Occurrence of antibiotics and their impacts to primary productivity in fishponds around Tai Lake, China

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HIGHLIGHTS

- Fishponds were tested for the presence of antibiotics.
- Sulfonamides contributed the majority of the antibiotic burden.
- Detection varied with sample area, time point, and species.
- Florfenicol was of highest risk to algae.
- Total antibiotics were detrimental to algae growth.

ABSTRACT

Antibiotics are widely used to improve the health and yields of farmed animals, including fish, but their use is accompanied by undesirable ecological effects. Relatively little is known about the water-body burden of antibiotics and their influence on primary productivity in aquaculture ecosystem. In this study, antibiotics usage within 24 fishponds, covering 4 areas, sampled 5 times, and having 5 fish species, was investigated surrounding Tai Lake in China. The study analyzed 15 antibiotics (including 5 sulfonamides, 2 quinolones, 3 β-lactams, 3 tetracyclines, 1 amphenicol, and 1 macrolide), and all of them were detected in water samples, with a detection frequency of 260%. Sulfonamides were the most prevalent, and concentrations of sulfamethoxazole, sulfamonomethoxine, and florfenicol being over 2000 ng L⁻¹ in some samples, while the other antibiotics levels ranged from ND (no detection) to 551.18 ng L⁻¹. Significant differences were observed in antibiotic burden among different regions for total antibiotics, sulfonamides, quinolones, and amphenicols; among time points for quinolones, β-lactams, and tetracyclines; and among species for quinolones and macrolides. Furthermore, basing on the risk quotient (RQ) method, the assessment revealed that florfenicol was of highest risk to algae with RQ values exceeding 0.1, while macrolide erythromycin posed the second highest risk. The partial correlation coefficient between total antibiotics and chlorophyll (a) was −0.035 that clearly indicated total antibiotics were detrimental to green algae growth, while the nutrient input and other physical — chemical factors...
were much more beneficial. Overall, holistic far-reaching measures of antibiotics control are recommended to preserve aquaculture ecosystem health.

1. Introduction

China has invested heavily in aquaculture technologies and is the largest producer of farmed fish worldwide (Naylor et al., 2000; Fishery Bureau, 2014). Significant input is needed to sustain high-density aquaculture, including feed, modifier, organic fertilizer, and pharmaceuticals such as antibiotics (Bosma and Verdegem, 2011) which help to prevent diverse bacterial diseases (Heuer et al., 2009). Much attention has been paid to the increased usage of antibiotics in aquaculture ecosystem (Seyfried et al., 2010; Xiong et al., 2015). According to the comprehensive evaluation of antibiotics emission and fate in the river basins of China, farming of food animals, including fish, was responsible for 13,700 tons in 2013, which was more than 80% of total antibiotics (Zhang et al., 2015).

Similar to salmon fish culture in Chile, medicated feed and direct splashing are the main methods of antibiotics administration in China (Shah et al., 2014). Digested or not digested antibiotics would end up in surrounding aquaculture environment (Cabello et al., 2013). Antibiotics burden in water would ultimately be a potential ecological risk to aquaculture ecosystems, such as inhibiting primary productivity that biogeochemical functioning of aquatic ecosystem relies on (Andrieu et al., 2015). Ecosystem services such as nitrogen assimilation, oxidation of organic matter might be hindered due to the loss of primary productivity (Celine and Anniet, 2015). However, recent researches were not well explored characteristic of antibiotics usage in aquaculture environment in China (Chen et al., 2015).

Primary productivity in fishponds is comprised of diverse phytoplankton, especially green algae, such as genus Chlorella, Scenedesmus, and Chlamydomonas, that produce dissolved oxygen by photosynthesis (Watson et al., 2014). Before raising filter feeding fish in ponds, green algae should be cultured as an additional feed to promote fish growth, and enhance the fish production (Chiu et al., 2013). Chlorophyll (a) is a crucial determinant for green algae growth correlates, such as biomass, diversity (Gilbert and Allen, 1972). The growth of green algae is equated to the assimilation of nutrient simultaneously (Hargreaves, 1998). Organic nitrogen input in the form of feed is inessancy for aquaculture (Olajiwola, 2015). Nitrogen cycling plays a pivotal role in metabolizing organic nitrogen into ammonia and nitrate, which can be absorbed by phytoplankton, e.g. green algae in fishpond (Hargreaves, 1998). The biodiversity of green algae facilitates to sustain the balance of aquatic ecosystem. However, nitrogen assimilation may be inhibited by the acute or chronic toxicological effects of antibiotics on green algae (Liu et al., 2011), however, the extent antibiotics influence the growth of the green algae in fishponds has remained poorly understood.

In the present study, we investigated the occurrence of antibiotics use in fishponds surrounding the Tai Lake in China, and checked if the antibiotics burden in surface water varied with some potential factors, such as sampling region, time and fish species raised in the system. Finally, we evaluated their impacts to green algae growth. The results would provide an in-depth new insight to take holistic measures to preserve aquaculture ecosystem.

2. Materials and methods

2.1. Fishponds

Fishponds surrounding Tai Lake (located in the middle and lower reaches of the Yangtze river region) were selected for sampling, since there has the largest fishpond area (Fishery Bureau, 2014), which was characterized by moderate climate and geography (Fig. 1) in China.

Four geographically distinct regions were sampled around Tai Lake from May to October in 2015: Changzhou city (CZ), having two bream ponds and two shrimp/crab ponds, Yixing city (YX), with two bream ponds and four shrimp/crab ponds, Huzhou city (HZ), two perch ponds and four shrimp/crab ponds, while Suzhou city (SZ), had two perch ponds, two Prawn ponds, and four shrimp/crab ponds (Fig. 1). In total, 24 fishponds, 5 fish species, and 5 sampling times across the entire aquaculture seasonal period were covered. Fish farmers in the above areas preferred polyculture of shrimp and crab to increase their incomes from each pond. Hence, the two species were combined in our experiments. Full sampling information is included in Table S1.

2.2. Chemicals

According to specifications and experience for antibiotics usage in aquaculture (Ministry of Agriculture, 2007a; Ministry of Agriculture, 2007b; Ministry of Agriculture, 2007c), 15 antibiotics were selected, including 5 sulfonamides (sulfadiazine, SDZ; sulfamethoxazole, SMX; sulfachloropyridazine, SCP; sulfamonomethoxime, SMM; sulfaminothiazine, SQX), 2 quinolones (norfloxacin, NOR; enrofloxacin, ENR), 3 β-lactams (cefalexin, CEA; cefadine, CER; cefotaxime, CEO), 3 tetracyclines (tetracycline, TC; oxytetracycline, OTC; chlorotetracycline, CTE), 1 amphenicols (florfenicol, FF), and 1 macrolides (erythromycin, ERY).

All antibiotic standards and internal standards (Sulfamethoxazole-d4, norfloxacin-d5, demeclocycline, and roxithromycin-d7) (Wang et al., 2015) were over 98% pure, and purchased from Dr. Ehrenstorfer (GmbH, Germany). Methanol and acetonitrile were chromatography – grade, and purchased from Merck (Germany). Antibiotic standards were dissolved in methanol and stored at –20 °C.

2.3. Sample collection, treatment, and analysis

Water samples 20 cm below surface were collected in reference to “five - sites method (water blended from five sites in a single pond)” (Ministry of Environmental protection, 2002), and immediately transported to the laboratory. Prior to extraction, samples were pre-filtered through a 0.45 μm glass fibre filter (Anpu, China) into a 47 mm stainless steel filter holder (Jingteng, China). Approximately 200 mL of filtered water was used for measuring total nitrogen and phosphorus, and 2 L water were acidified to pH 4 with 100 μM citrate buffer, while the antibiotics were extracted by solid phase extraction (SPE). The filtered residue was used to measure chlorophyll a (Chl a) as an indicator of the total biomass of green algae (Ministry of Environmental protection, 2002) and the pH and temperature were recorded at the time of sample collection.
Antibiotics extraction was conducted as previously described (Wang et al., 2015) using hydrophilic – lipophilic balance (HLB) SPE cartridges (500 mg/6 mL, Anpu, China) that were pre-conditioned with 6 mL methanol and 10 mL ultrapure water before sample loading. Samples were spiked with internal standards and the flow rate for loading ranged from 5 to 10 mL per minute. After loading, analytes were eluted from cartridges with 10 mL methanol concentrated to 1 mL by nitrogen gas evaporation prior to instrumental analysis.

An ultra performance liquid chromatography tandem mass spectrometry (LC-MS/MS, Agilent 6420, USA) was used to analyze the 15 selected antibiotics. All compounds were separated using a Luna C18 (2) HST column (100 mm × 2.0 mm, i.d. = 2.5 μm). The limit of quantification (LOQ) was defined as the concentrations corresponding to the signal/noise of 10. The LOQ and recoveries of antibiotics in water ranged from 0.06 to 1.53 ng L\(^{-1}\), and 63.1–123.4% respectively. Details of the elution condition and retention time are included in Tables S2 and S3.

2.4. Statistical analysis

Six antibiotic categories (sulfonamides, quinolones, \(\beta\)-lactams, tetracyclines, amphenicols, and macrolides) were generated by dividing 15 selected antibiotics in terms of antibacterial mechanism. The new category “all or total antibiotics” represents all 15 selected antibiotics.

Descriptive analysis of 15 antibiotics contents and 7 categories including mean, median, maximum and several percentiles, were performed. When calculating these statistical variables, Categories were the sum of respective represented antibiotics. The detection frequencies of antibiotics and categories were subsequently calculated. Categories meant represented antibiotics detected at least once.

The detection frequencies of antibiotic categories were also calculated by sampling area, time and fish species. Logistic regression model was used to estimate the association of antibiotic burden with the above contributing factors.
The risk quotient (RQ) was calculated to evaluate the potential risk of antibiotic burden and its correlation with adverse environmental effects (such as inhibition of algae growth) for each selected antibiotic. RQ was calculated using Equation (1).

$$RQ = \frac{MEC}{PNEC}$$

where MEC was the measured environmental concentration, and PNEC was the predicted no-effect concentration that is obtained from the ECOSAR model (US Environmental Protection Agency, 2011) and further literature (Gao et al., 2013; Xu et al., 2013; Yang et al., 2013).

Pathway analysis was performed based on AMOS structural equation modelling (Hodapp et al., 2015) to calculate the effects of environmental factors on algae growth. The environmental factors considered included: antibiotics content, nutrients (total nitrogen and phosphorus), climate (temperature at different time points), and physicochemical parameters (e.g. pH) (Table S4). A correlation matrix between all environmental factors was derived based on Pearson’s correlation coefficient. A partial correlation coefficient (PCC) was calculated by comparing the fit of models with the matrix using chi-square tests. PCC represented the direct dependencies between environmental factors and algae growth. The positive value of PCC indicated favourable effect to algae growth; conversely, the negative value indicated adverse effect.

The statistical software JMP (Version 7.0; SAS, Inc) was used to perform all statistical analysis, and a p-value of 0.05 was considered to be statistically significant.

3. Results

3.1. Descriptive analysis of selected antibiotics

Detection frequencies for the 15 selected antibiotics ranged between 2 and 60% (Table 1), and the overall detection frequency of total antibiotics was 84%. The order of the detection frequencies of antibiotic categories was revealed as: sulfonamides > macrolides > β-lactams > quinolones = tetracyclines > amphenicols, with values ranging between 8 and 81%. SCP and SMM, with detection frequencies of 60% and 45% respectively, were the predominant sulfonamides, and made the biggest contribution to total antibiotics. Together with three other sulfonamides and macrolide ERY, the six antibiotics were detected in more than 10% of all samples.

The concentration of total antibiotics ranged from the LOQ to 10,848 ng L⁻¹. Of antibiotic categories (Table 1), their maximum concentrations increased in the order of macrolides (48.80 ng L⁻¹) < quinolones (210.67 ng L⁻¹) < β-lactams (361.74 ng L⁻¹) < tetracyclines (551.18 ng L⁻¹) < amphenicols (2708.6 ng L⁻¹) < sulfonamides (10,242 ng L⁻¹). SMX, SMM, and FF were present at high concentrations (>2000 ng L⁻¹). Overall, sulfonamides category contributed the most among all antibiotics.

3.2. Antibiotic burden in fishponds around Tai lake

Logistic regression was used to correlate different antibiotic categories (based on antimicrobial mechanism) with sampling region, time and fish species raised in the system, to determine the antibiotic burden (Fig. 2). Significant sampling region differences were observed for total antibiotics (p = 0.009), sulfonamides (p = 0.0009), quinolones (p = 0.018), and amphenicols (p = 0.0003) adjusted for fish species and sampling time. The detection frequency of total antibiotics (57.1%), sulfonamides (46.4%), and quinolones (0%) were lowest in the Huzhou, a city to the south of Tai lake belonging to Zhejiang Province. The detection frequency of amphenicols (60%) was highest in Changzhou City (Fig. 2a). Regarding sampling time, the detection frequencies of quinolones (31.6%), β-lactams (42.1%), and tetracyclines (42.1%) were highest in June, and significant sampling time differences were found for these categories (p = 0.015, 0.0001 and 0.0003 respectively) adjusted for sampling regions and fish species (Fig. 2b). Adjusted for sampling region and time, significant fish species differences were found for quinolones (p = 0.009) and macrolides (p = 0.034). The detection frequency of quinolones (10%) was highest in prawn ponds, and macrolides ERY was only detected in Shrimp/ Crab ponds (Fig. 2c).
Fig. 2. Detection frequencies (%) of antibiotic categories with the association of sampling region, time and fish species raised in the system. (a) Differences with region adjusted for sampling time and fish species; (b) Differences with time adjusted for region and fish species; (c) Differences with fish species adjusted for sampling region and time. The symbols ‘*’ meant that significant detection frequency differences were observed in the category.
3.3. Potential impact of antibiotics on algae growth

Antibiotics may pose an acute or chronic toxicological threat to phytoplankton by inhibiting the growth of green algae, which can subsequently have adverse effects on biogeochemical processes in fishponds and other environments (Gonzalez-Pleiter et al., 2013; Yasser and Adli, 2015). We calculated PNEC values for different antibiotics classes using ECOSAR modelling or from literature values (Gao et al., 2013; Xu et al., 2013; Yang et al., 2013), and the revealed abundance order was macrolides < quinolones < tetracyclines = amphenicols < sulfonamides < β-lactams (Fig. 3). Macrolides ERY was the most detrimental for algae growth, followed by quinolones.

Together with the measured environmental concentration (MEC), individual RQ values were determined for the 15 selected antibiotics. An RQ value of more than 1 indicated a high risk of exposure to antibiotic burden, whereas a value of 0.1–1 and 0.01–0.1 indicated a medium and low risk, respectively (Lee et al., 2008; Yan et al., 2013), and the value below 0.01 indicated minimal or no risk to target organisms such as green algae (Fig. 4).

Among all antibiotics screened, the majority (8 out of 15 including all β-lactams, 4 sulfonamides, and 1 tetracycline) posed little or no risk to the growth of algae. SMX, TC, CTE and ERY posed a low risk, and FF posed a medium risk due to its high concentration and medium PNEC (Fig. 4).

The results of pathway analysis showed that nutrients (total nitrogen and phosphorus), climate (temperature), and physicochemical factor (pH) all promoted the growth of algae (Fig. 5). Total nitrogen and total phosphorus made major contributions with a PCC value of 0.303 and 0.424 respectively. Evidently, nutrient input is clearly the most crucial factor affecting the growth of algae, but total antibiotics posed an adverse effect on algal growth with a PCC value of −0.035. However, how antibiotics influence phytoplankton community was not explored in this study.

4. Discussion

In this study, 15 antibiotics were investigated, covering six different categories. The results showed that antibiotics were abundant in fishponds surrounding Tai Lake, which is unsurprising since they are used extensively as therapeutic tool to prevent bacterial diseases among aquaculture species. Sulfonamides were the most abundant category, and contributed the majority of the antibiotic burden in fishponds. Comparing with seawater fishponds (Chen et al., 2015), antibiotics were widely detected in water samples with the levels in the range of ND to 16,000 ng L⁻¹, tetracyclines were the predominant category. Whereas, in other aquaculture areas such as Thailand (Rico et al., 2014) and Vietnam (Andrieu et al., 2015), OTC and ENR are commonly used, the peak water concentration of which was 49,000 and 16,000 ng L⁻¹ respectively. It is well recognized that antibiotics have been absolutely necessary drugs in preventing disease in aquaculture. The differences might be due to their different usage among these regions. Comparing the levels in natural rivers, for example, in the Yangtze river, the categories investigated here were present in 100% of water samples (Yan et al., 2013), the levels were in the range of ND to 89.1 ng L⁻¹. National comparison of antibiotic concentrations in surface water indicated that the water burden in Pearl River was highest among all major rivers in China, the pear concentration was found in roxithromycin with the value of 2,260 ng L⁻¹ (Yan et al., 2013; Tang et al., 2015). The antibiotic concentrations were lower than that in fishponds, owing to direct usage in aquaculture.

The water burden of antibiotics in the environment has attracted
environmental side-effects, such as, the spread of antibiotic resistance genes. Evidences were provided by recent researches, for example, the levels of tetracycline residues were strongly correlated with absolute tet gene copies (Wu et al., 2010). This significant effect could be extended to other antibiotic categories. The number of antibiotic-resistant E. coli was strongly correlated with the concentrations of all detected antibiotics (tetracyclines, sulfonamides and quinolones) (Zhang et al., 2014).

In recent decades, the Chinese government has published several specifications for the application of antibiotic categories in aquaculture (Ministry of Agriculture, 2007a; Ministry of Agriculture, 2007b; Ministry of Agriculture, 2007c), and announced the maximum residue limit (abbreviated MRL, for example, 100 μg kg⁻¹ wet weight for total sulfonamides) for antibiotics to ensure levels are safe for food animals and aquatic products that are destined for human consumption (Ministry of Agriculture, 1999). Sulfonamides are particularly widely used generating sulfonamide residues in muscle tissue from some fish species inevitably exceeding the relative MRLs (our unpublished report). Authorities have attempted to ban several quinolones antibiotics from food production due to severe cross resistance in antibiotic classes. Indeed, exported tilapia fillets were returned because of high levels of sulfonamide residues that exceeded the MRLs of the EPA, even though they were below the guidelines announced by the agriculture ministry of China (Ministry of Agriculture, 1999).

In general, other than direct splashing, medicated feed is the main route through which antibiotics from fishponds reach the wider environment (Andrieu et al., 2015; Chen et al., 2015). To date, medicated feeds are mainly used to promote fish growth mainly, and prevent bacterial diseases secondly, it is called non-therapy usage at low dosage. Conversely, therapy usage at high dosage is to cure disease by medicated feed, sometimes direct splashing, and it is site specific. Non – therapy usage accounts for the most majority of the total antibiotic production in comparison with therapy usage (He et al., 2011). In this study, a high incidence of antibiotics does not always equate to high concentrations. For example, the detection frequency of SCP (60%) was higher than that of FF (8%), but the concentration of FF ranged from 199.2 to 2708.6 ng L⁻¹, while that of SCP was considerably lower at 1.2–328.08 ng L⁻¹. Thus, we concluded that the usage of SCP was preferred to medicated feed, and FF was preferred to direct splashing.

The distribution of antibiotics varied with sampling region, time and fish species. In terms of sampling region, the city of Huzhou in Zhejiang Province had a lower burden of total antibiotics (57.1%) than the three regions in Jiangsu Province, and levels of sulfonamides (46.4%) and quinolones (0%) were particularly low. This may be attributed to different habits, and aquaculture practices in different provinces (Aquaculture Station-Zhejiang, 2010). Significant differences in sampling event were apparent. The burden of quinolones, β-lactams, and tetracyclines were significantly heavy in June (during the early and intermediate aquaculture season), possibly due to therapeutic strategies to prevent fish diseases (Shah et al., 2014). The detection frequency of quinolones was highest in Prawn ponds, possibly because it is susceptible to bacterial diseases when farmed in fishponds (Ji et al., 2011). Interestingly, the study revealed significant species specific differences for Macrolides, a category only detected in shrimp/crab ponds, due to abundances of algae bloom usually occurring in shrimp/crab ponds, hypoxia caused by high density phytoplankton would influence fish growth (Hans and Timothy, 2013). Macrolides ERY is highly toxic to algae (Yang et al., 2013) and is splashed directly to water to control algal blooms, that often occur during high temperature periods in shrimp/crab ponds. ERY levels also peaked in August (Paerl and Otten, 2013).

Antibiotics not only cause toxic effects such as oxidative stress (Nie et al., 2013) and immune effects in aquatic animals (Wollenberger et al., 2000; Gust et al., 2012; Marx et al., 2015), but also inhibit the growth of phytoplankton and cause phytotoxiceffects to a diverse array of algal species (Gonzalez-Pleiter et al., 2013; Yasser and Adli, 2015). The results of this study showed that, although macrolides and quinolones had the lowest PNEC values against algae, FF posed the highest risk, with an RQ value exceeding 0.1, and ERY posed the next highest threat. This may be attributed to direct splashing into water bodies (Ferreira et al., 2007), resulting in
close high concentrations. Overall, total antibiotics did not influence the growth of algae in fishponds to a large extent, compared with nutrients and other important factors. However, the influence of different classes of antibiotics on phytoplankton structure are not well understood, and neither is their influence on biogeochemical processes or artificial ecological systems (Roose-Amsaleg and Laverman, 2016). These points should be addressed in future studies aimed at understanding the eco-environmental effects of aquaculture inputs on fishpond ecology.

5. Conclusions

In this study, the occurrence and distribution of antibiotics in fishponds was investigated, and antibiotic presence varied with sampling region, time, and fish species raised in the system due to different usage. The results of risk assessment based on RQ values and pathway analysis showed that all of antibiotics tested, FQ posed the highest risk to algae. Antibiotics were detrimental to the growth of algae in fishponds, but their influence was minor compared with nutrients and other factors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.07.096.

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