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Research on Florfenicol Residue in Coastal Area of Dalian (Northern China) and Analysis of Functional Diversity of the Microbial Community in Marine Sediment

Humin Zong · Deyi Ma · Juying Wang · Jiangtao Hu

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Abstract An analytical method based on high performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) has been developed to investigate florfenicol residues. Among 11 stations, florfenicol was detected in six water samples. The concentrations of florfenicol in the six samples were 64.2 μg L^{-1}, 390.6 μg L^{-1}, 1.1 × 10^4 μg L^{-1}, 29.8 μg L^{-1}, 61.6 μg L^{-1}, 34.9 μg L^{-1}, respectively. These results showed that high levels of florfenicol were observed in water samples collected from stations influenced by aquaculture discharges. However, no florfenicol residue was detected in the sediment samples. Furthermore, the functional diversities of microbial community in four marine sediment samples were analyzed by Biolog microplate. For the sediment samples from the stations where antibacterials had been used, the functional diversity of microbial community was much lower than those from the stations where antibacterials were not used.

Keywords Florfenicol · HPLC–MS/MS · Sediment · Functional diversity

Antibacterials are commonly used in aquaculture, both prophylactically and to treat disease. The undegraded antibacterials would be directly released into the environment (Rigos et al. 2004). The potential pressure on marine environment and ecosystem is of great concern in the last years, for antibiotics are suspicious environmental contaminants as they are biologically active, which obviously is a part of their nature (Wollenberger et al. 2000). Florfenicol, an antibacterial especially developed for veterinary, is used in many countries, because of its high efficacy in the control of bacterial infections in fish (Samuelsen and Bergh 2004). However, florfenicol as biologically active substance, its pollution of the marine environment in the vicinity of fish farms can have adverse ecological effects, including development of resistant bacterial populations and potential harmful effects on the aquatic organisms. Miranda and Zemelmanb (2002) reported that a high number of bacteria resistant to florfenicol was found in 4 Chilean salmon farms as florfenicol is the second largest antibacterial used in Chilean salmon farms. Our previous research showed that florfenicol resistance of heterotrophic bacteria developed in the areas near to aquaculture farms which used florfenicol before (Zong et al. 2008). Adverse effects of florfenciol on the aquatic organisms were also found by other authors (Lundén et al. 2002; Lai et al. 2009). Based on above, the environmental issues associated with florfenicol are rather neglected.

Since sediments play an important role in marine environment, the environmental impacts of antibacterials in sediments have been studied by many authors (Ma et al. 2006; Matyar et al. 2008). Microorganisms in natural habitats are sensitive to pollutants and the microbial assays could be utilized as the initial screening system to identify those abiotic factors that influence most the toxicity of different chemical pollutants (Babich and Stotzky 1983). Thus, the aim of the present study was to investigate the florfenicol residues in coastal area of Dalian and evaluate the effects of florfenicol on the functional diversities of microbial community in marine sediments.

H. Zong · D. Ma (✉) · J. Wang
Coastal Ecology Key Laboratory, National Marine Environmental Monitoring Center, 116023 Dalian, China
e-mail: nddk980610@hotmail.com

J. Hu
Testing Center of Food Quality, Ministry of Agriculture, 154007 Jia Mu Si, China

Materials and Methods

All environmental samples were collected during July to October, 2007, from Dalian Bay (Northern China) (Fig. 1). All samples were from the coastal seawater near the aquaculture farms (about 500 m). All water samples were collected in duplicate by grab sampling in precleaned 1 L amber glass bottles with polypropylene open-top screw caps and Teflon-silicone septa. The sampling containers were completely filled to eliminate any headspace. Surface sediment samples were collected with an Ekman grab and stored in precleaned black polyethylene bag. All sampling personnel wore latex gloves when collecting samples, and changed gloves between sites. After collection, the samples were labeled, wrapped with Parafilm, and immediately chilled. Water samples were filtered through 0.45 μm cellulose nitrate membranes and all samples were extracted and determined immediately after the sampling finished (Ye et al. 2007).

Water samples were prepared by liquid–liquid extraction. A volume of 80 mL ethyl acetate was added into 1,000 mL water sample, well mixed and allowed to stand for 5 min. Then, the supernatant was transferred to a flask. The analyte was eluted twice. The supernatants were combined and evaporated to dryness in a water bath (60°C) under a gentle stream of nitrogen. The dried residue was reconstituted in 1 mL of 50% methanol solution and the solution was filtered with 0.2 μm syringe filter before analysis with LC–MS/MS.

Sediment sample (20 g) was accurately weighed into a centrifuge tube. 20 mL ethyl acetate was added, vortexed and centrifuged for 10 min at 3,000 rpm. The supernatant was transferred to a flask. The sample was extracted twice. The supernatants were combined and evaporated to dryness in a water bath (60°C) under a gentle stream of nitrogen. The dried residue was reconstituted in 1 mL of 50% methanol solution and the solution was filtered with 0.2 μm syringe filter before analysis with LC–MS/MS.

LC experiments were conducted using a Finnigan Surveyor HPLC system (Thermo Electron, San Jose, USA). The separation was performed on a Shiseido C18 column with gradient elution using methanol (solvent A) and water containing 0.1% acetic acid and 10 mmol L^{-1} ammonium acetate (solvent B). The percent of A mobile phase was 10% from 0.0 min to 2.0 min and from 2.0 min to 4.0 min, it varied from 10% to 90%. After 1 min held at 90%, A switched to 10% at 6.0 min. At last, the column was recycled to 10% A and equilibrated for 6 min. The flow rate during the gradient separation was 0.25 mL min^{-1}.The LC column and autosampler temperature was set at 20°C and 10°C, respectively. The injection volume for all LC experiments was 10 μL. The entire LC effluent from the sample injections was directed to the mass spectrometer.

The system utilized was a triple stage quadrupole mass spectrometer with ESI (Thermo Finnigan TSQ Quantum Discovery MAX). The spectral acquisition was done in the negative electron spray ionization utilizing multiple reaction monitoring of three parent- daughter ion pairs: m/z 356/185, 356/219 and 356/336. The ion pair of m/z 356/336 was used for quantitation. Typical MS settings were: spray voltage 3,500 V, sheath gas pressure 49 arbitrary units, auxiliary gas pressure 22 arbitrary units, ion transfer capillary temperatures 320°C, tube lens offset −118 V,

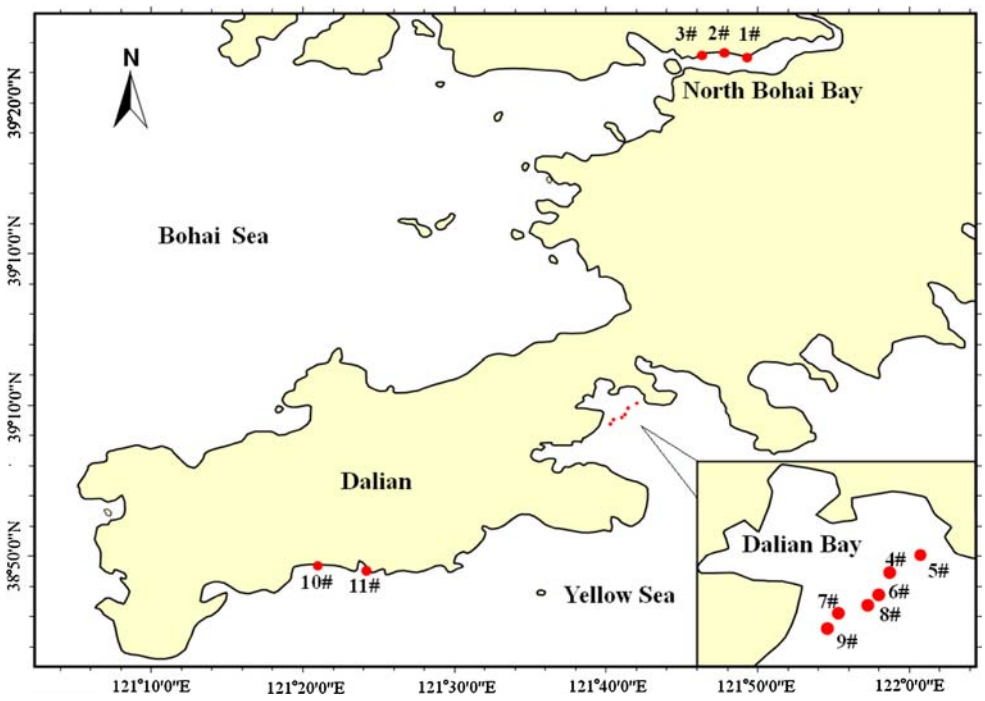

Fig. 1 Map of Dalian showing the locations

source CID Offset 12 V. The mass spectrometer selectivity was regulated by setting Q1 and Q3 resolution to a peak width of 0.7 Da fullwidth half maximum (FWHM).

Water collected from the area not contaminated fortified at nominal concentrations of 0.1, 1, 10, and 100 μg L^{-1} florfenicol. One control and three fortified samples were analyzed for florfenicol to determine accuracy and precision. The method accuracy was calculated as the percent of analyte recovered in the extract from the fortified samples. The precision of the method was calculated as the percent relative standard deviation (%RSD) for each fortification level. Sediments from not contaminated area were treated the same as the water samples to validate the method.

Four of the environmental sediment samples from four different stations were selected to analyze functional diversity of microbial community. The four samples were collected at stations where were clay mineralogy (similar physical and chemical properties) and 2 m depth. Station 1 and 3 were sea cucumber farm and nursery ground where florfenicol was employed before. Station 5 was prawn farm where florfenicol had not been applied while other antibacterials such as oxytetracycline, penicillin were used. Station 11 was a natural aquaculture farm where no antibacterials had been employed.

Functional diversity of microbial community in sediment sample was estimated by BIOLOG GN microplate method. There were three replicates for each sample. Sediment suspensions were prepared by suspending 10 g of sediment in 90 mL of sterile seawater. 150 μL of aliquots was pipetted to each well in the 96 wells of BIOLOG GN microplate which was prewarmed to 25°C. The plates were incubated at 25°C, and the absorbance reading at 590 nm after incubation of 0, 24, 48, 72, 96 and 120 h was determined periodically by using the microplate spectrophotometer. Overall color development in Biolog plates was expressed as average well color development (AWCD) and it was calculated according to Garland and Mills (1991), using the formula: $\mathrm{AWCD} = \frac{\sum (C-R)}{95}$, Where C is the absorbance of each, R is the absorbance of the control well. The functional diversity index of the sediment microbial flora was calculated according to the described method of Lin et al. (2007). Each parameter was calculated by using the following formulas: Shannon index $H' = -\sum (P_i \times \ln P_i)$; Shannon evenness $E = \frac{H'}{\ln S}$; McIntosh index $U = \sqrt{(\sum n_i^2)}$; McIntosh evenness $E = \frac{N-U}{N-N/\sqrt{S}}$. P_i is the ratio between the relative absorbance (C–R) of well i and the sum of the relative absorbance of the whole microplate; S is the number of wells with color changes; n_i is the relative absorbance (C–R) of well i; N is the sum of the absorbance of the whole microplate.

All the statistical analyses were performed with the statistical package SPSS 12.01. Treatment means were compared by means of one-way analysis of variance performed separately on each date of sampling. The comparisons were performed at set significance level of alpha = 0.05.

Results and Discussion

The recoveries of the method in water ranged from 82.2 to 101.4% with precision (%RSD) varying from 2.2% to 5.1% for all fortification levels. The calibration was highly correlated ($r = 0.9997$) and analysis of residuals between observed and predicted calibration standard values indicated uniform scatter and a good fit of the equation to the data. The limit of detection was influenced by sensitivity of the equipment and strongly dependent on the sample matrix, and it was 0.01 μg L^{-1} determined by extrapolating to an S/N of 3. The recoveries of the method in sediment ranged from 83.9% to 93.2% with RSDs varying from 2.4% to 7.8%. The correlation coefficient was $r = 0.9967$. The limit of detection was 0.1 μg kg^{-1} determined by extrapolating to an S/N of 3.

Florfenicol is widely used in marine aquaculture farms all over the world especially in the developing countries. However, Kim et al. (2008) prioritized veterinary pharmaceuticals in Korea by their usage, potential to enter the environment, and toxicological hazard. Twenty compounds were identified in the top priority class, most of which were antibiotics. Among the compounds, eight were identified as deserving more immediate attention, including florfenicol. And florfenicol also was found in the 18 top-priority veterinary pharmaceuticals list of Boxall et al. (2005). Using the methods of HPLC–MS/MS, environmental samples were detected. Among 11 stations, florfenicol was detected in water samples from stations 1#, 2#, 3#, 7#, 9# and 10#. The concentrations of florfenicol in the six water samples were 64.2, 390.6 μg L^{-1}, 1.1 × 10^4, 29.8, 61.6, 34.9 μg L^{-1}, respectively. The result showed that the frequently use of florfenicol in the marine aquaculture farms had already led to florfenicol residue in the marine environment and higher concentrations (390.6 and 1.1 × 10^4 μg L^{-1}) were even found in seawater.

Florfenicol has not detected in the sediment samples. This is mainly due to its short term half-life and adsorption character in the sediment. Florfenicol decreased rapidly in the sediment with a half-life of 1.7 days (Hu et al. 2007). The adsorption coefficient (Kf) of a compound is closely related to the octanol–water partition coefficient (Kow) and they were related by the log–log relationship $LgKf = aLgKow + b$ (Briggs 1981). It showed that compound with low Kow would not be expected to accumulate in the environmental matrices. The Kow value of florfenicol was low (Kow = 2.36), it means that the adsorption ability of florfenicol in the sediment was weak.

Even though it was not expected to accumulate in the sediment, just like other antibacterials (Herwig et al. 1997; Baquero 2001), florfenicol is the cause of general increasing concern as it can inhibit the beneficial bacteria and induce bacterial resistance, even at low concentration levels because of long-tern exposure. Because their continual input into the environment may lead to a long-term concentration and promote unnoticed adverse effects on aquatic and terrestrial organisms. Effects can accumulate so slowly that changes remain undetected until they become irreversible (Díaz-Cruz et al. 2003).

Garland and Mills (1991) have described a method that to allow observation of the functional potential of microbial communities using the Biolog system. This method shows the potential degrading abilities of a community in culture conditions. The main virtue of this substrate utilization assay is its ease of use, making it practical for thoroughly replicated large-scale studies. Other authors have demonstrated that these metabolic profiles can reveal differences among microbial communities that come from a variety of ecosystems (Gamo and Shoji 1999; Buyer et al. 2001; Grove et al. 2004). Average well color development (AWCD) could be a proper indicator of the whole microorganism activity, representing the total carbon utilizing capability of microbial community (Garland and Mills 1991). Different indices represented practically different profiles of functional diversity of sediment microbial community. The Shannon index was a comprehensive indicator of community species, individual numbers, and evenness, and was influenced by richness of community species, while the McIntosh index is an estimation of the uniformity of community species (Li et al. 2007).

Changes of AWCD values of microbial community in environmental sediment samples during the incubation can be seen in Fig. 2. From 48 h, the AWCD of microbial community in sample from station 11# was much higher than that of other samples and the difference was significant ($p < 0.05$). The AWCD of microbial community in sample from station 5# increased with the prolonging of the incubation time. It became higher than the samples from station 1# and 3# from 72 h. The differences between them were also significant. The AWCD of microbial community in samples from station 1# and 3# changed a little during the whole incubation and maintained in low level. Results from BIOLOG microplate methods showed that significant

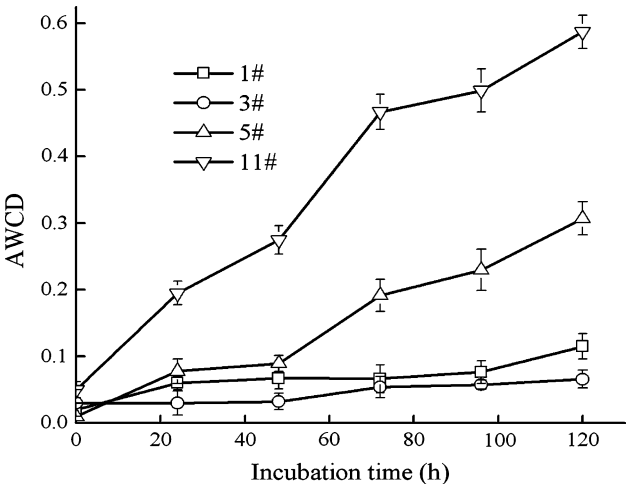

Fig. 2 Changes in average well color development during incubation. *Error bars* denote the range of three measurements

difference in AWCD occurred among different sediment samples in the order of 11#>5#>1# and 3#. It means that microorganism activities and functional diversities of sediment microbial community were different significantly between the samples from stations where have used florfenicol or other antibacterials (1#, 3# and 5#) and the sample from the station where no antibacterials had been employed (11#).

According to the curve of AWCD versus the culturing time, the AWCD values in 120 h culture were selected to calculate the functional diversity indices of microbial community. The Shannon indices and McIntosh indices of sediment microbial community were shown in Table 1. Among all the samples, the indices were in the same order as the AWCD values: 11#>5#>1# and 3#.

Oxytetracycline (OTC) on soil microbial community function was assessed with the Biolog method (Kong et al. 2006). The antibiotic OTC had significant negative effect on the microbial community function. Functional diversity, evenness and substrate utilization decreased significantly with increasing concentrations of oxytetracycline. The present study showed that the total carbon utilizing capability of microbial community in the sample from station 11# was much broader than that of other samples. The diversity index of microbial community in the sample from station 11# was consistent with the total carbon utilizing capability. It is much higher than that of other samples.

Table 1 Function diversity index of sediment microbe community

Different letters represent significant difference at 0.05 levels, and the same letters refer to no significant difference

Stations	Shannon index	Shannon evenness	McIntosh index	McIntosh evenness
1#	1.988 ± 0.032a	0.437 ± 0.006a	2.627 ± 0.011a	0.701 ± 0.002a
3#	1.957 ± 0.022a	0.430 ± 0.007a	2.533 ± 0.008b	0.691 ± 0.008a
7#	3.103 ± 0.011b	0.682 ± 0.010b	3.499 ± 0.007c	0.731 ± 0.006b
11#	4.010 ± 0.015c	0.881 ± 0.012c	5.431 ± 0.013d	0.961 ± 0.015c

Thus, lower diversity indices of sediment microbial community in the samples from stations 1#, 3# and 5# demonstrated the use of the antibacterials could limit microorganism growth, and caused a significant decrease in functional diversity index of sediment microbial community. It indicates that a long term and large amount of antibacterials application significantly changed the environment for microorganism growth, which would cause a catastrophic growth of some species and the death of others due to their inability to adapt. Florfenicol was detected in the water samples from stations 1# and 3# and the concentrations were 64.2 and 11,103 μg L^{-1}, respectively. It means that florfenicol residue in the environment presumably is one of the causes which lead to the change of the microbial community as the AWCD values and diversity indices of those samples were much lower than that of other samples. Then, the marine environment exposed to florfenicol will be impacted as the microorganisms play an important role in the marine circumstance.

Since the results of this paper indicated that florfenicol poses a potential risk to the microbial community in marine sediment, the aquaculture industry should use florfenicol chemotherapy more cautiously. Further studies are necessary to the potential influences of florfenicol on the marine environment thoroughly.

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References

Babich E, Stotzky G (1983) Developing standard for environmental toxicants: the need to consider abiotic environmental factors and microbe mediated ecological processes. Environ Health Perspect 49:247–260

Baquero F (2001) Low-level antibacterial resistance: a gateway to clinical resistance. Drug Resist Updat 4:93–105. doi:10.1054/drup.2001.0196

Boxall ABA, Fogg LA, Baird DJ, Lewis C, Telfer TC, Kolpin D, Gravell A, Pemberton E, Boucard T (2005) Targeted monitoring study for veterinary medicines in the environment. Science Report SC030183/SR. UK Environment Agency, Bristol

Briggs GG (1981) Theoretical and experimental relationships between soil adsorption, octanol water partition coefficients, coefficients, water solubilities, bioconcentration factors, and the parachor. Agri Food Chem 29:1050–1059

Buyer JS, Roberts DP, Millner P, Russek-Cohen E (2001) Analysis of fungal communities by sole carbon source utilization profiles. J Microbiol Methods 45:53–60

Díaz-Cruz MS, López de Alda MJ, Barceló D (2003) Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. Trends Anal Chem 22:340–351. doi:10.1016/S0165-9936(03)00603-4

Gamo M, Shoji T (1999) A method of profiling microbial communities based on a most-probable-number assay that use BIOLOG plates and multiple sole carbon sources. Appl Environ Microbiol 65:4419–4424

Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole carbon-source utilization. Appl Environ Microbiol 57:2351–2359

Grove JA, Kautola H, Javadpour S, Moo-Young M, Anderson WA (2004) Assessment of changes in the microorganism community in a biofilter. Biochem Eng J 18:111–114. doi:10.1016/S1369-703X(03)00182-7

Herwig RP, Gray JP, Weston DP (1997) Antibacterials resistant bacteria in surficial sediments near salmon net-cages farms in Puget Sound, Washington. Aquaculture 149:263–283

Hu JT, Zong HM, Wang JY, Ma DY (2007) The effect of environmental factors on the degradation of florfenicol in marine sediment. China Environmental Science 27:748–751 (in Chinese)

Kim Y, Jung J, Kimb M, Park J, Boxall ABA, Choi K (2008) Prioritizing veterinary pharmaceuticals for aquatic environment in Korea. Environ Toxicol Pharmacol 26:167–176. doi:10.1016/j.etap.2008.03.006

Kong WD, Zhu YG, Fu BJ, Marschner P, He JZ (2006) The veterinary antibiotic oxytetracycline and Cu influence functional diversity of the soil microbial community. Environ Pollut 143:129–137. doi:10.1016/j.envpol.2005.11.003

Lai HT, H JH, Su CI, Chen CL (2009) Effects of chloramphenicol, florfenicol, and thiamphenicol on growth of algae *Chlorella pyrenoidosa*, *Isochrysis galbana*, and *Tetraselmis chui*. Ecotoxicol Environ Saf 72:329–334. doi:10.1016/j.ecoenv.2008.03.005

Li ZP, Wu XC, Chen BY (2007) Changes in transformation of soil organic C and functional diversity of soil microbial community under different land uses. Agricultural Sciences in China 6:1235–1245

Lin RY, Rong H, Zhou JJ, Yu CP, Ye CY, Chen LS, Lin WX (2007) Impact of allelopathic rice seedlings on rhizospheric microbial populations and their functional diversity. Acta Ecologica Sinica 27:3644–3654

Lundén T, Lilius EM, Bylund G (2002) Respiratory burst activity of rainbow trout (*Oncorhynchus mykiss*) phagocytes is modulated by antimicrobial drugs. Aquaculture 207:203–212

Ma DY, Hu YY, Wang JY, Ye S, Li A (2006) Effects of antibactericals use in aquaculture on biogeochemical processes in marine sediment. Sci Total Environ 367:273–277. doi:10.1016/j.scitotenv.2005.10.014

Matyar F, Kaya A, Dinçer S (2008) Antibacterial agents and heavy metal resistance in Gram-negative bacteria isolated from seawater, shrimp and sediment in Iskenderun Bay, Turkey. Sci Total Environ 407:279–285. doi:10.1016/j.scitotenv.2008.08.014

Miranda CD, Zemelmanb R (2002) Antimicrobial multiresistance in bacteria isolated from freshwater Chilean salmon farms. Sci Total Environ 293:207–218

Rigos G, Nengas I, Alexis M, Troisi GM (2004) Potential drug (oxytetracycline and oxolinic acid) pollution from Mediterranean sparid fish farms. Aquat Toxicol 69:281–288. doi:10.1016/j.aquatox.2004.05.009

Samuelsen O, Bergh Ø (2004) Efficacy of orally administered florfenicol and oxolinic acid for the treatment of vibriosis in cod (*Gadus morhua*). Aquaculture 235:27–35. doi:10.1016/S0044-8486(03)00446-0

Wollenberger L, Halling-Sørensen B, Kusk KO (2000) Acute and chronic toxicity of veterinary antibiotics to *daphnia magna*. Chemosphere 40:723–730

Ye S, Yao ZW, Na GS, Wang JY, Ma DY (2007) Rapid simultaneous determination of 14 sulfonamides in wastewater by liquid chromatography tandem mass spectrometry. J Sep Sci 30:2360–2369. doi:10.1002/jssc.200600539

Zong HM, Ma DY, Wang JY, Hu JT (2008) Effects of florfenicol on microbial activity in marine sediment. Chin J Appl Environ Biol 14:408–412 (in Chinese)