Sensitivities of seven algal species to triclosan, fluoxetine and their mixtures

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Increasing release of pharmaceuticals and personal care products (PPCPs) into aquatic ecosystems is a growing environmental concern. Triclosan and fluoxetine are two widely used PPCPs and frequently detected in aquatic ecosystems. In this study, the sensitivities of 7 algal species from 4 genera to triclosan, fluoxetine and their mixture were evaluated. The results showed that the inhibitory effect on algal growth (EC50-96h) of triclosan varied with 50 times differences among the 7 algal species. Chlorella ellipsoidea was the least susceptible species and Dunaliella parva was the most sensitive species to triclosan. The inhibitory effect of fluoxetine was less variable than triclosan. Slightly higher toxicity of fluoxetine than triclosan was shown in the 7 tested algal species. No consistent pattern of the effects from mixture of triclosan and fluoxetine was observed among the 7 algal species and among the 4 genera. Additive effects of the mixture occurred in 4 species and antagonistic effects in the other 3 species but no synergistic effect was detected. The algal species might show some sign of phylogenetic response to triclosan, as evidenced by the wide range of differences in their sensitivity at the genus level. This study provides important data which could be beneficial for biomonitoring programs on the ecological risk (algal species diversity) of these two chemicals.

Pharmaceuticals and personal care products (PPCPs) are a large group of chemicals including antibiotics, hormones, anti-inflammatory drugs, disinfectants, insect repellants, and UV-filters, etc.1,2. Considered as emerging contaminants, PPCPs have been receiving increasing attention in recent years for their occurrence in waters and effects in aquatic organisms3–5. In 2011, the global annual production of PPCPs has been estimated at approximately 13 million tons1. Due to incomplete removal in wastewater treatment plants (WWTPs), some PPCPs in domestic, industrial and hospital sewages after WWTPs treatment will have the chance to enter the receiving aquatic environments. PPCPs and their metabolites have been detected in various waters at levels that are generally in the ng/L range, sometimes can be found in μg/L level, and some of the PPCPs may be accumulative in aquatic organisms3,6–8.

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a synthetic antimicrobial chemical and applied in a variety of consumer healthcare products, soaps and plastics9. It has been used as a disinfectant for several decades10. It is one of the most frequently detected PPCPs in surface water worldwide8. The concentration of triclosan was found in the range of 0.011 to 2.7 μg/L in surface water6,11, and the mean concentration was approximately 10 μg/L in untreated wastewaters12. After treatment in WWTPs, triclosan concentrations in typical effluents average 0.78 μg/L13, which may cause adverse effects in many aquatic organisms14,15. Triclosan has a logKow value of is 4.8 at pH 7.5 and is likely to be photodegraded in water. However, its degradation products (e.g. 2,4-DCP and 2,8-DCDD) are persistent and more toxic than triclosan itself16,17. The hydrophobic property of triclosan increases its potential for bioaccumulation and trophic transfer through food web18. The effects of triclosan have been studied in a variety of aquatic organisms19. The EC50-96h values range from 0.53 to 800.0 μg/L for algae and...
LC50-96h values range from 184.7 to 3000 µg/L for aquatic invertebrates19–21. Triclosan blocks the lipid synthesis by inhibiting the enzyme enoyl-acyl carrier protein reductase (ENR)22,23 and destabilizing the cell membrane24. This may increase disturbance of the permeability-barrier functions on the membrane25.

Fluoxetine (N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine) is a selective serotonin reuptake inhibitor (SSRI), among the most often prescribed drugs for the treatment of depression and some compulsive disorders for more than two decades26. It has been found that fluoxetine concentration was up to 0.012 µg/L in streams in the U.S and concentration was from 0.013 µg/L to 0.099 µg/L in WWTPs effluent27. Fluoxetine can be rapidly metabolized to norfluoxetine28. Fluoxetine has a logKow value of approximately 4.5 in surface water and is hydrodynamically and photolytically stable in water. It can be rapidly dissipated from water phase as a result of adsorption in the particulate matter or sediment in natural water29. It is persistent in aquatic environments30,31. Due to its enantiospecific effects, it may cause potential deleterious effects to aquatic organisms at even low concentrations31. EC50-96h values of fluoxetine range from 16.0 to 900 µg/L in freshwater algae and LC50-96h values range from 234 to 820 µg/L in aquatic invertebrates32–34. In addition, fluoxetine can be transferred from the lower trophic level to the higher trophic level in a laboratory-demonstrated three-level aquatic food chain35.

Exposure to fluoxetine in algae results in cell deformities and smaller sizes at concentrations over 13.6 µg/L36. In addition, fluoxetine has also been found to inhibit efflux pumps in cell membrane37.

Algae form the base of aquatic food webs and play important roles in energy and nutrient transfer to upper trophic level species. In addition, algae have proved to accumulate many pollutants from the water which can be transferred to species at higher trophic levels38–40. Algae have a fast reproduction rate and high sensitivities to environmental disturbance and pollution. Some algal species can be either used as environmental indicator or capable of removing pollutants. For example, species of Chlorella and Scenedesmus are relatively tolerant to environmental contaminants and are highly efficient in removing heavy metals from wastewater32,41. Chlamydomonas sp. is effective for phosphate removal42. Dunaliella are salt tolerant species found in salt lake and marine environment and are often used as model test species for marine and estuary environment43,44.

No single species in general can be expected to represent all other species from the same biological classification unit (i.e., at the order or genus levels) in the response to environmental stressors. Different algae species may have different sensitivity to environmental pollutants. Studies of the PPCPs toxicity to different algae species among different genera may increase our understanding of triclosan and fluoxetine toxicity to freshwater algae. However, the field has many detected concurrently in waters receiving effluents from WWTPs47–49. In this study, the individual and mixture effects of these two chemicals were determined for 7 algal species from four different genera by growth inhibition bioassays. The objectives of this study were to determine the sensitivity of different algal species to triclosan and fluoxetine, and to determine the joint actions of these two chemicals to the different algal species. The results from this study will broaden our knowledge on the general toxicity of these two chemicals to algae and improve our understanding of the different sensitivities in different algal species among different genera. The outcome of this work will help us understand the ecological risk of the two chemicals.

Material and Methods

Chemicals. Triclosan (purity >97%) and fluoxetine (purity >98%) were purchased from Aladdin Industrial Corporation (Shanghai, China) and Sigma-Aldrich (St. Louis, MO, USA) respectively. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. All glassware and other containers were acid washed, rinsed with deionized water, air-dried, and autoclaved before use.

Culturing of Algae. Algal strains in this study were purchased from the Freshwater Algae Culture Collection (FACHB-collections) at the Institute of Hydrobiology, Chinese Academy of Sciences. Seven species were studied from Chlorella (C. pyrenoidosa and C. ellipsoidea), Scenedesmus (S. obliquus and S. quadricauda), Dunaliella (D. salina and D. parva) and Chlamydomonas (C. microsphaera) genera. The algae were cultured in 250 ml flasks with approximately 100 mL of medium prepared according to the OECD guideline for 5 of the species (C. pyrenoidosa, C. ellipsoidea, S. obliquus, S. quadricauda, C. microsphaera) (OECD 201) (see Table S1). The recipe of the culture medium from OECD 201 for the recipe of the medium), and in flasks with a medium according to FACHB for two of the species (D. salina and D. parva) (see Table S2). The recipe of the Dunaliella medium for the recipe of the medium). The cultures of all algal strains were maintained in the lab by re-inoculating in freshly sterilized flasks with freshly prepared medium at 1:20 (v/v) at least once a week. The purity of the algae stock was frequently examined under a microscope connected to a computer with software aiding in algal species counting and identification (Shinseo Algacount-Sx, Hangzhou, China).

Exposures to triclosan and fluoxetine. Preliminary experiments were conducted to determine the appropriate range of exposure concentrations for the determination of EC50 for each algal species. The growth inhibition tests for each algal species were performed in 96-well microplates according to the method by Petersen, et al., with some modifications. The nominal exposure concentration range was 0–2000 µg/L for triclosan and 0–1280 µg/L for fluoxetine with 9 concentration gradients including solvent control (i.e., DMSO at approximately 0.1% (v/v) of the exposure volume), respectively. Prior to the exposure, algae cultures were incubated in the growth medium for 4 to 6 days to ensure the cultures to be at the stage of exponential growth with a cell density reaching approximately 105–106 cells/mL. The relationships between absorbance (optical density at 450 nm on a Multiskan FC spectrophotometer, Thermo Scientific, China) and algal concentration (cell density) were assessed for all strains (in all cases, the straight line had a R² > 0.90). In addition, algal density in the stock and the diluted solutions was estimated using a hemocytometer and counted with the aid of the computer software for algal growth.
counting (Shineso Algacount-Sx, Hangzhou, China) to ensure the comparability between absorbance and cell density. Results from both methods were comparable.

For all exposures (including individual and mixture), the outer wells of a microplate were filled with 200 µL of growth medium to counteract the edge-specific evaporation from the microplate (Thermo, China). For each algal culture, the cell concentration was adjusted to 5 × 10^4 cells/mL from the algal stock in a 10 mL centrifuge tube. For each culture, ten 10 mL tubes were prepared, each containing the diluted algal solution for a treatment in the exposure (see below). Freshly prepared stock solutions of the chemical (triclosan or fluoxetine) were transferred to each culture tube to achieve the nominal exposure concentrations. After that, approximately 200 µL from each tube was transferred directly to a well in a microplate. The exposure for each species was conducted using at least four plates (therefore four replicates). Each plate had 10 exposure concentrations including a control (growth medium only), a solvent control, and 8 concentrations of the chemical (i.e., C_{Fi} for fluoxetine and C_{Ti} for triclosan, where i = 1 to 8, representing 8 concentrations used in the exposure). The wells from the second to the sixth row on the plates were used for the exposure (therefore, on each plate, there were five replicates for each concentration). The wells of the seventh row on the plate contained growth medium and exposed chemical without algal cells. This row was used to monitor the change of absorbance due to the chemical alone during the 96 h exposure (for more direct visualization of the arrangement of the wells in each plate, please see Fig. S1). Each plate was sealed with a plate cover and then wrapped with parafilm before incubated on a microplate shaker (Leopard, China) at 200 rpm. A continuous illuminance was maintained at 1700 ± 100 lux and temperature was maintained at 20 ± 2 °C. After 96 h of exposure, the absorbance of the algal culture was measured at 450 nm on a Multiskan FC spectrophotometer.

For the binary mixture exposure, similar exposure regime was employed except for the chemicals used. Each of the 8 exposure concentrations for the mixture was a simple addition of the concentration of each chemical in its individual exposure (i.e., C_{Mix} = C_{Fi} + C_{Ti}, where, i = 1 to 8, representing 8 concentrations used in the exposure; C_{Mix} is the ith concentration used in the mixture, Fi is the individual concentration of fluoxetine, Ti is the concentration of triclosan) (for more direct visualization of the arrangement of the wells in the plate for mixture exposure, please see Fig. S2). After 96 h of exposure, the absorbance of the algal culture was monitored at 450 nm.

Statistical analyses. All data were expressed as mean ± standard error unless otherwise stated. The concentration-response curve was set from the experimental data for each algal and each chemical exposure (single substance or binary mixture). For the inhibitory effects of individual chemicals, the relative algal growth in each well was fitted to the exponential growth function, using the Graphpad Prism software (Version 5, San Diego, CA, USA). Since there was no intra-plate effect, the measured absorbance values of the five replicated wells in each microplate were combined to generate an arithmetic mean, which was used as a single datum point. Since four plates were used for each algal species, the number of replicates was four. The differences in absorbance for each concentration among the four replicated plates were determined using One-way analysis of variance (ANOVA). Genus, treatment, and species parameters were used as fixed effects to test if the inhibitory effects were significantly different for the fixed factors. P < 0.05 was considered to be significantly different.

To determine whether the effect of the binary mixture is additive, antagonistic or synergistic, the statistical method of Marking and Dawson was adopted using the Eq. (1):

\[ S = \frac{A_{mix}}{A_i} + \frac{B_{mix}}{B_i} \]

where A and B are the two chemicals, i and m are the toxicities (EC_{50}'s) of the individual chemicals and the mixture, respectively, and S is the sum of the toxicities. S > 1.0 indicates synergistic effect. S > 1.0 indicates antagonistic effects.

Ethical approval. This article does not contain any studies with animals performed by any of the authors.

Results

Inhibitory effects of individual chemicals in seven algal cultures. In general, the concentration response (CRC) data on the relative growth of each algal strain were fitted well to the non-linear regression line (R² > 0.90, p < 0.001 in all cases) for both chemicals (Figs 1 and 2). For triclosan, the no-observed effect concentrations (NOEC) in the seven algal species ranged from 6.2 µg/L in S. quadricauda to 100 µg/L in C. pyrenoidosa and C. ellipsoidea (Table 1). The lowest observed effect concentrations (LOEC) spanned from 18.6 µg/L in C. microsphaera and 600 µg/L in C. pyrenoidosa (Table 1). There was a significant difference in the susceptibility to triclosan among the four genera (ANOVA, F_{(3,12)} = 23.8, p < 0.0001). The susceptibility to triclosan for the four genera ranked as Dunaliella ~ Scenedesmus > Chlorella ~ Chlamydomonas. The EC_{50}-96h varied with a factor of approximately 50 times among the seven algal species (Fig. 3). C. ellipsoidea was the least susceptible species with an EC_{50}-96h of 1441.5 ± 52.8 µg/L, while D. parva was the most sensitive species with an EC_{50}-96h of 39 ± 0.1 µg/L (Fig. 3). Algal species in the same genera showed different susceptibilities to triclosan in 96 h exposure. C. pyrenoidosa, S. quadricauda, D. salina were more susceptible than C. ellipsoidea, S. obliquus and D. parva, respectively (Fig. 3).

For fluoxetine, the NOEC in the seven algal species varied from 6.2 µg/L in C. pyrenoidosa and D. parva to 40.2 µg/L in S. obliquus and D. salina (Table 1). The LOEC had a range from 18.6 µg/L in C. pyrenoidosa and D. parva to 80.4 µg/L in C. ellipsoidea and S. quadricauda (Table 1). There was a significant difference among different genera in the susceptibility to fluoxetine (ANOVA, F_{(3,12)} = 64.1, p < 0.0001), with Chlorella being the least sensitive genus, followed by Chlamydomonas. The EC_{50}-96h values were less variable than those of triclosan, with approximately 13 times of differences among the seven algal species (Fig. 4). C. ellipsoidea and C. pyrenoidosa...
were the two least susceptible species with EC$_{50}$-96h values of 640.0–773.3 µg/L, followed by *C. microsphaera*. The other four species had similar susceptibility to fluoxetine. There was no significant difference in algae responses in fluoxetine within the same genera, except that *D. parva* was more susceptible than *D. salina* to fluoxetine in 96 h exposure time (Fig. 4).

Finally, the EC$_{50}$-96h values of fluoxetine were generally lower than those of triclosan in the 7 algal species. At the species level, fluoxetine showed higher inhibitory effects than triclosan ($p < 0.001$) in six of the seven tested species, except for *D. parva*, which had similar EC$_{50}$ values for the two chemicals (Figs 3 and 4).
Effects of binary mixture of triclosan and fluoxetine in seven algal cultures. The sum of toxicity ($S$) of the mixture of triclosan and fluoxetine in the 7 algal species ranged from 0.9–2.0 (Table 2). In general, no consistent pattern of the effects of the binary mixture was observed among the 7 algal species and among the 4 genera studied. Both antagonistic and additive effects were noticed for the binary mixture in the 7 species. However, no synergistic effect was observed in all the seven algal species.

Figure 2. Concentration-response curves of 7 algal species to fluoxetine. The growth rate of 7 algal species exposed to concentration gradients of fluoxetine for 96 h relative to that of the control (mean ± standard error, $n = 4$).
Genera | Species     | Triclosan (µg/L) | Fluoxetine (µg/L) |
|--------|-------------|-----------------|-------------------|
|        |             | NOEC  | LOEC  | NOEC  | LOEC  |
| Chlorella | C. pyrenoidosa | 100   | 600   | 6.2   | 18.6  |
|         | C. ellipsoidea  | 100   | 500   | 18.6  | 80.4  |
| Scenedesmus | S. obliquus | 49    | 100   | 40.2  | 49.5  |
|         | S. quadricauda  | 6     | 49    | 18.6  | 80.4  |
| Dunaliella | D. salina     | 41    | 49    | 40.2  | 46.4  |
|         | D. parva       | 20    | 29    | 6.2   | 18.6  |
| Chlamydomonas | C. microsphaera  | n.d.  | 9     | 18.6  | 40.2  |

Table 1. The 96h NOEC and LOEC values based on growth inhibition of 7 algal species exposed to a concentration gradient of triclosan or fluoxetine. n.d.: data was not available.

Figure 3. The EC50 of 7 algal species exposed to triclosan for 96 h. And mean ± standard error, n = 4. Asterisk (*) indicated significant difference (P < 0.05) within the same genus. The letters indicated significant differences (P < 0.05) among genera.

Figure 4. The EC50 of 7 algal species exposed to fluoxetine for 96 h. And mean ± standard error, n = 4. Asterisk (*) indicated significant difference (P < 0.05) within the same genus by Turkey’s test. The letters indicated significant difference (P < 0.05) among genera.
The tested algal species showed inconsistent sensitivities to triclosan among species and genera. Moreover, the inhibitory effects for triclosan in 5 of the 7 algal species (except for *C. Pyrenoidosa* and *S. obliquus*) have never been reported in the literature. The EC<sub>50-96h</sub> values for triclosan in algae have a relatively wide range from 0.53 (in *Pseudokirchneriella subcapitata*) to 800 μg/L (in *C. pyrenoidosa*). Metabolic pathway of triclosan may include biodegradation, hydroxylation, methylation, glycosylation and xylosylation in different organisms, where the major pathway for triclosan is biodegradation in *C. pyrenoidosa*, and biotransformation in *S. obliquus*. Other factors, such as algal species, culture medium, pH, light intensity would also affect the inhibitory effect of triclosan on the growth of algae. Since triclosan targets lipid synthesis, it is likely that fatty acid metabolism in plants might also be disrupted by the same mechanism due to the toxicity of triclosan.

### Table 2. Sum of toxicity (S) of the mixture of triclosan and fluoxetine in the 7 algal species. The effect of the two chemicals in mixture is supposed to be additive when S is not different from 1.0, synergistic when S is less than 1.0, and antagonistic when S is greater than 1.0 (n = 4).

| Genera       | Species        | S (mean and 95% CI) | Interactions |
|--------------|----------------|---------------------|--------------|
| *Chlorella*  | *C. pyrenoidosa* | 1.3 (0.96e+01–1.66) | antagonistic |
|              | *C. ellipsoidea* | 1.0 (0.73–1.20)     | additive     |
| *Scenedesmus*| *S. obliquus*   | 2.0 (1.64e-02–2.42) | antagonistic |
|              | *S. quadricauda*| 1.1 (0.46–1.73)     | additive     |
| *Dunaliella* | *D. salina*     | 1.5 (1.26–1.73)     | antagonistic |
|              | *D. parva*      | 0.9 (0.57–1.24)     | additive     |
| *Chlamydomonas*| *C. microphaera*| 1.2 (0.58–1.78)     | additive     |

### Discussion

In this study, both triclosan and fluoxetine showed inhibitory effects in the seven algal species within 96 h of exposure. The inhibitory effects of triclosan had a relatively wide range (50 times of differences) among the seven species. The inhibitory effects for triclosan in 5 of the 7 algal species (except for *C. Pyrenoidosa* and *S. obliquus*) have never been reported in the literature. The EC<sub>50-96h</sub> values for triclosan in algae have a relatively wide range from 0.53 (in *Pseudokirchneriella subcapitata*) to 800 μg/L (in *C. pyrenoidosa*). Metabolic pathway of triclosan may include biodegradation, hydroxylation, methylation, glycosylation and xylosylation in different organisms, where the major pathway for triclosan is biodegradation in *C. pyrenoidosa*, and biotransformation in *S. obliquus*. Other factors, such as algal species, culture medium, pH, light intensity would also affect the inhibitory effect of triclosan on the growth of algae. Since triclosan targets lipid synthesis, it is likely that fatty acid metabolism in plants might also be disrupted by the same mechanism due to the toxicity of triclosan.
mixture can be species specific. For example, in a study on the effects of eight mixtures of herbicides (with different sites of action) in six plants and algal species, it was shown that only two of the mixtures were consistently antagonistic across all species studied, while for the remaining six mixtures, the joint effect depended on the species tested.

Finally, the susceptibility of the seven algae to triclosan showed a relatively wide range (but less to fluoxetine) and both chemicals showed significant differences in susceptibility at the genus level but similar at species level. Previous studies have shown that sensitivity to chemical pollutants (including organic pollutants and metals) might show phylogenetic signals in aquatic organisms. For example, significant phylogenetic signal was demonstrated in the sensitivity to four herbicides (atrazine, terbutryn, diuron, and isoproturon) among 14 diatom species representative of a freshwater lake. Phylogenetic signal on the susceptibility to pollutants can help explain the field observations in the contaminated environments. For example, Buchwalter et al. showed that susceptibility to Cd among 21 aquatic insects showed a significant phylogenetic signal in a laboratory study. The absence and presence of aquatic insects along the metal gradients in the mining impacted Clark River, USA, corresponded to the sensitivities of these species to Cd in their respective clades assessed in the laboratory, with the most Cd sensitive mayfly species that dissappeared earlier than the less susceptible caddisfly species. Due to the requirement of large database to detect the phylogenetic signal in sensitivity to chemical pollutants in organisms, it could not be concluded from this study whether the sensitivity to these two chemicals had a phylogenetic basis. Meanwhile, the use of a phylogenetic framework to improve biomonitoring remains an unexplored field, and few data are available for freshwater algae. Based on the wide range of differences in sensitivity of the 7 algal species to the two chemicals (especially for triclosan) in this study, it will be very interesting to include more species in future work to test whether a phylogenetic signal exists for the susceptibility to these two chemicals in algae. It is suggested that approaches that integrate phylogeny and ecotoxicology can provide information for biassessment tools operating at a larger taxonomic scale and thus increase the effectiveness of biomonitoring. Up to now, the lack of quality datasets based on multiple species with a wide range of sensitivities has hindered this type of research. Such quality datasets may also bring valuable sensitivity data to build relevant risk assessment models such as Species Sensitivity Distribution in order to predict efficiently effects of PPCPs and their mixtures.

This study demonstrated that both fluoxetine and triclosan can inhibit the growth of seven algae. Fluoxetine seemed to have higher inhibitory effects on the growth of the algae than triclosan. The binary mixture showed additive and antagonistic effects depending on the algal species. Future research could focus on the mechanisms of effects (inhibition of growth and other detrimental effects) of the two chemicals in algae and on the determination of the sensitivity to these two chemicals using more species from different genera in order to understand the response of algal species to the chemical exposure and consequent change in structure and function of phytoplankton community, in order to predict the risk of PPCPs to the aquatic ecosystems.

Data Availability
The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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**Acknowledgements**

We thank to the National Natural Science Foundation of China (Grant 41401582, 31270549 and 41501548), China Postdoctoral Science Foundation (Grant 2018M632471), Department of Science and Technology of Guangdong Province (Grant 2011B050300026), Guangdong Natural Science Foundation (Grant S2011030005257).

**Author Contributions**

R.B., X.Z., L.H., W.L., P.L., L.X. and A.B. contribute to study concept and design. L.M., H.C., D.L. and J.T. contributed to data collection and interpretation. R.B., H.C., L.X., A.B. contributed substantially to the statistical analysis. All the authors have approved the manuscript and agree with the submission to Scientific Reports.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-33785-1.

**Competing Interests:** The authors declare no competing interests.

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