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Toxicities of sulfadimethoxine to five aquatic organisms

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ABSTRACT

Bioassays were conducted with five species of aquatic organisms to assess toxicities of sulfadimethoxine (SDM), a veterinary sulfonamide antibiotic commonly used in aquaculture industry. For the growth inhibition of microalgae, SDM had the 72-h EC_{50} values of 50.6 mg L^{-1} for the freshwater *Chlorella vulgaris* and 39.3 mg L^{-1} for the marine *Isochrysis galbana*. For the survival of cladocerans, SDM had the 48-h LC_{50} of 180 mg L^{-1} for *Daphnia magna* and 427 mg L^{-1} for *D. similis*. For the brook production of the cladocerans in chronic toxicity test, SDM had the 21-d EC_{50} of 10.4 mg L^{-1} for *D. magna* and 127.1 mg L^{-1} for *D. similis*. No significant toxicity of SDM was found to the medaka *Oryzias latipes* at a concentration as high as 1000 mg L^{-1}. This study demonstrated that the potential adverse effect of SDM on aquatic organism. The microalgae showed higher sensitivities to SDM than the cladocerans. Therefore, the use of the SDM on aquatic organisms should be elucidated for carefully evaluation of wastewater treatment after use and reduction of ecological impacts.

Keywords: antibiotics, sulfadimethoxine, bioassay, toxicity

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INTRODUCTION

The use of antibiotics in many countries is increasing and also is an important role in diseases treatment and prevention for animals in modern livestock and aquaculture industries [1-3]. They are usually administered to the animals through feed, but often resulting in releasing of their residues from the medicated feed, feces of animals, and even water runoff from manure-treated farmlands, that contaminate surrounding surface water [4]. The antibiotic residues may cause direct toxic effects on microflora and microfauna [5], develop resistant bacterial populations [6], and possibly transfer antibiotic resistance to human pathogenic microbes [3].

Sulfonamide antibiotics (SAs) are widely used in medication of farm animals and occupy a high proportion of the total global usage of antibiotics today [3]. They have high excretion rates in urine and feces of treated animals as parent compounds or metabolites that are easily transferable from their contaminated sites to surrounding water because of their low sorption rates in soils and sediments [3,7]. Residues of SAs have been detected in fish ponds [8,9] and their effluents and downstream water [10-13].

Sulfadimethoxine (SDM) is an broad-spectrum SAs commonly used in aquacultures [14]. However, SDM have been detected in water and effluents of aquaculture ponds [13], and also in sludge of sewage (wastewater) treatment plants [15] and manure of farm animals [13,16]. A concentration as high as 100 μg L^{-1} was detected for both antibiotics in the sewage sludge [15]. For these two antibiotics, SDM is the most frequently detected antibiotics in aquaculture water. It has been also detected in groundwater [17] and in influents and effluents of sewage treatment plants [10,18]. A concentration as high as 36 μg L^{-1} of SDM was detected in water of a fish hatchery during the medication period [8]. Despite the intensive uses of SDM in aquaculture and their wide contamination on surface water, the information on toxicity of SDM to aquatic life was scarce. Furthermore, there were discrepancies in toxicities of SDM to aquatic organisms that had been reported. A few studies reported that SDM exhibited significant toxic effects [19-21], while some were not [22]. The discrepancies might be due to difference in sensitivity of the test organisms and criteria used for defining the risk [3].

This study was intended to conduct bioassays to investigate acute and chronic toxicities of SDM to aquatic organisms belonging to various trophic levels in the ecosystem. With the results obtained, environmental hazards of SDM were evaluated, and the aquaculture effluents management concerning the antibiotic was discussed.

MATERIAL AND METHODS

Test antibiotics

SDM (4-amino-N-(2,6-dimethoxypyrimidin-4-yl) benzenesulfonamide, CAS 122-11-2) with a purity as high as 98% or higher were purchased from Sigma-Aldrich (St. Louis, MO, USA). SDM was dissolved in a 0.03 M NaOH solution to make a stock solution of 5000 mg L^{-1}. Tape water was distilled and then deionized with the Milli-Q Plus analytical deionization system (Bedford, MA, USA). This deionized water was used in making the NaOH solution and preparing the stock and test solutions of SDM. All chemicals and solvents used in this study were HPLC grade.

Test organisms

Five species of aquatic organisms were used as test animals, including freshwater microalga (*Chlorella pyrenoidea*), marine microalga (*Isochrysis galbana)*, two freshwater cladocerans (*Daphnia similis* and *D. magna*), and freshwater medaka fish (*Oryzias latipes*). The two microalgae have been commonly used as indicator species for primary producers in aquatic food chains and ecosystems. As they have high protein and fatty acid contents, they are also used as feed in larval culture of aquatic organisms [23]. The cladocerans are effective bio-indicators of toxicity because of their high sensitivity and short reproductive cycle [24]. *O. latipes* is a common fresh water fish that used in acute toxicity tests [25].

Stock cultures

Algae

C. pyrenoidosas was obtained from the Institute of Marine Biology, National Taiwan Ocean University, Keelung, Taiwan in 2007 [5]. *I. galbana* was from the Tungkang Biotechnology Research Center, Fisheries Research Institute, Pingtung, Taiwan. In the laboratory, both algae were cultured in a 1.5 L conical flask, containing 1000 mL of culture media. The medium for *C. vulgaris* was prepared with the deionized water following guideline of OECD [26], and the Walne medium prepared with seawater at 34‰ salinity for *I. galbana* [27]. The seawater was prepared by mixing artificial sea salt (TAAM, Camarillo, CA, USA) in the deionized water.

The culture media were UV-irradiated for 30 min and then filtered with a 0.2 µm filter before use. After the inoculation, the cultures were gently aerated at the temperatures of 25±1°C under a continuous illumination at 6 Klux. The pH values were 7.2-7.8 for *C. vulgaris* while 6.8-7.0 for *I. galbana* in the culture. The cultures were renewed weekly.

Cladocerans

D. magna was obtained from the Environmental Analysis Laboratory of Environment Protection Administration, Taoyuan, Taiwan. *D. similis* was from the Freshwater Aquaculture Research Center, Fisheries Research Institute, Chupei, Taiwan. Both daphnids were separately cultured in 10-L glass jars containing 8 L of the dechlorinated tap water (hardness 146 mg L^{-1} as $CaCO_3$, conductivity 534-585 μS cm^{-1}), at a constant temperature of 25±1 °C and a 16-h light (L):8-h dark (D) period under 600 lux of white fluorescent light in accordance to the OECD guidelines [24,25]. The culture water and the glass jars were renewed once a week. They were fed with the green alga *C. vulgaris* once a day at an average density of approximately 3×10^5 cells mL^{-1}.

Medaka fish

O. latipes was obtained from the Freshwater Aquaculture Research Center, Fisheries Research Institute, Chupei, Taiwan. The fish was kept in 20-L aquaria containing 15 L of the dechlorinated water. The culture temperature, light intensity, and light periods were similar to those used for the cladocerans. The fish were fed with freshly hatched *Artemia* nauplii and artificial feed (Trifish BP, Omega, Kaohsiung, Taiwan) once a day. A day before the test, the feeding was stopped for those to be used in the test.

Acclimation

In this study, the culture water and media used in stock cultures of test organisms was similar to those used for the test solutions in the toxicity. Also, culture conditions (temperature, light intensity, and light-dark periods) of the stock cultures were kept similar to those in the toxicity tests. Therefore, the organisms in the stock cultures were considered to be acclimated and used directly in the tests.

Individual toxicity tests

For each of the individual toxicity test, a two-step experiment was conducted [21]. The first step was a range-finding experiment. It was made with four nominal concentrations (1, 10, 100 and 1000 mg L^{-1}) to obtain an estimate of the median effective or lethal concentration (E/LC_{50}). Based on this estimate, five test concentrations were made by diluting a small amount of the stock solution in a geometric series (ratio=2) with the culture medium for the microalgae and dechlorinated water for the cladocerans and the fish. The second step was the determination experiment. It used a control and five test concentrations obtained from the range-find experiment. The test concentrations were adjusted, if necessary, to obtain more precise data.

Algal growth inhibition tests

For each of the toxicity tests of the algae, a 72-hour (h) growth inhibition test was conducted with three replicates, each with five test concentrations and a control, in accordance with the guidelines of the OECD [26]. The test tanks were 125 mL conical flasks, each containing 50 mL culture medium similar to those used in the stock culture. The test concentrations of SDM were 2.5, 5, 10, 15 and 20 mg L^{-1} for *C. pyrenoidea* and 5, 10, 20, 50 and 100 mg L^{-1} for *I. galbana*. The initial algal density inoculated was approximately 10^4 cells mL^{-1} for each of the flasks. During the test period, the flasks were gently shaken, and their positions were changed randomly twice a day to ensure equal irradiating light intensity of 6 Klux. The test conditions were at a constant temperature of 25±1 ºC and pH of 7.4±0.2 for both *C. pyrenoidea* and *I. galbana*, similar to those for the stock cultures.

The number of algal cells in each of the flasks was counted daily with the image analysis software (Image-Pro Plus, Media Cybernetics, Acton, MA, USA). The count was further confirmed with those from the hemocytometry and microscopy. The growth curves were established and compared between the test concentrations and the control.

Cladoceran survival and brook production toxicity tests

A 48-h acute lethal toxicity test and a 96-day (d) chronic brook production toxicity test were conducted for SDM to each species of the cladocerans, in accordance with the guidelines 202 and 211 of OECD [28,29].

For the 48-h lethal toxicity test, four replicates were used, each with five test tanks and one control tank. The tanks were 100 mL glass beakers, each containing 50 mL of the solutions. For SDM, the test concentrations of SDM were 100, 200, 400, 800, and 1000 mg L^{-1} for both *D. magna* and *D. similis*. For each of the tanks, five neonates less than 24-h old were added as the test animals. No feeding during the test period. The test conditions were at a constant temperature of 25±1 ºC, a 16L:8D cycle of 600 lux illumination, dissolved oxygen (DO) concentrations of 7.6±0.1 mg L^{-1} without aeration and pH of 8.0±0.3. The number of immobile individuals in each of the flasks was counted as the dead and recorded daily.

For the 96-day (d) chronic brook production toxicity test, ten replicates were used, each having a control tank and five test tanks. The tanks were 100 mL glass beakers, each containing 50 mL solutions and *D. magna* or *D. similis* daphnids that less than 24-h old. The test concentrations were the same for both test species: 3, 6, 12, 24 and 48 mg L^{-1}. The test was carried out with the semi-static bioassay that renewed the culture medium in each of the beakers three times a week. The test conditions were similar to those for the stock cultures at a constant temperature of 25±1°C, DO concentration of 7.7±0.1, and pH of 7.4±0.2. They were fed daily with the green algae *C. vulgaris* (3×10^5 cells/mL in average). The numbers of broods produced by the females were recorded daily as the reproductive outputs.

Fish survival test

The 96-h lethal toxicity tests of SDM were conducted to juveniles of medaka *O. latipes* (1.3±0.2 cm in length and 25±5 mg in weight), according to the guidelines of OECD 203 [30]. For each of the tests, five replicates, each with six 200 mL glass beakers, were used: five for the test solutions and one for the control solution. Each beaker had 100 mL solution. The concentrations of the test solutions were 1, 10, 100, and 1000 mg L^{-1} for SDM. A single medaka juvenile was transferred to each of the beakers. The test conditions were at a constant temperature of 25±1 ºC and a 16L: 8D cycle of 2 Klux illuminations similar to those for the cladocerans, and DO of 7.1±0.5 mg L^{-1}, and pH of 8.4±0.2. During the test period, there was no feeding, and death of the fish was checked daily. The handling of the fish was in accordance with national and institutional ethical guidelines for the protection of animal welfare.

Antibiotics and water quality analyses

For each of the tests, the concentrations of SDM were analyzed and the water qualities (temperatures, dissolved oxygen and pH) were recorded for both control and test solutions at the beginning and end of the test periods.

The antibiotic concentrations were measured with a high-performance liquid chromatography (HPLC). The mobile phase was consisted of a mixture, 2:3 vol vol^{-1}, of methanol (Merck, Darmstadt, Germany) and 25 mM sodium phosphate (Shimakyu's Pure Chemical, Osaka, Japan) at a flow rate of 1.0 ml min^{-1} and a temperature of 40 ºC. All detections were performed using UV absorption at 270 nm, compared to authentic standards [31]. Water Temperatures, dissolved oxygen and pH were recorded with a multi-function instrument (Lutron, Taipei, Taiwan).

Statistical tests

The E/LC50 values in each test were calculated with linear regression analysis of the test concentrations versus percentage of the test organism affected. Significant differences between the tests and control , as well as the concentration of SDM between start and end of test were determined with the Mann-Witney U test with a significantly level at $p<0.05$ (SPSS 20.0, 2012).

RESULTS AND DISCUSSION

Antibiotics concentrations

The SDM exhibited high chemical stability during the test periods. At the end of the tests, the average residues of SDM were 98%-99% of the initial study concentrations. Therefore, the E/LC50 in the toxicity tests for the SDM were obtained by the nominal concentrations since no significant degradation found during the experiment.

Individual toxicities

Toxicity to algae

SDM in this study was toxic and inhibited growth of the two species of microalgae. SDM substantially inhibited the growth of C. vulgaris at all treatment concentrations, including 15, 30, 60, 120, and 250 mg L-1, in the determination experiment (Fig. 1). SDM also inhibited the growth of I. galbana at all treatment concentrations: 10, 20, 50, 80, and 100 mg L-1 (Fig. 1). The 72-h EC50 values of SDM to C. vulgaris and I. galbana were 50.6 and 38.3 mg L-1, respectively (Table 1). The toxicity of SDM to both algae, EC50s of 40.7~59.9 mg L-1, can be classified as harmful to aquatic organisms and may cause long-term adverse effects in the aquatic environment which according to a range of acute toxicity to algae at 10~100 mg L-1 [32]. Additionally, some studies indicated that SDM had high toxic levels to a number of microalgae. For example, SDM exhibited EC50 values of 2.3 and 11.2 mg L-1 to Selenastrum capricornutum and C. vulgaris , respectively [19], and 9.9 mg L-1 to Scenedesmus vacuolatus [33]. The toxicity level varies with the two microalgae that used in the study. A few reports indicated that SDM showed higher toxicity effects to duckweeds than the microalgae. The 7-d EC50 to Lemna gibba and L. minor were 0.25 and 0.02 mg L-1, respectively [33,34], which were significantly lower than the EC50 to the microalgae in this study, 38.3 and 50.6 mg L-1 and in the other studies, 2.3~11.2 mg L-1. Therefore, a more complete evaluation of the toxicity effects to aquatic plants is required as using SDM in chemotherapy of aquatic animals.

Table 1: Median effective and lethal concentrations (E/LC_{50}, mg L^{-1}; mean and the 95% confidence intervals in parentheses) of SDM for five species of test aquatic organisms. in acute and chronic toxicity tests involving *Chlorella vulgaris*, *Isochrysis galbana*, *Daphnia magna*, *Daphnia similis* and *Oryzias latipes*.

Test organisms	Assessment endpoint	E/LC_{50} (95% CI)	N
Algae			
C. galbana	72-h EC_{50}, Growth	50.6 (39.1~64.4)	3
I. galbana	72-h EC_{50}, Growth	38.3 (33.2~44.2)	3
Cladocerans			
D. magna	48-h LC_{50}, Survival	180 (155~209)	4
	21-d EC_{50}, Reproduction	10.4	10
D. similis	48-h LC_{50}, Survival	427 (347-527)	4
	21-d EC_{50}, Reproduction	127.1	10
Fish			
O. latipes	96-h LC_{50}, Survival	> 1000	5

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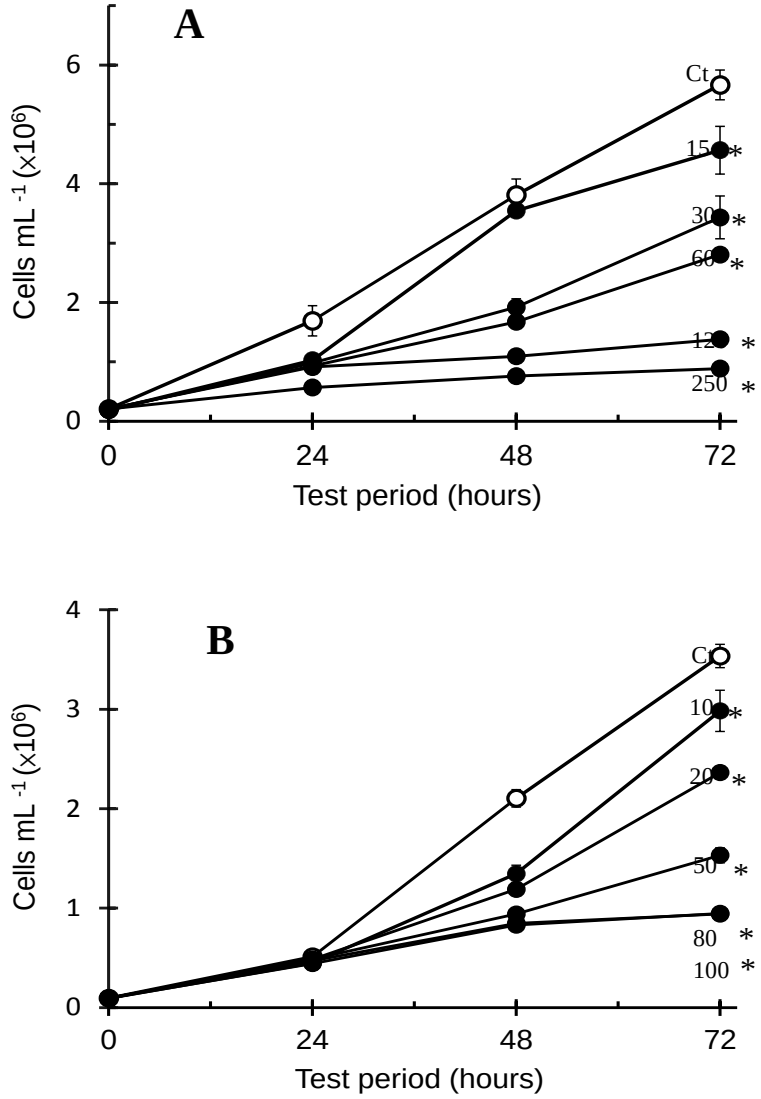

Figure 1: Growth curves of the algae *Chlorella vulgaris* (A) and *Isochrysis galbana* (B) in the control (open circles) and the five test solutions (solid circles, mg L^{-1}) of sulfadimethoxine (SDM) during the 72 hour test period (asterisks, significant difference from control).

Toxicity to cladocerans

In addition, SDM also had acute toxic effects to the D. magna and D. similis. The Figure 2 shows the survivals of two Daphnia during the 48-h test period. SDM substantially reduced the number of surviving neonates at concentrations of 200, 400, 800, and 1,000 mg L-1 to D. magna whereas no significant differences were found among concentrations of 100 mg L-1 and the control (Fig. 2). SDM also reduced the neonates of D. similis at concentrations of 400, 800, and 1000 mg L-1, and no differences found among 100, 200 mg L-1, and the control (Fig. 2). The 48-h LC50 values of SDM to D. magna and D. similis were 180 and 427 mg L-1, respectively (Table 1). Previous studies indicated that the LC50 of SDM to D. magna were 248-270 mg L^{-1} that are higher to the result obtained in the present study, 180 mg L^{-1} [35,36]. It might due to the water temperature in this study was kept at 25 ºC in average that higher than the temperature in the former studies that study temperature was 18~22 ºC according to the guideline of OECD [29]. The study temperature of 25 ºC for *D. magna* is acceptable by US EPA [37] and also in consist to the ambient temperature conditions. Moreover, the toxicity of SDM to *D. magna* in the present study is similar to the result for another zooplankton, *Moina macrocopa*, with LC_{50} 183.9 mg L^{-1} [36].

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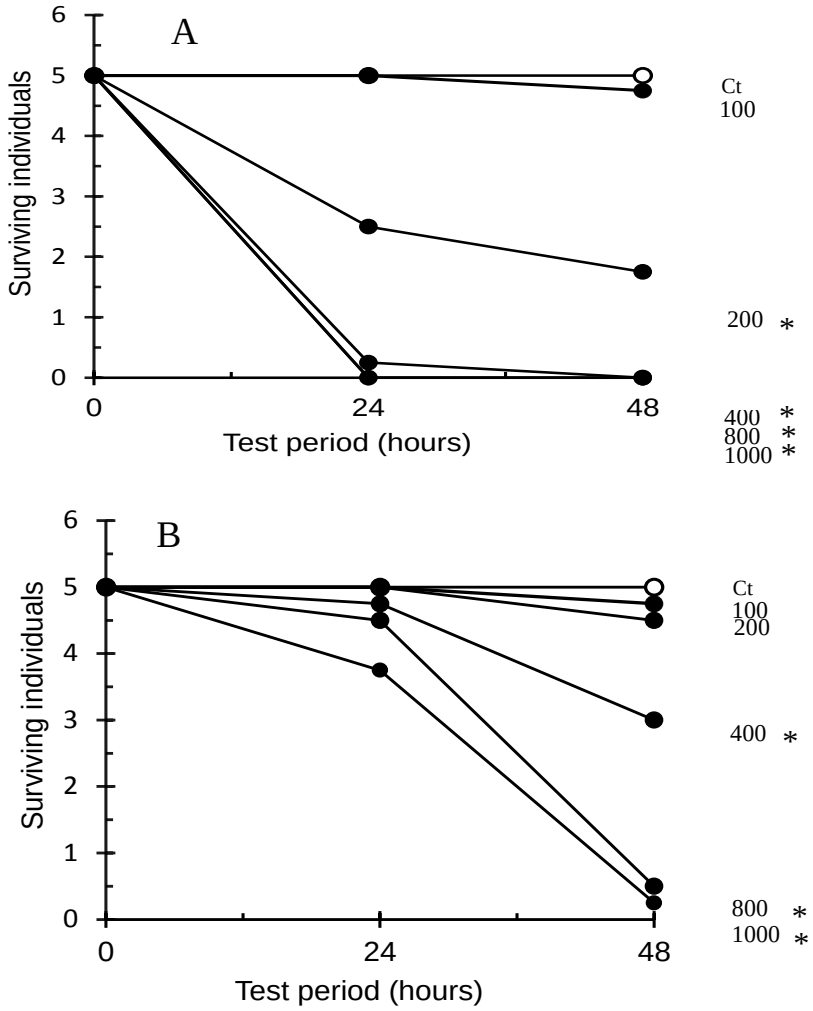

Figure 2: Numbers of the surviving individuals (mean±SD, n=4) of cladocerans *Daphnia magna* (A) and *D. similis* (B) in the control solution and the five test solutions of sulfadimethoxine (SDM) for the 48 hour test period (asterisks, significant difference from control).

SDM also showed chronic toxicities to the daphnids and significantly decreased the reproduction of the tested Daphnids. The reproduced neonates of D. magna and D. similis decreased in treatments that the concentrations of SDM higher than 12.5 and 200 mg L-1, respectively (Fig. 3). The EC50 of SDM to D. magna and D. similis were 10.4 and 127 mg L-1, respectively (Table 1). The chronic toxicities of SDM to the two Daphnids were much lower than the acute toxicities. It indicates that the released SDM at low concentrations might still upset the aquatic ecosystem even no directly acute toxicity observed. The other veterinary SAs were reported having higher toxicities to the Daphnid than the present study. For example, two SAs, sulfaquinoxaline and sulfaguanidine, were also found to inhibit the neonate reproduction of *D. magna* and the EC_{50} were of 3.47 and 0.87 mg L^{-1}, respectively [20]. In another study, the EC_{50} of sulfamethazine to reproduction of *D. magna* was 4.25 mg L^{-1} [35]. Up to our best knowledge, the present study is the first one reporting the chronic toxicity of SDM to Daphnids. SDM showed a lower chronic toxicity to *D. magna* than the other SAs. Furthermore, the *D. magna* had much higher sensitivities for both acute and chronic toxicity to SDM and is suitable for monitoring the toxic effect of SDM in water.

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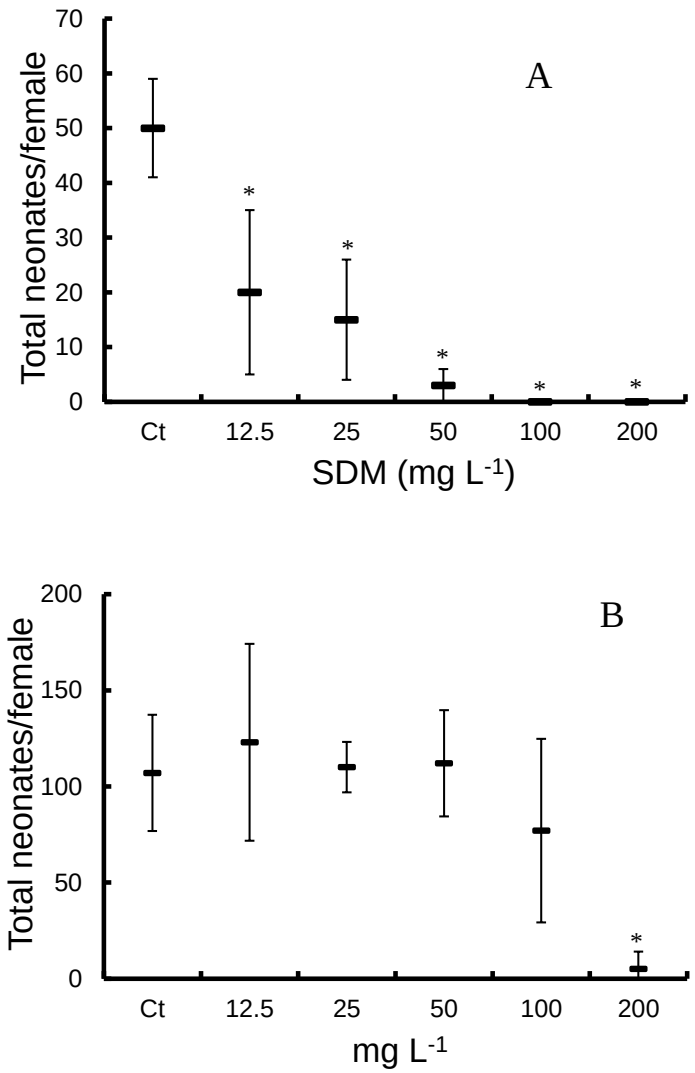

Figure 3: Average numbers of neonates (mean±SD, n=10) produced by female cladocerans *Daphnia magna* (A) and *D. similis* (B) in the control solution and the five test solutions of sulfadimethoxine (SDM) during the 21-d tests period (asterisks, significant difference from control).

Toxicity to fish

SDM exhibited no remarkable acute toxicity to the larvae of medaka fish at any of the tested dose in the present study. The 96-h LC_{50} values of SDM to *O. latipes* were > 1,000 mg L-1 (Table 1). Therefore, no further chronic toxicity study of the SAs was performed for the medaka fish. In another studies, the acute toxicities of SDM to medaka was also higher than 100 and 500 mg L^{-1} [36,38], suggesting that it had no significant acute toxicity to medaka and is consistent with the results of the present study.

Comparison of toxicity sensitivity

The aquatic organisms tested in this study showed various toxicity sensitivities to SDM. Results indicate that the two algae were more sensitive than the *Daphnia*, whereas medaka fish was the least sensitive to SDM. The EC_{50} values of the two algae *C. vulgaris* and *I.* galbana of SDM were 72% and 79% lower than the LC50 of D. magna, and 88% and 91% lower than the LC50 of D. magna (Table 1). In addition, a comparison of the two microalgae shows that I. galbana was more sensitive to SDM than C. vulgaris is, which had a 32% lower EC50 (Table 1). Additionally, D. magna showed higher sensitivities in either acute or chronic toxicity tests than D. similis. In acute and chronic toxicity tests, the LC50 of D. magna were 58% and 92% lower than that of D. similis for SDM. In summary, the results indicated that the microalgae can be superior bioindicator organisms than *Daphnia* and medaka for monitoring SDM contaminations.

In fact, the SA antibiotics were only considered potential micro-pollutants because they are usually present at low concentrations (in the ppb range or less) in the field aquatic environments [39] and being considered to be no effect to the aquatic populations at environmentally realistic concentrations [35]. However, the required concentrations inducing toxic effects on the microalgae and cladocera reported in this study may occur in exceptional conditions in the closed environment. For example, antibiotics may arrive in aquatic environments at higher concentrations from sources of contamination (e.g., animal manure, runoff from contaminated soils, aquaculture therapy in closed waters, overflow of treated aquaculture ponds, and domestic, industrial, and hospital effluents) [1,16,39]. Consequently, the use of SDM in aquaculture and by veterinarians could still be a potential source of pollution that affects aquatic environments of ponds and discharged areas. Because microalgae and cladoceran play basic roles in aquatic ecosystems, the results of this study suggest that future research should investigate the environmental effect of SDM after routine mass treatments of cultured animals and their chronic toxicity to aquatic organisms. This approach would allow a more exhaustive assessment of the potential environmental effects of SDM. [10,34,40][10,34,40][10,34,40][10,34,40][10,34,40]

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REFERENCES

[1] Sarmah AK, Meyer MT, Boxall ABA. Chemosphere 2006; 65: 725-759.
[2] Cabello FC. Environ Microbiol 2006; 8: 1137-1144.
[3] Baran W, Adamek E, Ziemiańska J, Sobczak A. J Hazard Mater 2011; 196: 1-15.
[4] Nikolaou A, Meric S, Fatta D. Anal Bioanal Chem 2007; 387: 1225-1234.
[5] Lai HT, Hou JH, Su CI, Chen CL. Ecotoxicol Environ Saf 2009; 72: 329-334.
[6] Kümmerer K. Chemosphere 2009; 75: 435-441.
[7] Sukul P, Spiteller M. Rev Environ Contam Toxicol 2006; 187: 67-101.
[8] Dietze JE, Scribner EA, Meyer MT, Kolpin DW. Int J Environ An Ch 2005; 85: 1141-1152.
[9] Le TX, Munekage Y. Mar Pollut Bull 2004; 49: 922-929.
[10] García-Galán MJ, Díaz-Cruz MS, Barceló D. Talanta 2010; 81: 355-366.
[11] Tamtam F, Mercier F, Le Bot B, Eurin J, Tuc Dinh Q, Clement M, Chevreuil M. Sci Total Environ 2008; 393: 84-95.
[12] Luo Y, Xu L, Rysz M, Wang Y, Zhang H, Alvarez PJJ. Environ Sci Technol 2011; 45: 1827-1833.
[13] Lin AY-C, Yu T-H, Lin C-F. Chemosphere 2008; 74: 131-141.
[14] Arthur JR, Lavilla-Pitogo CR, Subasinghe RP. Use of Chemicals in Aquaculture in Asia, in, Southeast Asian Fisheries Development Center, Tigbauan, Iloilo, Philippines, 2000.
[15] Okuda T, Yamashita N, Tanaka H, Matsukawa H, Tanabe K. Environ Int 2009; 35: 815-820.
[16] Zhao L, Dong YH, Wang H. Sci Total Environ 2010; 408: 1069-1075.
[17] García-Galán MJ, Garrido T, Fraile J, Ginebreda A, Díaz-Cruz MS, Barceló D. J Hydrol 2010; 383: 93-101.
[18] Choi K, Kim Y, Park J, Park CK, Kim M, Kim HS, Kim P. Sci Total Environ 2008; 405: 120-128.
[19] Eguchi K, Nagase H, Ozawa M, Endoh YS, Goto K, Hirata K, Miyamoto K, Yoshimura H. Chemosphere 2004; 57: 1733-1738.
[20] De Liguoro M, Di Leva V, Gallina G, Faccio E, Pinto G, Pollio A. Chemosphere 2010; 81: 788-793.
[21] Isidori M, Lavorgna M, Nardelli A, Pascarella L, Parrella A. Sci Total Environ 2005; 346: 87-98.
[22] Lin CL, Shyong WJ, Kuo SR, Chen SN. J Fish Soc Taiwan 1993; 20: 357-366.
[23] Spolaore P, Joannis-Cassan C, Duran E, Isambert A. J Biosci Bioeng 2006; 101: 87-96.
[24] OECD 202. Paris, France 2004.
[25] OECD 203. Paris, France 1992.
[26] OECD. Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, in, OECD Publishing, 2011.
[27] Walne PR. Culture of Bivalve Molluscs: fifty years experience at Conwy, in, Whitefriars Press Ltd., London, 1974, pp. 173.
[28] OECD. Test No. 211: Daphnia magna Reproduction Test. OECD Publishing, 2012.
[29] OECD. Test No. 202: Daphnia sp. Acute Immobilisation Test, in, OECD Publishing, 2004.
[30] OECD. Test No. 203: Fish, Acute Toxicity Test, in, OECD Publishing, 1992.
[31] Lai HT, Hou JH. Aquaculture 2008; 283: 50-55.

[32] Carlsson C, Johansson A-K, Alvan G, Bergman K, Kuhler T. Sci Total Environ 2006; 364: 67-87.
[33] Bialk-Bielinska A, Stolte S, Arning J, Uebers U, Böschen A, Stepnowski P, Matzke M. Chemosphere 2011; 85: 928-933.
[34] Brain RA, Johnson DJ, Richards SM, Sanderson H, Sibley PK, Solomon KR. Environ Toxicol Chem 2004; 23: 371-382.
[35] De Liguoro M, Fioretto B, Poltronieri C, Gallina G. Chemosphere 2009; 75: 1519-1524.
[36] Park S, Choi K. Ecotoxicology 2008; 17: 526-538.
[37] US EPA. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. 4th ed. ed., U.S. Environmental Protection Agency, EPA-821-R-02-012, 2002; 5th ed., 2002.
[38] Kim Y, Choi K, Jung J, Park S, Kim P-G, Park J. Environ Int 2007; 33: 370-375.
[39] Ferreira CSG, Nunes BA, Henriques-Almeida JMdM, Guilhermino L. Ecotoxicol Environ Saf 2007; 67: 452-458.
[40] Migliore L, Brambilla G, Grassitellis A, Dojmi di Delupis G. Int J Salt Lake Res 1993; 2: 141-152.