WATER RESEARCH 42 (2008) 307–317

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Determination of penicillin G and its degradation products in a penicillin production wastewater treatment plant and the receiving river

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ARTICLE INFO

Article history:
Received 27 February 2007
Received in revised form
11 July 2007
Accepted 14 July 2007
Available online 20 July 2007

Keywords:
Penicillin G
Degradation products
Production wastewater
Degradation pathway
LC–ESI–MS

ABSTRACT

To investigate the fate of penicillin G (PEN G) in the wastewater from a PEN G production facility and the receiving river, an analytical method was developed for the simultaneous detection of PEN G and five degradation products using liquid chromatography–electrospray ionization mass spectrometry (LC–ESI/MS). PEN G had already undergone transformation before entering into the wastewater treatment plant (WWTP), with concentrations of 153 ± 4 μg/L in raw wastewater. Most of the PEN G could be eliminated following successive treatments of anaerobic, hydrolysis, and two aerobic units under a hydraulic residence time (HRT) of 30 h, and the final concentrations were 1.68 ± 0.48 μg/L in treated water. In the receiving river, the concentration of PEN G decreased from 0.31 ± 0.04 μg/L at the discharging point to under the detection limit (0.03 μg/L) at the last sampling site (about 30 km from the discharging point). The main PEN G degradation products in surface water were found to be penilloic acid, penicilloic acid and isopenillic acid, which occupied 65.8%, 20.4% and 12.9%, respectively, of the total concentration at the last site. This is the first study on the behaviors of PEN G and its main degradation products in wastewater treatment processes and the aquatic environment.

1. Introduction

Large amounts of antibiotics are produced, consumed and applied to treat bacterial diseases in humans and to promote animal growth (Kümmerer, 2003), and bacterial resistance to antibiotics has become a serious problem encountered frequently in clinical treatment (Fluit et al., 2000). Abuse of antibiotics and the existence of residual antibiotics in the environment have been linked with the formation of antibiotic resistance (Boxall et al., 2003; Silver and Bostian, 1993). The occurrences of several kinds of antibiotics like macrolides and sulfonamides have been reported in many environmental samples such as municipal wastewater (McArdell et al., 2003), surface water, groundwater (Batt and Aga, 2005), sludge and sediments (Lindberg et al., 2005).

Penicillin G (PEN G) ($pK_a = 2.75$) was the first antibiotic found by humans and applied for human bacterial disease (Clarke et al., 1949), as it could bind to penicillin-binding proteins (PBPs) of bacteria which are involved in cell wall synthesis and thus inhibit bacterial cell wall synthesis (Hou and Poole, 1971). It belongs to the β-lactam class of antibiotics, which all contain a β-lactam ring, and is a cause for concern due to its large production, mostly common usage in clinical treatment and widely appearing PEN G-resistant bacteria in medical treatment and the environment (Hou and Poole, 1971; Bush et al., 1995). However, in most surveys until

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doi:10.1016/j.watres.2007.07.016

now, PEN G and many other β-lactam antibiotics have not yet been detected in environmental samples (Hirsch et al., 1999; Sacher et al., 2001), mainly due to the chemically unstable β-lactam ring, which is highly sensitive to pH, heat and β-lactamase enzymes (Clarke et al., 1949; Hou and Poole, 1971; Bush et al., 1995). Laboratory experiments have shown that with the opening of the β-lactam ring through an intermediate of penicillenic acid in weak acidic or neutral solutions (Blaha et al., 1976), PEN G quickly transforms to penicilloic acid, which is also the degradation product of PEN G in alkaline media (Clarke et al., 1949) and can easily convert to penilloic acid under acidic conditions (Clarke et al., 1949; Blaha et al., 1976), or penillic acid, which could be formed from PEN G in strong acid and convert to isopenillic acid in alkaline media (Clarke et al., 1949), depending on the pH of the solutions. The end hydrolytic degradation products of PEN G are reported to be penicilloaldehyde and penicillamine (Hou and Poole, 1971; Blaha et al., 1976). Besides the chemical degradation process, β-lactamase enzymes released from resistant bacteria can also transform PEN G to penicilloic acid by opening the β-lactam ring (Bush et al., 1995), and some products like penicilloic acid have been detected in human bodies as the metabolites of PEN G after taking medicine (Pcole et al., 1973). These PEN G degradation products have not been detected in the environment until now, and most of theirantibiotic abilities have been lost as the β-lactam ring is opened (Hou and Poole, 1971); however, it has been reported that penicillin allergic reactions in 3–5% of patients are linked with these compounds to some extent (Blaha et al., 1976), with their environmental influence still unknown.

From the view of discharging sources, antibiotic production wastewater cannot be neglected because it contains high concentrations of antibiotics, as exemplified by the sulfonamide contamination in groundwater under the former waste dumpsite of a pharmaceutical production facility (Holm et al., 1995), and up to 50 mg/L oxytetracycline in the outflow of an oxytetracycline production facility (Qiting and Xiheng, 1988), which was 4–6 orders higher than those reported in the secondary effluents from municipal wastewater treatment plants (WWTPs) (Kolpin et al., 2002). Such high concentrations of antibiotics in production wastewater have been reported to significantly influence the treatment of wastewater, and improving the removal efficiency of antibiotics in WWTPs has also attracted increased emphases now (Kim et al., 2005). The fates of antibiotics during different treatment processes and their environmental behaviors should be illuminated first.

China has become a top maker and consumer of many antibiotic classes, and in 2004 the output of PEN G reached 35.6 thousand tons. In this study, to determine the occurrences and fates of PEN G and its degradation products in production wastewater and receiving surface water, a liquid chromatography–electrospray ionization mass spectrometry (LC–ESI/MS) method for simultaneous identification of PEN G and its five main degradation products, penicilloic acid, penilloic acid, penillic acid, isopenillic acid, and penicilloaldehyde, had been developed and applied in a biological system for treating wastewater from the PEN G production facility of the North China Pharmaceutical Group Corporation (NCPGC) and the receiving river, the Wangyang River in Hebei Province, China. To the best of our knowledge, this is the first description of a methodology for simultaneous quantitative analysis of PEN G and its five degradation products in environmental samples, and the first instance where PEN G and its degradation products were observed in the aquatic environment.

2. Materials and methods

2.1. Chemicals and materials

PEN G (sodium salt) and penicillin V (PEN V) (potassium salt) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Benzylpenicilloic acid, benzylpenilloic acid and benzylpenillic acid were bought from Sandoz (Sandoz GmbH, Kundl, Austria). Benzylpenicilloaldehyde and benzylisopenillic acid were prepared by recrystallization according to the methods described previously (Clarke et al., 1949). Stock solutions of all standards were made by dissolving 1 mg of each compound in 1 mL of water/acetonitrile (1:1, v/v). The stock solutions were stored at 4 °C in the dark and were prepared monthly. Working dilutions were prepared freshly on the day of use.

Formic acid of p.a. grade and ammonium acetate of HPLC grade were purchased from Merck (Darmstadt, Germany) and Fluka (Steinheim, Germany), respectively. Purified water (18.2 MΩ cm) was prepared by PURIC-MXII (Organo, Japan). Acetonitrile of HPLC grade was from Fisher Scientific (Fair Lawn, NJ, USA). Oasis HLB solid extraction cartridges (500 mg) were obtained from Waters (Milford, MA, USA).

2.2. Sample collection

NCPGC is the top producer of PEN G in Asia, with the annual production of about 7000 tons ranging around 20% of China's total output. PEN G production wastewater from its factory in the city of Shijiazhuang is discharged into the Wangyang River following successive biological treatments: anaerobic treatment, hydrolyzation and acidification, primary aerobic treatment and secondary aerobic treatment. Prior to these treatments, raw wastewater is stored in a tank with the temperature raised to 75 °C to recover the extraction solvent of PEN G, and then diluted with sewage wastewater and some other chemical production wastewater including avermectins, ivermectin and several kinds of semisynthetic antibiotics before and after the anaerobic treatment. In the two aerobic systems, short fibers fixed on plastic rings are used as biological support media. The average hydraulic residence time (HRT) for each unit is about 30 h. The annual output of excess sludge from the WWTP, which has been in operation since the 1990s, is 1200 tons in dry weight.

Wastewater and river water samples were taken in December 2004, April and August 2005, with the sampling sites in the WWTP (W1–W5) and the Wangyang River (R1–R5) shown in Fig. 1. Wastewater samples included raw wastewater and the effluent of each treatment unit, and river water samples were taken from five sites distributed along the river (R1 at the upstream). For each season, the sampling campaign lasted 3 successive days and triplicate samples were taken from each site every day. No rain event had taken place in the previous

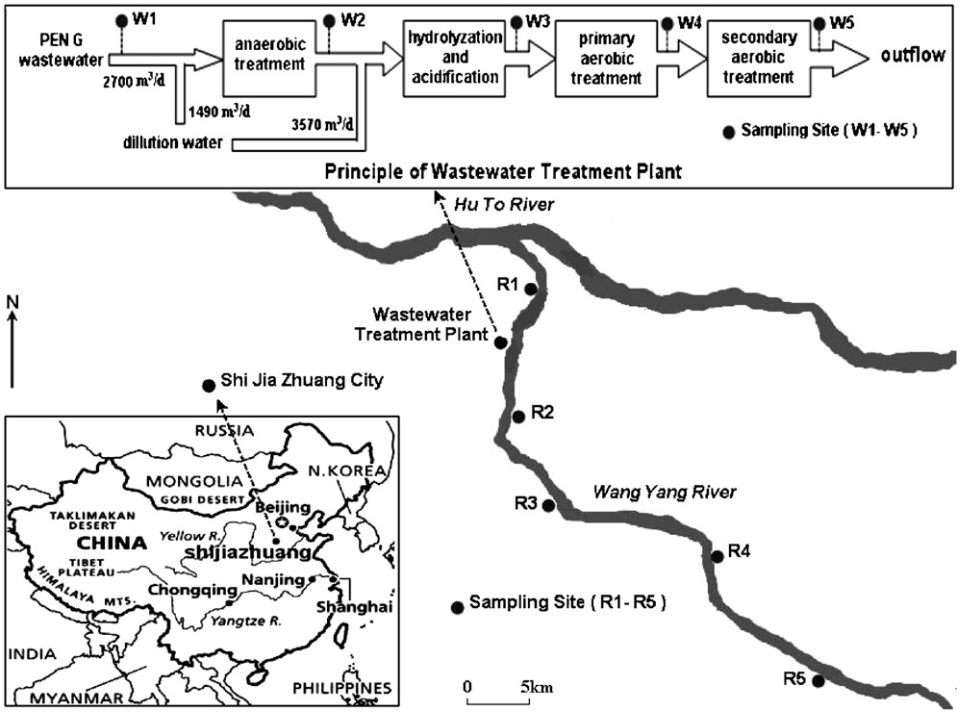

Fig. 1 – Sampling sites of Wangyang River and schematic diagram of the WWTP. Sites W1–W5 were in the WWTP for the PEN G production wastewater treatment and sites R1–R5 were distributed along Wangyang River, with R1 located at about 5 km upstream of the discharge point of the WWTP.

week of the campaign or during sampling days. Water samples were collected in 4-L brown glass bottles that had been successively washed with tap water, ultrapure water and hexane, and then kept at 4 °C in the dark for at most 2 days. The degradation of the target analytes during storage has been checked in triplicate by the analysis of the compounds in raw wastewater, the effluent of the WWTP and river water taken from R2 at an interval of 2 days kept at 4 °C on the dark in the laboratory. PEN G had been spiked to the river water sample at 20 μg/L because of its low concentration in river water samples. The detailed characteristics of water samples with standard deviations are summarized in Table S1 (Supporting Information).

2.3. *Sample preparation for wastewater and surface water*

Prior to analysis, 10-fold serial dilutions of the water samples had been made with ultrapure water, as the concentrations of most analytes in raw samples had exceeded the higher limits of their calibration curves (100 μg/L for PEN G and 500 μg/L for other analytes, as described in Section 2.5). The samples were then analyzed with LC–ESI/MS after being filtered through 0.2-μm polyethersulfone syringe filters (Whatman, Puradisc, 25 AS, England) and added with PEN V as an internal standard (IS) at a concentration of 10 μg/L. PEN V is usually used as IS for determining β-lactam antibiotics in some analytical methods (Fagerquist et al., 2005; Sørensen et al., 1999), and it has been confirmed to be undetectable in all water samples of this study.

Because of the low concentrations in all river water samples (lower than 0.93 μg/L, the instrumental limit of quantification, LOQ, of PEN G), enrichment with SPE was performed for PEN G in all of the river water samples after spiking with PEN V at a concentration of 1.0 μg/L, using a procedure described by Holstege et al. (2002) in which the stability of PEN G was carefully monitored during extraction: briefly, 0.1 L of water sample filtered through a 0.45-μm polyethersulfone syringe filter (Whatman, England) was extracted by an Oasis HLB SPE column. The analytes were eluted with 6 mL of 40% acetonitrile in water and evaporated with a nitrogen stream, and then 2 mL of ammonium acetate buffer (pH = 6.7) was immediately added to avoid the degradation of PEN G.

2.4. *Chemical analysis*

The HPLC system consisted of an Alliance liquid chromatography 2695 (Waters, USA) and a Waters SymmetryShield™ RP18 column (150 × 2.1 mm i.d., particle size 5 μm) operated at 25 °C, and the flow rate was 0.2 mL/min. The mobile phase consisted of 0.1% formic acid in water with 1 mM ammonium acetate (solvent A, pH 2.7) and acetonitrile (solvent B). The initial composition was 100% B and was linearly decreased to 50% in 9 min, held for 2 min and then brought to 100% in 3 min. The injection volume was 20 μL for each sample.

Mass spectrometry was carried out with a single-quadruple mass spectrometer ZQ 4000 (Micromass, Manchester, UK). Electrospray ionization (ESI) was conducted in the negative ion mode. The capillary voltage was 3.5 kV, and the cone

voltage was set at 20 and 60 V in the multi-channel mode. Source and desolvation temperatures were set at 105 and 250 °C, respectively. Nitrogen gas was used as the desolvation gas with a flow rate of 300 L/h and as cone gas with a flow rate of 50 L/h. Selected ion recording (SIR) mode was chosen for quantification of the analytes using $[M–H]^-$ ions recorded in each case.

2.5. Quantification and quality assurance

The five-point matrix-matched internal standard calibration curve for each analyte was established by fortifying blank river water from site R1 with different levels of the analyte (1, 10, 20, 50, 100, 150 and 200 μg/L for PEN G and 10, 100, 200, 300, 500, 700 and 1000 μg/L for the other analytes), which could compensate for potential signal loss due to matrix-induced ion suppression. The blank river water had been checked without containing any detectable target analytes (less than LOQ) using LC–ESI/MS. The area ratios of the SIR signal response for the target analytes versus IS were plotted against their respective concentration ratios (Fig. S1, Supporting Information), and higher limits of the linear range of the ratios were found to be 100 μg/L for PEN G and 500 μg/L for the other analytes, which were used as higher limits of the calibration curves for the analytes. Recovery studies were carried out for PEN G and PEN V using blank river water from site R1 fortified at the 0.1, 1 and 10 μg/L levels. For each concentration six replicates were injected and the recovery was their average.

Matrix effects, which have been found in complex samples and lead to ion signal suppression or enhancement especially in the ESI mode (Lindberg et al., 2004), were investigated for water samples of this study using standard addition. Raw wastewater and the effluent of the WWTP were first diluted with ultrapure water 1000 times, and river water from site R5 was diluted 100 times, in which the procedure was the same as that for detecting PEN G degradation products in real water samples. Then the analytes were spiked in the diluted water samples at concentrations more than 3 times those already in the samples, and determined with LC–ESI/MS before and after fortification. We have measured the matrix effects by determining the ratio of the SIR trace area for each analyte added in wastewater and river water samples versus that for standards in ultrapure water at the same concentration level with each sample injected 6 times.

3. Results and discussion

3.1. Qualification and quantification

Of the operational conditions of the ESI interface for analyzing analytes, cone voltage is the most important parameter influencing the fragmentation of the analytes. We have set the cone voltage from 20 to 80 V at an interval of 20 V, and the maximum structural information was obtained at 60 V for all the analytes except for penilloic acid and penicilloaldehyde, which were obtained at 80 V as shown in Table 1. It has been reported that fragmental ions of PEN G were *m/z* 160 and 176 in the positive ion mode (Riediker and Stadler, 2001), and *m/z* 192 and 289 in the negative mode (Becker et al., 2004) using LC–ESI/MS/MS. In this study, fragmental ions were found to be *m/z* 192 and 289 for PEN G, the same as that reported using LC–ESI/MS (Straub and Voyksner, 1993). The sensitivity at 20 V was about 6 times higher than that at 60 V, considering the maximum structural information obtained at 60 V for most of the analytes; all the water samples were subsequently analyzed in multi-channel mode, with quantification performed at 20 V using $[M–H]^-$ ions and qualitative analysis performed simultaneously at 60 V for all the analytes. To make sure that no overlapping peaks from other components in water samples exist, the confirmation ratio of the peak areas of molecular ion and two fragment ions has been evaluated for each analyte in standards and in all water samples including each wastewater compartment and river water samples, and the variability of the ratio between water samples and standards was all within 15% as shown in Table S2 (Supporting Information).

It has been reported that LOQ for PEN G was 15–20 ng/L using LC–ESI/tandem MS (Hirsch et al., 1999; Sacher et al., 2001), while in this study the instrumental LOQ for PEN G fortified in ultrapure water was estimated to be 0.93 μg/L as shown in Table 2, based on the concentration corresponding

Table 1 – Structural information of PEN G and its degradation products in standard solutions in negative ion mode

Analyte	MW	*m/z* (%, relative abundance)[a]		
		20 V	60 V	80 V[b]
PEN G	334	192(15), *333(100)*	192(100), 289(35), *333(25)*	
Penicilloic acid	352	307(75), *351(100)*	229(85), 307(100), *351(70)*	
Penilloic acid	308	229(20), *307(100)*	229(70), 233(20), *307(100)*	134(60), 229(100), 233(35), *307(70)*
Penillic acid	334	289(20), *333(100)*	157(30), 289(100), *333(50)*	
Isopenillic acid	334	255(20), *333(100)*	211(80), 255(100), *333(65)*	
Penicilloaldehyde	177	146(40), *176(100)*	134(75), 146(55), *176(100)*	119(65), 134(100), 146(60), *176(75)*

[a] Ions with relative abundance lower than 10% are not listed in this table. The molecular ions are shown in italics.
[b] The maximum structural information was obtained at 60 V for PEN G, penicilloic acid, penillic acid and isopenillic acid, and at 80 V for penilloic acid and penicilloaldehyde.

Table 2 – Summary of the recoveries during sample storage, matrix effects of wastewater and river water, and the LOQs for PEN G and its degradation products

Analyte	Sample storage[a], recovery, % ($n = 6$)			Standard addition,[b] ratio,[c] % ($n = 6$)			LOQ[d] (μg/L)
	Raw wastewater	Effluent	River water	Raw wastewater	Effluent	River water	
PEN G	69±7	73±8	78±7	71±9	87±7	91±8	0.93[e] 0.031[f]
Penicilloic acid	82±8	86±6	85±5	74±8	79±9	88±6	0.64
Penilloic acid	94±6	95±7	97±5	86±9	85±8	96±4	1.05
Penillic acid	91±7	88±5	86±5	72±6	84±5	90±7	0.41
Isopenillic acid	95±9	98±8	102±7	78±8	80±9	95±5	0.96
Penicilloaldehyde	96±10	97±6	95±8	81±7	84±6	89±8	1.36

[a] Raw wastewater, the effluent of the WWTP and river water taken from R2 were kept at 4 °C in the dark in the laboratory for 2 days to determine the degradations of the analytes during storage. PEN G had been only spiked to the river water sample at 20 μg/L because of its low concentration in river samples.
[b] Standard additions were performed to determine the matrix effects of raw wastewater, the effluent and river water.
[c] The ratio was calculated by deducting the SIR trace area of the analytes in non-spiked water samples from the SIR trace area of the analytes after fortification, and then comparing it to the area of standards in ultrapure water at the same concentration as the fortification.
[d] LOQ was defined as the concentration corresponding to the signal at the y-intercept plus 10 times its standard deviation, with all the analytes fortified in ultrapure water.
[e] The instrumental LOQ.
[f] LOQ after enrichment.

to the signal at the y-intercept plus 10 times its standard deviation (Batt and Aga, 2005; Lindberg et al., 2005). The LOQs for all the analytes in river water were also determined in the same way by fortifying all the analytes in blank river water from R1. Since LOQs were derived from calibration solutions of ultrapure and blank river water, a signal-to-noise ratio of 10 was used for all the analytes in wastewater samples according to the American Chemical Society (ACS) Committee on Environmental Improvement (1980). The recoveries for extracting PEN G spiked at 0.1, 1 and 10 μg/L in the blank river water samples with HLB columns were 93±6%, 98±5% and 97±5%, respectively, and 94±6%, 99±8% and 93±7% for extracting PEN V at the corresponding levels, and the LOQ for PEN G after enrichment with SPE was 0.031 μg/L.

Matrix effects were observed in the analysis of the samples as shown in Table 2. The signal suppressions were found to be more significant for all analytes in raw wastewater, and the ratio of the SIR trace area for each analyte added in wastewater and river water samples versus that for standards in ultrapure water ranged from 71±9% to 96±4% for all objective compounds. In this study, the use of matrix-matched calibration curves could compensate the ion suppression of the analytes in river water samples. For wastewater samples, the analytical results have not been modified with the matrix factors.

Significant degradations of β-lactam antibiotics such as PEN V, amoxicillin and ampicillin during sample storage have been reported by Lindberg et al. (2005) with in-field fortifications. In this study, during sample storage at 4 °C for 2 days, all of the PEN G degradation products demonstrated a relatively high recovery (82±8%–102±7%) except for PEN G, whose recoveries were 69±7%, 73±8% and 78±7% in raw wastewater, effluent and river water, respectively (Table 2).

3.2. *Determination of analytes in the WWTP*

PEN G and its degradation products were detected in all wastewater samples, and typical SIR LC–ESI/MS chromatograms for the influent and effluent of the WWTP are shown in Figs. 2(a) and (b). Considering the dilution of the wastewater at sites W2 and W3, the load of each analyte was obtained by multiplying the concentration with the flow rate at each sampling site (Table S1, Supporting Information), and the role of each wastewater treatment process in the removal of the analytes from wastewater was investigated by percent removal calculation, which was obtained as the ratio of the load of each analyte in the effluent versus that in the influent of each treatment process. The median results with standard deviations for three sampling campaigns are shown in Table 3. In raw wastewater, the concentration of PEN G was 153±4 μg/L, and the concentrations of its degradation products were 389±10, 75.3±0.6, 23.5±2.0, 8.49±0.61 and 1.05±0.09 mg/L for penilloic acid, penicilloaldehyde, penillic acid, penicilloic acid and isopenillic acid, respectively. The molar concentration of PEN G (0.458±0.01 μmol/L) only accounted for about 0.03% of the total molar concentration of all analytes including PEN G and five degradation products (1786±44 μmol/L). Compared with its degradation products, such a low concentration of PEN G in raw wastewater indicated that before entering the WWTP, most of the PEN G had undergone chemical degradation processes at 75 °C and a pH of 3.9±0.3 during solvent recovery (Table S1). Penilloic acid, which was reported to be easily transformed from penicilloic acid under acidic conditions (Clarke et al., 1949), was found to be the dominant compound, with the molar concentration (1263±32 μmol/L) accounting for 70.7% of the total molar concentration in raw wastewater, and this result was in accordance with that obtained by Blaha et al. (1976) in which penilloic acid was also the main

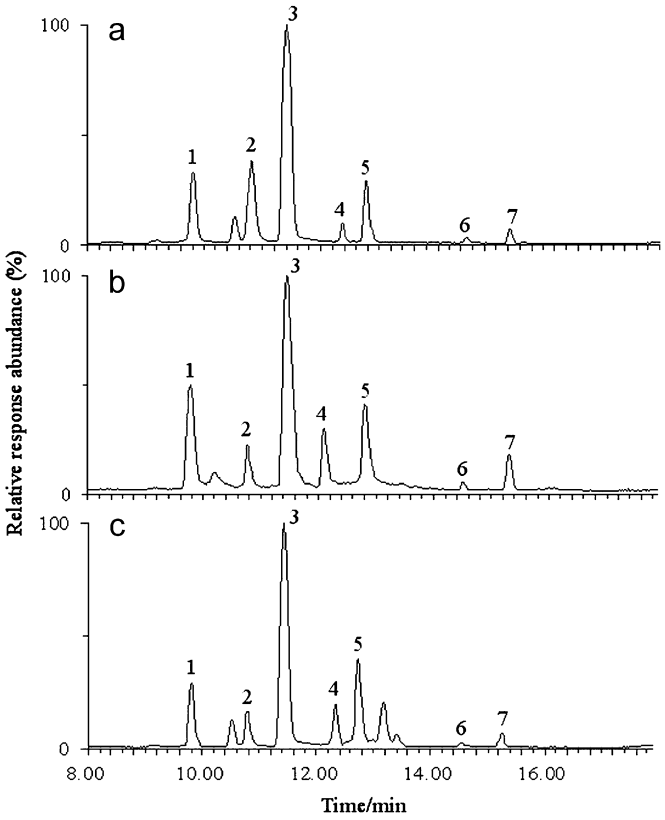

Fig. 2 – Time-scheduled SIR chromatograms of all the analytes in water samples collected in December 2004: (a) raw wastewater; (b) effluent of the WWTP; and (c) river water. (1) Penicilloaldehyde; (2) penillic acid; (3) penilloic acid; (4) isopenillic acid; (5) penicilloic acid; (6) PEN G; and (7) PEN V: IS.

Table 3 – Concentrations and percent removals of PEN G and its degradation products in the WWTP

Analyte	Concentration (mg/L)		Percent removal (%)			
	Site W1[a]	Site W5[b]	Anaerobic treatment[b]	Hydrolysis treatment[b]	Two aerobic treatments[b]	Total[c]
PEN G	0.153±0.004	0.00168±0.00048	26.1±5.3	41.5±1.9	92.7±1.6	96.8±1.0
Penicilloic acid	8.49±0.61	3.70±0.43	−61.5±3.4[d]	20.1±6.2	2.4±1.0	−25.9±9.5[d]
Penilloic acid	389±10	44.5±2.5	32.2±3.0	36.3±6.2	22.9±5.0	67.0±0.9
Penillic acid	23.5±2.0	5.49±0.68	17.8±3.0	0.1±8.5	17.8±2.9	32.5±6.1
Isopenillic acid	1.05±0.09	0.146±0.105	−46.6±6.1[d]	61.4±24.7	30.9±4.5	60.9±25.0
Penicilloaldehyde	75.3±0.6	6.77±1.91	6.5±1.9	8.9±3.8	69.4±8.9	74.0±7.3

[a] The temperature was 75 °C.
[b] These treatments were at ambient temperatures.
[c] The removal of the overall process.
[d] The minus values indicate the increase of the loads of the analytes.

degradation product and was identified to be transformed from PEN G through the intermediate of penicilloic acid under acidic conditions in laboratory experiments. Additionally, it was reported that penicilloaldehyde could be formed from penilloic acid as the end hydrolytic degradation product of PEN G (Hou and Poole, 1971; Blaha et al., 1976), and this was partly supported by the fact that penicilloaldehyde was the second most abundant product in raw wastewater, with the molar concentration (425±3 μmol/L) accounting for more than 23% of the total molar concentration. On the other hand, the existence of penillic acid in raw wastewater, whose molar concentration (70.4±6.0 μmol/L) ranged up to 3.94% of the total concentration of all the analytes, was also in accordance with the report that a low percent of PEN G would also convert to penillic acid as the other degradation pathway of PEN G in acidic media simultaneously with the transformation to penilloic acid (Blaha et al., 1976).

During the treatment of wastewater, it should be noted that the load of isopenillic acid increased significantly by 46.6±6.1% during the anaerobic treatment process as shown in Table 3. The fact that isopenillic acid can be formed from penillic acid at alkaline conditions (Clarke et al., 1949), while the pH of wastewater was 7.4±0.2 during the anaerobic process (Table S1), suggested that isopenillic acid was mainly formed from penillic acid under anaerobic conditions. In the hydrolysis process, the loads of isopenillic acid and penicilloic acid decreased with percent removals of 61.4±24.7% and 20.1±6.2%, respectively, indicating that the hydrolysis process was more efficient for the removal of these two compounds compared with biological treatment processes, including anaerobic and aerobic treatments, with percent removals also shown in Table 3. PEN G was also obviously removed during this process (percent removal of 41.5±1.8%), while the load of penillic acid was found to be almost steady with a percent removal of only 0.14±8.44%, indicating that little chemical degradation of penillic acid occurred during the hydrolysis process. Then aerobic treatments were found to be especially efficient for removing PEN G and penicilloaldehyde from wastewater, with percent removals up to 92.7±1.6% and 69.4±8.9%, respectively. β-Lactamase enzymes released from resistant bacteria should significantly contribute to the degradation of PEN G in aerobic biological processes, and the efficient removals of PEN G by hydrolysis and aerobic treatments were matched with the removing results for amoxicillin, another kind of β-lactam antibiotic, in batch experiments (Andreozzi et al., 2004). For penicilloaldehyde, the high removal suggested that it can be further degraded under aerobic conditions compared with the slight removals during anaerobic and hydrolysis processes. On the other hand, it was found that penicilloic acid increased during anaerobic processes; one possible reason may be due to the transformation from its isomeric form, penamaldic acid, which was reported to chemically equilibrate with penicilloic acid by the reaction of isomerization (Blaha et al., 1976). Unfortunately, penamaldic acid was not the target analyte in this study, and further study is necessary. On the basis of the above results, degradation pathways for PEN G in wastewater treatment processes are proposed, as shown in Fig. 3, which include hydrolyzation and biotransformation, and the pathways due to thermal treatment (75 °C) before biological treatments or at ambient temperatures were also indicated. As raw wastewater was treated thermally as a very first step, the investigation of degradation processes at the ambient temperature in the environment was limited, and the significance of degradation processes at the ambient

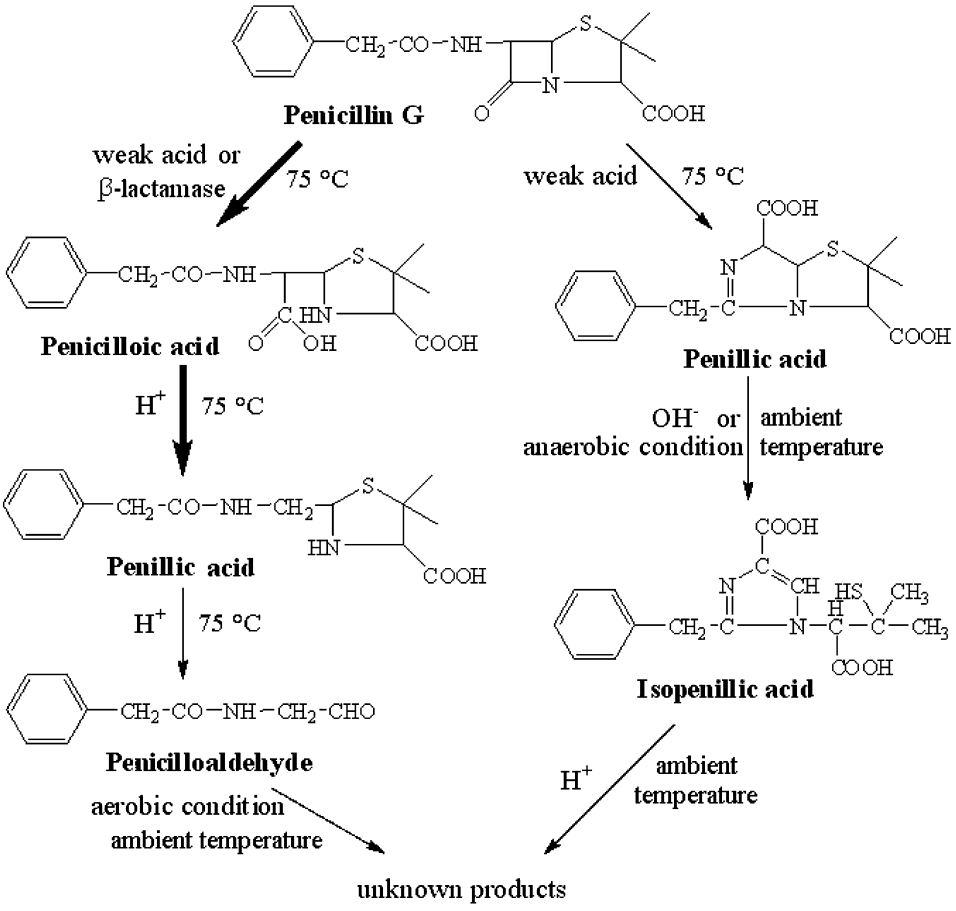

Fig. 3 – Proposed degradation pathways of PEN G during the wastewater treatment processes. The main degradation pathway in raw wastewater is shown by the bold arrows.

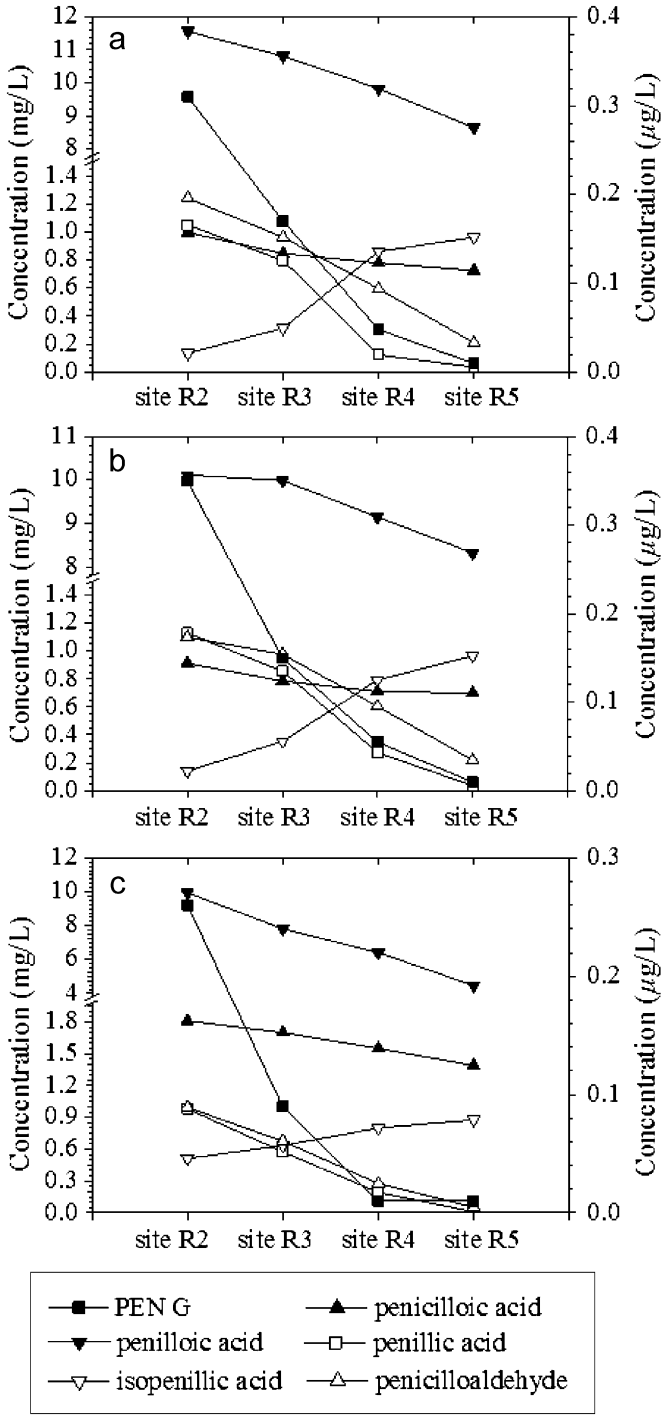

Fig. 4 – Concentrations of the analytes in the Wangyang River for three sampling campaigns. (a) December 2004; (b) April 2005; and (c) August 2005. The y-axis on the right shows the concentration of PEN G, and the y-axis on the left shows the concentrations of the other compounds. For PEN G, the concentration value was set to half of its LOQ at the sites where PEN G was undetectable.

temperature cannot be assessed; however, some degradation products in this study should help to elucidate the fate of PEN G in the environment.

About 96.7% of PEN G had been eliminated in the WWTP, with the concentration of 1.68 ± 0.48 μg/L in the effluent discharged into the receiving surface water. Penilloic acid was still the dominant compound in the effluent at up to 44.5 ± 2.5 mg/L, the molar concentration of which (145 ± 8 μmol/L) accounted for 68.8% of the total molar concentration (210 ± 22 μmol/L). While, due to the comparably efficient removal in aerobic treatments, the abundance of penicilloaldehyde became a little lower in the effluent, about 18.2% of the total molar concentration, it still was the second abundant degradation product (Fig. S2, Supporting

Information). Considering the sum of molar amounts of PEN G and its five degradation products in the raw wastewater and the effluent, the removal efficiency for PEN G and its five degradation products during the WWTP was calculated to be 66.0%. The residues of the analytes discharged into the environment via the effluent were estimated to be annually about 4.78, 127,000, 19,300, 15,600, 10,500 and 415 kg for PEN G, penilloic acid, penicilloaldehyde, penillic acid, penicilloic acid and isopenillic acid, respectively.

3.3. *Determination of analytes in the receiving river*

The Wangyang River receives effluent from the WWTP, and all the analytes in river water samples were determined using LC–ESI/MS, with the results shown in Fig. 4. Except for isopenillic acid, the concentrations of all analytes decreased obviously in surface water with increasing distance from the discharging point, and the total concentrations of all analytes were calculated to be 14.2 ± 0.8, 12.7 ± 1.3, 11.0 ± 1.8 and 9.2 ± 3.6 mg/L from sites R2 to R5, respectively, while the fate for individual compounds showed divergent behavior. PEN G was detected with the maximum concentration of 0.35 μg/L at R2 in April, and was undetectable (less than the LOQ, 0.031 μg/L) at R5 for all three seasons. The dominant species was still penilloic acid in all river water samples, with the mean concentration ranging from 10.54 to 7.14 mg/L; the ratio of its molar concentration versus the total molar concentration remained at 69.8–77.9%, similar to that in the WWTP. However, the ratios of the molar concentrations of isopenillic acid and penicilloic acid increased significantly along the river from 2.7% to 10.1% and from 10.5% to 17.4%, respectively, suggesting that these two compounds are also important PEN G degradation products in aquatic environment. It should be noted that the increase of isopenillic acid was due to its concentration increase along the river, from 0.263 ± 0.25 mg/L at R2 to 0.935 ± 0.057 mg/L at R5, which was possibly due to the conversion of penillic acid. While the increase of penicilloic acid was possibly the result of its relatively tardy removal (percent removal of $24.6\pm2.7\%$) in surface water compared with other compounds like penicilloaldehyde, the ratio of its molar concentration versus the total molar concentration decreased fast along the river (from 12.7% at R2 to 1.5% at R5) and percent removal was up to $85.9\pm8.8\%$, just like the result in aerobic treatment processes.

Predicted environmental concentration (PEC) of PEN G in this study was also calculated to compare with the actual measured environmental concentration (MEC) in the receiving river. During the production in NCPGC, PEN G was extracted from the production solution using butyl acetate, and about 10% of PEN G still remained in the fermentation solution after the extraction. Considering the annual production quantity of 7000 tons, PEN G residues discharged into wastewater were about 700 tons/year. By assuming that PEN G only has the five hydrolysis products investigated in this study and more than 99.9% of PEN G had been transformed to the five degradation products at site W1 (Table 3), only about 0.215 tons of PEN G still remained in the raw wastewater per year and about 0.218 mg/L of PEN G was expected in the raw wastewater, which was in a comparable range with the measured concentration (about 0.153 mg/L shown in Table 3).

PEC of PEN G in the receiving river was calculated according to the following equation (EC, 2003):

$$\mathrm{PEC\ (g/L)} = [A(100 - R)]/(365PVD \times 100),$$

where A is the quantity of PEN G residues in raw wastewater, which was calculated to be 0.215 ton/year; R is the removal percentage during wastewater treatment, which is about 96.7% (Table 3); PV is the volume of wastewater per day, which is around 7800 m^3/d (Table S1); and D is the dilution factor of final effluent to receiving river water, which is about 3.85 (Table S1). Thus, PEC of PEN G in this study was 0.65 μg/L, in a comparable range with MEC of PEN G, which was from 0.26 to 0.35 μg/L obtained at site R2, about 5 km from the discharging point (Fig. 1), and PEC of PEN G could be more closely matched with MEC of PEN G considering the degradation of PEN G in the surface water and the existence of other hydrolysis products of PEN G, which were not target analytes of this study.

The minimal inhibition concentration (MIC) of PEN G has been reported to be lower than 60 μg/L for 30.1% susceptible clinical isolates of *Streptococcus pneumoniae* collected from all over Lebanon (Uwaydah et al., 2006), lower than 30 μg/L for *Staphylococcus aureus* isolates (Bonfiglio and Livermore, 1994), and 15 μg/L for mode-sensitive *Streptococcus pneumoniae* strains (Schito and Felmingham, 2005). These MICs of PEN G were in a comparable range with the concentration levels of PEN G detected in this study. For PEN G degradation products, most antibiotic abilities have been lost as the β-lactam ring is opened (Hou and Poole, 1971); however, it has been reported that these compounds are linked with penicillin allergic reactions to some extent in patients, as these degradation products may react with the amino group of the macromolecular carrier protein of the human body to form haptens of antigens, giving rise to sensitizing conjugates that are responsible for sensitization (De weck, 1962; Klaus and Fellner, 1973). The biodegradability and environmental toxicity of these degradation products have not been investigated until now. Considering the high concentrations and relative environmental persistence of some compounds like penilloic acid, penicilloic acid and isopenillic acid compared with PEN G in the receiving surface water of this study, more research about the degradation products is needed.

4. Conclusions

Most of the PEN G could be eliminated following successive treatments of anaerobic, hydrolysis, and two aerobic units under a HRT of 30 h in the WWTP, and the concentration of PEN G decreased in the receiving river along with the distance from the discharging point. Of the PEN G degradation products, penilloic acid was the dominant species in all water samples. High concentrations of the PEN G degradation products were discharged into the surface water, some of which, like penilloic acid, penicilloic acid and penillic acid, were found to be relatively environmental persistent in this study. These results suggest the PEN G production wastewater as an important source of PEN G and its degradation product residues in the environment, with potential environmental risks, be evaluated in future.

Acknowledgments

Financial support from the National Natural Science Foundation of China (20477056, 20610103 and 50525824) is gratefully acknowledged. We are particularly grateful to anonymous reviewers for helpful comments to improve the manuscript.

Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.watres.2007.07.016.

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