC and ammonia than a secondary effluent because secondary treatment is designed to remove biodegradable organic matter and a portion of ammonia via activated sludge process.

After chlorination, the three effluents were tested for developmental effects using the improved method. Figure 1a illustrates the relationship between the percent normal development and the chlorinated wastewater percentage in seawater. Within the low wastewater percentage range, percentages of normal development of the three effluents declined slightly and were quite close to control samples (0% wastewater). When the wastewater percentage increased beyond 25%, normal development declined sharply and samples began to deviate from one another. As shown in Table S2, an ANOVA test provided the lowest wastewater percentages of the effluent samples that induced a significant reduction in normal development as compared to the control. The percent normal development was zero when the wastewater percentages were 44.0%, 56.0%, and 62.5% for the freshwater secondary effluent, the saline primary effluent and the saline secondary effluent, respectively, revealing that the freshwater secondary effluent presented the highest developmental toxicity among the three types of chlorinated wastewater effluents. As for the two chlorinated saline wastewater effluents, the primary one exhibited more severe developmental toxicity than the secondary one. With Sigmaplot 12 software, we estimated the EC_{50} values of the freshwater secondary effluent, the saline primary effluent and the saline secondary effluent to be 36.5%, 42.8%, and 51.7%, respectively. The three chlorinated wastewater effluents collected in a different season exhibited the same order of development toxicity (Figure 1b). Interestingly, the three unchlorinated wastewater effluents collected in either season also exhibited the same order of development toxicity (Figure 1c–d). The EC_{50} difference between the effluents collected from the same wastewater treatment plant in different seasons was generally much smaller than that between the effluents collected from different wastewater treatment plants in the same season (Table S2), suggesting that the toxicity order of the three effluents was irrelevant to season. (As a comparison, Jha et al.[37] evaluated the developmental toxicity of a primary freshwater effluent collected from the Buckland Sewage Treatment Works in UK with the same polychaete. Their EC_{50} value (18%) was lower than our freshwater secondary effluent (32.0–36.5%) most likely because their primary effluent contained a higher level of organic matter than our secondary effluent.)

Notably, once the wastewater effluents are discharged to the receiving marine water, they will be in contact with seawater and diluted by seawater to different wastewater percentages, thus the ranking order of development toxicity of the three effluents should represent the toxicity order or direct impact of the effluents to the marine species. The fact that the chlorinated saline wastewater effluents (containing Br- and I-DBPs) were less toxic than the chlorinated freshwater wastewater effluent was kind of surprising. Also, the EC_{50} values indicated that the unchlorinated effluents were about as toxic as their chlorinated counterparts (Table S2). The question is why?

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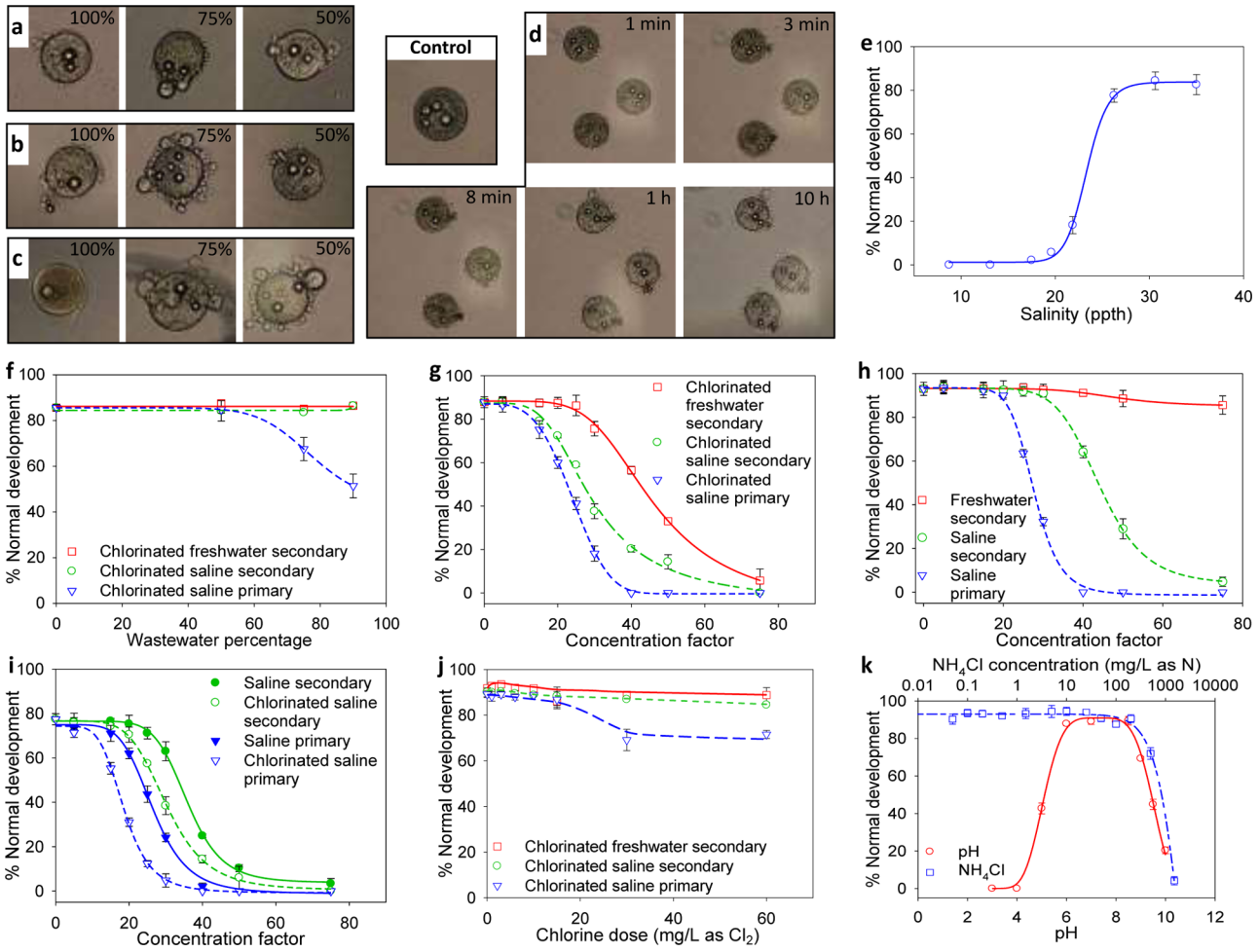

Figure 2. (a–c) Morphology of embryos exposed for 900 min in the chlorinated saline primary effluent, chlorinated saline secondary effluent, and chlorinated freshwater secondary effluent, respectively; these samples were also diluted with seawater to different wastewater percentages. (d) Morphology of embryos exposed for different periods of time in the chlorinated saline secondary effluent. (e) Variation of the percent normal development with the salinity of seawater that was diluted with ultrapure water. (f) Developmental toxicity of the chlorinated wastewater effluent samples that were diluted with seawater to different wastewater percentages and then adjusted to salinity 35 ppth. (g–i) Developmental toxicity of the organic matter that was isolated from each chlorinated or unchlorinated wastewater effluent sample and dissolved in seawater. (j) Developmental toxicity of the chlorinated wastewater effluents with different chlorine doses (After a 30 min contact time, all chlorinated wastewater effluents were dechlorinated by $Na_2S_2O_3$; prior to the toxicity test, the chlorinated freshwater secondary, saline secondary and saline primary effluents were diluted with seawater to wastewater percentages of 90%, 90%, and 62.5%, respectively, and then adjusted to salinity 35 ppth). (k) Variation of the percent normal development with pH or ammonia concentration in seawater. The three unchlorinated wastewater effluents were collected on July 6, 2010; unless otherwise specified, they were chlorinated by dosing 15 mg/L NaOCl as Cl_2 for a 30 min contact time and then dechlorinated by dosing $Na_2S_2O_3$ to produce the three chlorinated wastewater effluents.

Factors Responsible for the Abnormal Development of the Marine Polychaete *P. dumerilii*. Further observation of the morphology of embryos placed in the chlorinated saline wastewater effluents diluted to different extents (Figure 2a and b) revealed that cytoplasm spill, the spilling of cellular content outside the cellular membrane, occurred when wastewater percentages were ≥50%. Embryos in 50% and 75% freshwater wastewater samples (Figure 2c) showed more severe cytoplasm spill, whereas embryos in 100% freshwater wastewater became opaque and died in less than 3 min. It has been reported that marine invertebrates can survive under lower salinity by maintaining their body fluids hypertonic to the external medium. Under the stress of hypotonicity, the larvae exhibit initial shock with temporary immobility, then a slow recovery, followed by irreversible death when the stress is extreme.[41] Moreover, cell rupture became more severe with time (Figure 2d). This observation might explain the toxicity order of the three chlorinated effluents: under unbalanced osmotic pressure, excessive water containing little salts (especially as in the case of the freshwater effluent) flows into the cell membrane to induce cell rupture, causing embryo deaths.

To verify whether low salinity is the principal factor leading to abnormal development, we tested seawater diluted with ultrapure water to different salinities less than that in seawater (35 ppth). Embryos exposed to seawater diluted with ultrapure water appeared to develop normally when salinity was 26 ppth or higher (Figure 2e). However, a dramatic drop occurred as salinity decreased further, with the EC_{50} corresponding to a salinity of 23.3 ppth. At low salinities (8.8 to 21.9 ppth), the embryo abnormalities were very similar to embryos placed in high wastewater percentage effluents, and both were accompanied by cytoplasm spill. Because the salinities of both saline and freshwater wastewater effluents were lower than 23.3 ppth (Table S1), abnormal development occurred mainly from

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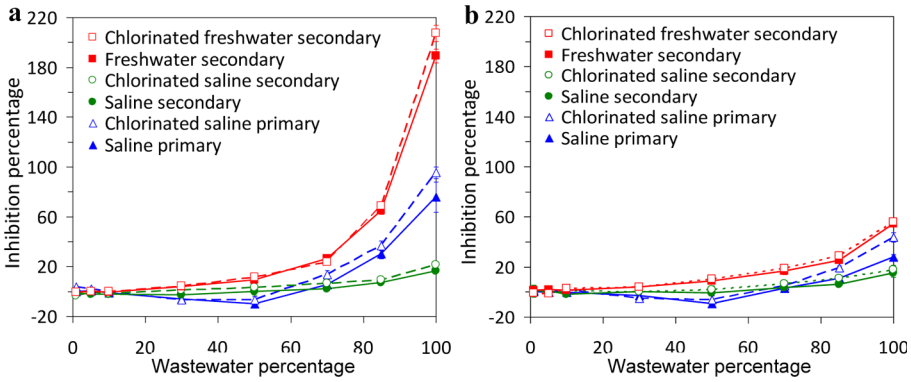

Figure 3. Algal inhibition percentages of the chlorinated and unchlorinated wastewater effluents at various wastewater effluent percentages: (a) without salinity adjustment, and (b) with salinity adjusted to 35 ppth. The three unchlorinated wastewater effluents were collected on January 12, 2011; they were chlorinated by dosing 15 mg/L NaOCl as Cl_2 for a 30 min contact time, and then dechlorinated by $Na_2S_2O_3$ to produce the three chlorinated wastewater effluents.

relatively low salinity levels in the chlorinated wastewater effluents. Garcês and Pereira[42] have demonstrated that the growth rate of juveniles of the polychaete *Marphysa sanguinea* decreased considerably as the salinity was outside 25−35 ppth. Freitas' group[43,44] has recently reported that the polychaete *Diopatra neapolitana* exposed to low salinity regenerated less chaetigers after amputation. Interestingly, by adjusting the wastewater effluents' salinities to 35 ppth with sea salt, all three types of chlorinated wastewater effluents exhibited a dramatic increase in normal development (Figure 2f), compared with effluents without salinity adjustment.

After the effect of salinity was eliminated, other factors responsible for the abnormal development may be revealed. When the salinities of all three effluents were adjusted to 35 ppth, the chlorinated saline primary effluent showed stronger adverse effects than the chlorinated saline secondary effluent or the chlorinated freshwater secondary effluent, for example, when wastewater percentage was 90%, the percent normal development of the chlorinated saline primary effluent (51.4%) was substantially lower than that of the chlorinated saline secondary effluent (86.5%) or chlorinated freshwater secondary effluent (86.4%). It has been reported that wastewater contains carbohydrates, free and bound amino acids, fatty acids, soluble acids, esters, and trace amounts of amino sugars, amide, and creatinine.[45] A higher level of DOC in the wastewater effluent means a higher content of organic matter, which might pose adverse effects on the embryos directly or indirectly. A primary wastewater effluent generally contains a substantially higher DOC level than a secondary wastewater effluent. As shown in Table S1, the DOC level in the saline primary effluent was much higher than that in the saline or freshwater secondary effluent, which matched well with the developmental toxicity order (the chlorinated saline primary effluent was much more toxic than the chlorinated saline or freshwater secondary effluent), suggesting that the organic matter content might be partially responsible for the developmental toxicity of the chlorinated saline primary effluent. To amplify the differences in toxicity effects among the three effluents, the organic matter was isolated from a given volume of each chlorinated/unchlorinated wastewater effluent and redissolved in smaller volumes of seawater to investigate the effect of organic matter at different concentration factors. Figure 2g indicates that the toxicity order of the isolated/concentrated organic matter from the chlorinated effluents was chlorinated saline primary effluent > chlorinated saline secondary effluent > chlorinated freshwater secondary effluent, which is coincident with the toxicity order of the three effluents with salinity adjustment to 35 ppth (Figure 2f). The isolated/concentrated organic matter from the unchlorinated effluents also exhibited the same toxicity order (Figure 2h) as the isolated/concentrated organic matter from the chlorinated effluents (Figure 2g). The ranking order of the DOC levels of the three effluents (i.e., saline primary effluent > saline secondary effluent > freshwater secondary effluent) well matched the toxicity order of the isolated/concentrated organic matter of the three effluents (i.e., the concentrated effluent samples). All these implicate that, once the salinity effect was eliminated, the organic matter (or the DOC level) of a wastewater effluent took over in determining the developmental toxicity of it. Furthermore, the organic matter from each chlorinated effluent was more detrimental on the embryos than the corresponding unchlorinated counterpart (Figure 2i). As aforementioned, numerous halogenated DBPs have been detected in the chlorinated saline wastewater effluents. Therefore, the formation of halogenated DBPs might explain the increased adverse effects after chlorination.

Effects of chlorine dose, pH, and ammonia on the embryos were also investigated because they could be partially responsible for the abnormal development. Figure 2j shows the developmental toxicity of the effluents with different chlorine doses. Percentages of normal development exhibited slightly descending trends as the chlorine dose increased from 0 to 60 mg/L. Previous studies[6,26] have shown that for the saline primary effluent, the levels of total organic bromine (TOBr, an indicator for all Br-DBPs) and total organic chlorine (TOCl, an indicator for all Cl-DBPs) increased from 8.0 to 110 μg/L as Br and 14.9 to 84.8 μg/L as Cl respectively as the chlorine dose increased from 0 to 15 mg/L; the total organic iodine (TOI, an indicator for all I-DBPs) level increased from 7.23 to 13.6 μg/L as I as the chlorine dose increased from 0 to 18 mg/L, and it basically remained the same as the chlorine dose further increased (Figure S2a−c). For the saline secondary effluent, the TOBr and TOCl levels increased from 8.8 to 402 μg/L as Br and from 20.9 to 64.3 μg/L as Cl respectively as the chlorine dose increased from 0 to 10 mg/L, and they became relatively stable as the chlorine dose further increased from 10 to 15 mg/L; the TOI level did not increase significantly as the chlorine dose increased from 0 to 18 mg/L (Figure S2d−f). Therefore, the overall concentrations of Br-DBPs, I-DBPs, and Cl-DBPs exhibited increasing trends in both chlorinated saline primary and secondary wastewater effluents with increasing the chlorine

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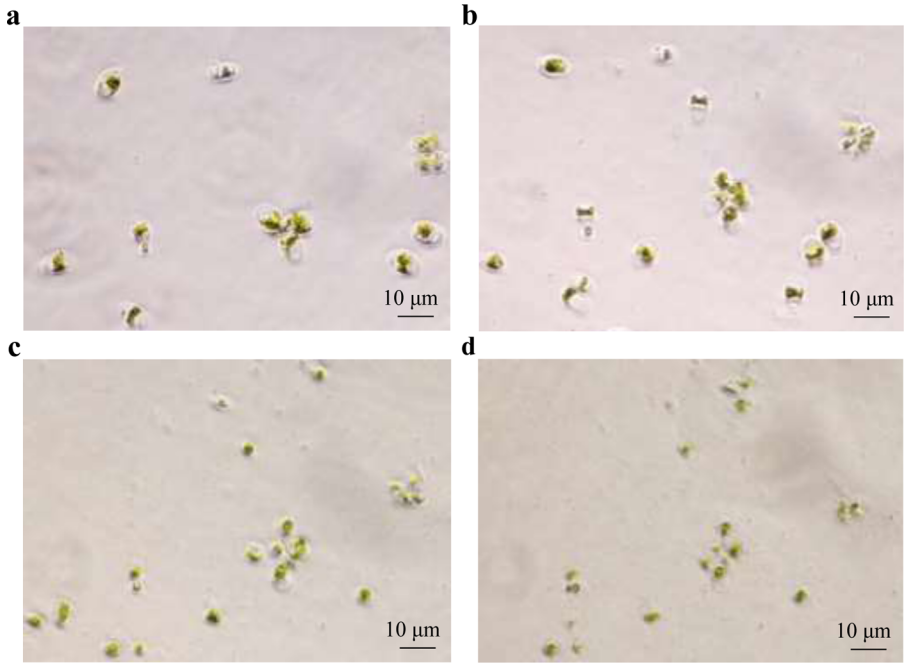

Figure 4. Morphological changes of *T. marina* exposed to 100% of the unchlorinated freshwater secondary effluent for (a) 4 min, (b) 4 h, (c) 12 h, and (d) 48 h.

dose, which might explain the slightly growing toxicity observed in the chlorinated saline wastewater effluents. As the chlorine dose increased from 0 to 60 mg/L, overall reductions in normal development were 2.9%, 3.1%, and 20.1% for freshwater secondary effluent, saline secondary effluent, and saline primary effluent, respectively, also indicating the overall low magnitude of the dependency on the chlorine dose.

By testing seawater adjusted to different pH values, we found that any pH value between 6.0 and 8.5 was harmless (Figure 2k). Because the pH of the chlorinated/unchlorinated wastewater effluents did not vary beyond that range, it should not have contributed to the adverse effects on the embryos. Seawater spiked with different NH_4Cl levels did not show any evidence of dose-related decreases in normal development in experiments with NH_4Cl levels <100 mg/L as N (Figure 2k). The ammonia concentration in 100 mg/L NH_4Cl solution was considerably higher than in any of the three effluents, indicating that the ammonia in the wastewater effluents should not result in adverse effects.

Growth Inhibition of the Chlorinated and Unchlorinated Wastewater Effluents to the Marine Alga *T. marina*. Figure 3a shows the algal growth inhibition percentages of unchlorinated and chlorinated wastewater effluents at different wastewater percentages. At low wastewater effluent percentages (<50%), the freshwater and saline secondary effluents basically exhibited no or low inhibition on algal growth, and the saline primary effluent exhibited slight stimulation to algal growth due to its high ammonia content (Table S2). It has been reported that ionized ammonia could serve as a nutrient for algal growth.[46] Figure S3 shows the effect of ammonia on algal growth. Growth stimulation occurred when the ammonia concentration was in 3−20 mg/L as N, and the maximum stimulation was observed at 9−15 mg/L as N (equal to the ammonia levels in the test samples containing 30−50% of the saline primary effluent). At high wastewater effluent percentages (≥50%), no matter whether the effluents were chlorinated, the freshwater secondary effluent presented substantially higher algal inhibition percentages than the saline primary or secondary effluent. As shown in Figure S4, the alga grew normally in the salinity range of 25−60 ppth, and algal inhibition increased sharply as the salinity decreased below 25 ppth. The salinities of the three wastewater effluents were well below 25 ppth, suggesting that low salinity was the major contributor to the inhibition effect of all the chlorinated and unchlorinated effluents, especially the freshwater secondary effluent.

Compared with the normal algal growth in the control sample (Figure S5), morphological changes of *T. marina* exposed to the freshwater secondary effluent were recorded. In 100% of the freshwater secondary effluent (Figure 4), the vacuole in each algal cell swelled significantly and extruded cytoplasm and other organelles in the first 4 min. This continued until 4 h. At 12 h, some algal cells died. At 48 h, most cells died and began to decompose. Swollen vacuole is a response of unicellular and multicellular algae to a surrounding hyposaline condition,[47] for example, swollen vacuoles were observed in the red alga *Porphyra umbilicalis* and the marine flagellate *Tetraselmis subcordiformis* in hyposaline environments.[48,49] Algae can recover via osmotic acclimation, in which osmotically active substances are excreted or transferred to inactive storage, but excretion and biotransformation of osmotically active substances are energy requiring processes. In 100% of the freshwater secondary effluent, the difference between intracellular and external osmotic stresses was so large that some algal cells died due to overexpansion and rupture of the vacuoles. For the cells that could maintain the structure, the energy was exhausted in excretion and biotransformation of osmotically active substances, and the basic metabolisms could not be supported. Finally, these cells failed to survive either. Thus, the inhibition percentages of the unchlorinated and chlorinated freshwater secondary effluents reached around 200%, indicating that not only growth inhibition but also death occurred to the initial alga. Swollen vacuoles in algal cells were also observed in 85% and 70% of the freshwater secondary

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effluent, but these were not as significant as that occurred in 100% of the freshwater secondary effluent (Figures S6 and S7). After salinity adjustment to 35 ppth, inhibition percentages of all chlorinated/unchlorinated wastewater effluents were reduced dramatically (Figure 3b).

The pH values of all unchlorinated and chlorinated effluents were in the tolerance range of the alga (6.5−9.0, as shown in Figure S8). Inhibition percentages of all three effluents increased slightly after chlorination (especially for the saline primary effluent) (Figure 3a and b), suggesting that the halogenated DBPs formed during chlorination also exerted certain adverse effects on the marine alga. Further experiments using isolated/concentrated organic matter from the chlorinated and unchlorinated saline primary effluents (SI Figure S9) also verified that the organic matter from the chlorinated effluent exhibited higher inhibition percentages on the alga than the unchlorinated counterpart, that is, halogenated DBPs in the concentrated chlorinated effluent did show certain adverse effects on the algal growth.

Our findings reveal that, in terms of the "direct" acute detrimental effect of wastewater effluents to the marine species near the discharging point (i.e., by "directly" exposing the effluents to the marine polychaete and alga), chlorinated saline wastewater effluents presented lower toxicity than chlorinated freshwater wastewater effluents. The toxicity of a chlorinated saline or freshwater wastewater effluent to the marine species was dominated by its relatively low salinity compared to the salinity in seawater. The organic matter content in a chlorinated saline or freshwater wastewater effluent might be partially responsible for the toxicity. The adverse effects of halogenated DBPs on the marine species were observed pronouncedly only in the "concentrated" chlorinated wastewater effluents. With respect to the preservation of the marine ecosystem, we see the possible potential for adopting seawater for toilet flushing to relieve the increasing shortage of freshwater resources. In 2003, 2.35×10^8 m^3 of seawater was supplied to about 80% of Hong Kong's 6.8 million people for toilet flushing.[4] By 2020, the world's population will grow to over 7.5 billion.[50] If Hong Kong's practice of using seawater for toilet flushing can be promoted, 75% of the world's population (living within 60 km of the coast) in 2020 will save over 2.43×10^{11} m^3 of freshwater annually, which is equal to 43% of the total freshwater use in the United States (5.66×10^{11} m^3 in 2005).[51]

Finally, it needs mentioning that this study does not represent the end of this important topic. Because chlorinated wastewater effluents are continuously discharged into the marine environment (the receiving water body), marine species may be exposed to low levels of Br-/I-DBPs, as well as Cl-DBPs persistently. At this point, we cannot address the long-term effects of low levels of halogenated DBPs on marine species, and further chronic toxicological studies may be needed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03796.

Additional details, Figures S1−S9, and Tables S1 and S2 (PDF)

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Notes

The authors declare no competing financial interest.

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