Behavior of antibiotics and antiviral drugs in sewage treatment plants and risk associated with their widespread use under pandemic condition

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（下水処理場での抗生物質と抗ウイルス剤の挙動とパンデミック発生時のその多用に伴うリスク）

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ABSTRACT

The concern for pharmaceutically active compounds (PhACs) as contaminants in the environment and the need to assess their environmental risk have greatly increased since the early nineties. Among PhACs, antibiotics and antiviral drugs are of important concern due to their role in growing antibiotic and antiviral drugs resistance among pathogenic bacteria and influenza viruses, respectively. Besides resistance issue, the compounds may upset sensitive ecosystems as they are designed to be highly bioactive. Clinically-important antibiotics are virtually ubiquitous contaminants in sewage water and surface water. Notably, recent emergence of novel influenza and use of anti-influenza drugs (specially Tamiflu®) during seasonal influenza、 influenza epidemics and for future pandemic are of emerging concern. Every year seasonal influenza epidemic causes tens of millions of respiratory illnesses and 250,000 to 500,000 deaths worldwide. WHO (World Health Organization) recommend the use of antiviral drug Tamiflu® during pandemic, as they are easy to use. Currently only Japan uses over eighty percent of Tamiflu® prescribed globally during common seasonal influenza.

It is a fact that a huge amount of antiviral drugs and antibiotics (for post infection cure of respiratory illness) will be used during an influenza pandemic and will arrive to sewage treatment plants (STPs).Unfortunately, these compounds behaviors are mostly unknown in both conventional and advanced STPs. The exposure of antiviral drug in the wild fowl gut and its implications for hastening the generation of antiviral-resistance in avian influenza viruses are also an emerging issue.

The major objective of this thesis work was to investigate the occurrence of antibiotics and antiviral drugs in sewage treatment plants and their fate in different sewage treatment plants. The specific objectives were as follows: (a) to established appropriate analytical method for the selected antibiotics and antiviral drugs in sewage treatment plants, (b) to investigate the occurrence and removal of antibiotics and antiviral drugs in sewage treatment plants differ in technology and operation conditions; and (c) to predicts environmental concentration of the target compounds during a pandemics and appraisal of appropriate technology to reduce the risk associated with widespread use under pandemic conditions.

In this study we selected twenty antibiotics: one beta-lactam: ampicillin; four macrolides: azithromycin, clarithromycin and roxithromycin; five quinolones: ciprofloxacin, enrofloxacin, levofloxacin, nalidixic acid and norfloxacin; two tetracycline: tetracycline and oxytetracycline; five sulfonamides: sulfadimethoxine, sulfadimizine, sulfamerazine, sulfam-

ethoxazole and sulfamonomethoxine; and four others: lincomycin, novobiocin, salinomycin and trimethoprim. Oseltamivir Carboxylate (OC), the active metabolite of oseltamivir phosphate (Tamiflu®) and amantadine (AMN) were selected as antiviral drugs.

This dissertation consists of nine chapters: Chapter I describe the background and objective of the study and chapter II represent a brief literature review. In Chapter III, analytical methods for selected antibiotics and antiviral drugs (for the first time) in water and wastewater were described. In Chapter IV, the occurrences and fate of antibiotics in sewage treatment plants were investigated in Japan and China. Clarithromycin was detected in the highest concentration in influent (1129 to 4820 ng/L), followed by azithromycin (160 to 1347 ng/L), levofloxacin (255 to 587 ng/L) and norfloxacin (155 to 486 ng/L) and sulfamethoxazole (159 to 176ng/L) in Japan. Ozonation as tertiary treatment of secondary effluent for wastewater reclamation provided significant elimination of antibiotics. Fifty present of the selected antibiotics were removed over eighty percent during ozonation. There was no elimination of antibiotics in dissolve phase during ultra filtration.

From Chapter IV a hypothesis was drawn on antibiotics removal and its relation with longer sludge retention time (SRT) in STPs and in Chapter V the role of nitrifier in antibiotics removal was evaluated to verify the hypothesis established from Chapter IV. Nitrifying activated sludge (NAS) can biodegrade the tested antibiotics with different biodegradation rate between 2.74 to 9.95 L/gSS/d. Sulfamethoxazole and sulfamerazine degraded faster than trimethoprim, clarithromycin and enrofloxacin.

In Chapter VI, occurrence of antiviral drugs in sewage water discharge and in river water in Japan was conducted during seasonal influenza epidemic and their fate in different sewage treatment facilities were evaluated in Chapter VII. This is the fist findings of antiviral (anti-influenza) drugs in the environment in the world and for the first time the removal mechanism in STPs was elucidated. Finally, it was observed that only primary and secondary treatment processes in STPs were not sufficient to remove these compounds significantly. Overall OC and AMN removal in STP with ozonation as tertiary treatment was 90% and 96% respectively. In ozonation batch experiment, Chapter VIII, (feed ozone gas concentration 4.0mg/L, ozone gas flow rate 0.23L/min to maintain ozone feed rate of 0.6 mg/L/min), it was observed that AMN and OC concentration decreased linearly with time in all the experiments conducted and it can be, therefore, said that the degradation reactions follow pseudo first-order reaction. The k'_{O3} (pseudo first-order rate constant for O_3) of AMN was 0.596/min (0.00993/sec), and OC was 0.524 /min (0.008725/sec) and over 99% removal within 10min.

Chapter VIII described the predicted OC and antibiotics concentration in STPs influent, secondary effluent, after advance tertiary treatment (ozonation) and receiving water during a pandemic with three expected infection scenario (according to US CDC FluAid model 2.0) in Kyoto city. Both antiviral drugs and antibiotics pose an environmental risk associated to there widespread use during a future pandemic. Ozonation as tertiary treatment can provide a technological solution to reduce the ecotoxicological effect of antibiotics and antiviral drugs uses during a pandemic. In a full scale STP, the antiviral drugs (OC and AMN) reduction were over 90% from secondary effluent after ozonation during seasonal influenza outbreak in Kyoto city in 2008/2009.

Finally, (1) analytical methods for commonly used antibiotics and antiviral drugs in water sample was developed with an excellent precision and accuracies, (2) both antibiotics and antiviral drugs were detected in environmental sample, and their behavior in STPs were elucidated. Antivirals in this study were the first time findings in sewage water. This study will provide a surrogate for planning a pandemic preparedness action plan for sewage treatment pants for ecotoxicological risk management.

Acknowledgements

CHAPTER I
INTRODUCTION

1.1 **Research Background**

The concern for pharmaceutically active compounds (PhACs) as contaminants in the environment and the need to assess their environmental risk have greatly increased since the early nineties. Several reviews dealing with the exposition and effect of pharmaceuticals have been published recently (Halling-Sorensenet et al., 1998; Daughton and Ternes, 1999; Jorgensen and Halling-Sorensen, 2000; Kummerer, 2001; Ternes et al., 2001; Heberer, 2002; Petrovic et al., 2003; Larsen et al., 2004; Janex- Habibi et al., 2004; Jones et al., 2004; Kuster et al., 2004; Zwiener and Frimmel, 2004; Garric and Ferrari, 2005; Hernando et al.,2005; Fent et al., 2006; Zuccato et al., 2006). These reviews allow identifying more than one hundred PhACs from various therapeutic classes measured in sewage treatment plants (STPs) in European, Asia, South America, North America and Australia, and a few studies compared PhACs removal efficiencies among sewage treatment plants (STPs) differ in technologies and operation conditions. STPs effluents are the main source of PhACs in the environment. These PhACs include a wide range of drugs according to their uses, such as analgesics and anti-inflammatory drugs, antibiotics, anti-epileptics, beta-blockers, blood lipid regulators, contrast media, cytostatics, hormones (including oral contraceptives), antidepressants and anxiolitics, musk fragrances, disinfectants and antiseptics. To the best of my knowledge, at present there is no published data on anti-influenza drugs in the environment.

Among PhACs, antibiotics are of important concern due to their role in growing antibiotic resistance among pathogenic bacteria. Besides resistance issue, antibiotics may upset sensitive natural ecosystem as they are designed to be highly bioactive. Clinically-important antibiotics are virtually ubiquitous contaminants in sewage water (Ghosh et al., 2009; Okuda et al., 2008). Presence of antibiotics at substantial concentration in influent of centralized or decentralized STPs may cause adverse effects to sensitive biological processes such as nitrification, which is still hindered by the lack of information. Inhibition of nitrification under uncontrolled conditions may lead to accumulation of ammonia and/or nitrite in the effluent which is regulated.

Recently uses of anti-influenza drugs (especially Tamiflu®) during seasonal influenza、 influenza epidemics and future use during a pandemic are of emerging concern. Seasonal influenza epidemics causing tens of millions of respiratory illnesses and 250,000 to 500,000

deaths worldwide each year (WHO 2003). World Health Organization (WHO) recommends the use of antiviral drug Tamiflu® during pandemic, as they are easy to use and effective for both influenza virus A and B (Ward *et al.,* 2005). According to WHO guideline, many countries developed their pandemic preparedness action plan and stockpiling anti-influenza drugs. In Japan, the pandemic influenza preparedness action plan was formulated by the initiative of the Ministry of Health, Labour and Welfare, and approved at the Inter Ministerial Committee on November 14, 2005 (MHLW 2007). The pandemic influenza preparedness action plan of Japan determined the amount of antiviral drug to be secured and stockpiling based on the FluAid model of US Center for Disease Control (CDC) (around 25% of total population). Target amount of stockpile of Tamiflu® for total number of patients requiring treatment is doses for 25 million patients (amount reserved by the government and prefectures: doses for 21 million patients; and amount of domestic circulation: doses for 4 million patients, dose for one adult patient is 2 capsules (75 x 2 mg) daily for 5 days, total 10 capsules) which corresponds to several tons of drugs, additionally several tons of antibiotics drugs will be used to protect post infection .

Currently Japan uses over eighty percent of Tamiflu® prescribed globally during common seasonal influenza (Fick et al., 2007; Singer et al., 2008). The above target stockpile amounts shall be increased as necessary from the viewpoint of crisis management, considering the possibility of viruses obtaining tolerance to Tamiflu®, and referring to the status of surveillance on drug-resistance strains. So, it is clear that a huge amount of antiviral drugs and antibiotics (to protect or cure from post influenza infections) will be used during an influenza pandemic condition, and will arrive to sewage treatment plants (STPs). Unfortunately, these compounds behaviors are mostly unknown in existing STPs. As they are design to be highly bioactive, these compounds may have significant environmental health impact on non target organism, if they pass through the STPs. The waterfowl are the natural reservoir of influenza viruses (Olsen et al., 2006). The exposure of antiviral drug in the wild waterfowls gut and its implications for hastening the generation of antiviral-resistance in avian influenza is also an emerging issue due to wide spread use of these drugs as well as antibiotics resistance (Singer *et* al., 2007) .

1.2 Selection of antibiotics and antiviral drugs investigated in this study

The presence of antibiotics in the environment are of emerging concern, since they are designed to be highly bioactive and cause specific effects in human or animals and more importantly spread and maintenance of bacterial resistance. The high number and diversity of

antibiotic compounds and their different physico-chemical properties made it necessary to choose a particular substance group for this investigation. Criteria for this selection were the amount used, the environmental behavior and the analytical feasibility.   Most often the antibiotic substances are representatives of the following substance classes: (a) beta lactams, (b) tetracycline, (c) fluoroquinolone (d) macrolides and (e) sulfonamides. In this study I selected twenty antibiotics: one beta lactam: ampicillin; four macrolide: azithromycin, clarithromycin and roxithromycin; five quinolones: ciprofloxacin, enrofloxacin, levofloxacin, nalidixic acid and norfloxacin; two tetracycline: tetracycline and oxytetracycline; five sulfonamides: sulfadimethoxine, sulfadimizine, sulfamerazine, sulfamethoxazole and sulfamonomethoxine; and four others: lincomycin, novobiocin, salinomycin, and trimethoprim.

At present, two groups of anti-influenza drugs have become available for the treatment of influenza infections are the neuraminidase inhibitors (*e.g.* Tamiflu®) and the M2 ion channel inhibitors (*e.g.* Amantadine). Oseltamivir phosphate (OP) which is marketed as Tamiflu®, is recommended by World Health Organization (WHO) for both treatment and prophylaxis of influenza, and considered an important first-line defense in the event of a future influenza pandemic. OP is a prodrug which is rapidly and extensively hydrolyzed *in vivo* to its active metabolite oseltamivir carboxylate (OC). In this study I selected two anti-influenza drugs oseltamivir carboxylate and amantadine.

1.3 Research objectives

The major objective of this research work was to investigate the occurrence and fate of antibiotics and antiviral drugs in different STPs. Finally, predict the environmental concentration of   antibiotics and antiviral during a future pandemic including their risk associated with widespread use. The specific objectives were as follows:

1. To establish appropriate analytical methods for the selected antibiotics and antiviral drugs in sewage treatment plants.
2. To investigate the occurrence and removal of antibiotics and antiviral drugs in sewage treatment plants differ with technology and operation conditions, and investigate the nitrifier role in antibiotics degradation.
3. To predict environmental concentration of the target compounds during a pandemic and appraisal of appropriate technology to reduce the risk associated with widespread use under pandemic conditions.

1.4 Research structure

This dissertation consists of nine chapters. The structure of this research work is described

bellow with a general outline of each Chapter.

A background of the research with research objectives and structure is described in Chapter I. In Chapter II a literature review was summarize based on the available knowledge on antibiotics and antiviral drugs are available on their occurrences and fate in STPs, and their potential use during a pandemic.

In Chapter III, analytical methods for selected antibiotics and antiviral drugs in wastewater were established and verified. In Chapter IV, occurrences and fate of antibiotics in sewage treatment plants were investigated in Japan and China. From Chapter IV a hypothesis was drawn on antibiotics removal in STPs and in Chapter V the role of nitrifier in antibiotics removal was evaluated to establish the hypothesis.

In Chapter VI, occurrence of antiviral drugs in STPs discharge and in river water in Japan was conducted during seasonal influenza epidemic, and their fate in different sewage treatment facilities were evaluated in Chapter VII.

In Chapter VIII, antibiotics and antiviral drugs concentrations were predicted in sewage and receiving water with the knowledge obtained from the previous Chapters and literature. Environmental risk assessment of these pharmaceuticals was conducted with different concentration derived from predicted scenario. Finally ozonation as tertiary treatment technology was proposed to reduce the risk associated with widespread use in a pandemic with the help of survey information and laboratory scale batch experiments.

Chapter IX summarized the major results obtained from this research works and provides some recommendations for future works.

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CHAPTER II

LITERATURE REVIEW

2.1 Antibiotics: source and fate in the environment

2.1.1 Background

The first antibiotic was accidentally discovered by Fleming in 1928, observed inhibition of bacterial growth on exudates from the mold *Penicillium notratum*. In the late 1940s, penicillin, which had been found to be extremely useful in curing infection diseases, became commercially available.

Over the last fifty years, public awareness of the long-term effects of antibiotics has increased due to the anticipation of adverse human and ecological health effects. Industrial activities, and their resulting by-products in wastewater, have long been studied and regulated by the governments for this reason. Research has shown that many chemicals manufactured and used today enter the environment, disperse, and persist for much longer than originally expected (Koplin 2002). However, little research is available on the effects that humans, in everyday life and activities, are having on the pollution problem.

Household chemicals (e.g. detergents, deodorizers, degreasers), pharmaceuticals (e.g. hormones, steroids, antibiotics), and other personal care products (e.g. fragrances) are being washed down sinks and flushed down toilets all over the world without a second thought. Most of these chemicals are not regulated in any way and their potential health effects and acute toxicities in the environment are not known (Halling-Sorensen *et al*. 1997). In the past it was thought that the key to dealing with this type of waste was merely to dilute it by releasing the contaminated water into streams, rivers, or out to sea. However, as the human population continues to rise so to does our dependence on the earth's limited freshwater supplies. With more contaminants being released into this resource every year, the world has started to think about the long-term effects of this action.

Antibiotic usage has received a lot of attention in the media in the last several years due to the increasing numbers of pathogenic bacteria becoming resistant to traditional treatments. According to the Center for Disease Control (CDC), approximately 70 percent of infections that people get while hospitalized are now resistant to at least one antibiotic. Antibiotics are not entirely processed by our bodies when we take them for medical purposes. Some are expelled as waste and wind up in wastewater treatment plants where even the best tertiary treatment does little to disable antibiotic activity (World Health Organization 1997). This wastewater is

then released into our waterways where it gets transported to larger areas. Microbial populations in the water and sediments change when exposed to antibiotics and antibacterial agents. In some cases some tropic levels may be completely wiped out causing community structure to be remarkably changed (Wollenberger *et al.* 2000). This effect can make its way up the food chain and may be a cause of the trends we are now seeing towards lower biodiversity.

2.1.2 Fate of pharmaceuticals in body before entering the environment

Most pharmaceuticals are metabolized to phase I or phase II metabolites before being retrieved from the body with the urine and may be exposed to the environment as such. Phase I reactions usually consist of oxidation, reduction or hydrolysis, and the products are often more reactive and sometimes more toxic than the parent drug. Phase II reactions involve conjugation, which normally results in inactive compounds. Both phase I and II reactions change the physico-chemical behavior of the substance because metabolisation always renders metabolites more water soluble than the parent compounds. Berger et al., (1986) showed that *N*-4-acetylated sulphadimidine, both phase II metabolites, was converted to sulphadimidine in samples of liquid manure, and thus reactivated, the phase II metabolites into the parent compounds. Figure 2-1 shows an overview of the metabolisation of parent compound into phase I and phase II metabolites. Thus, often it is not only the parent compound which should be the subject for a risk assessment but also the main metabolites.
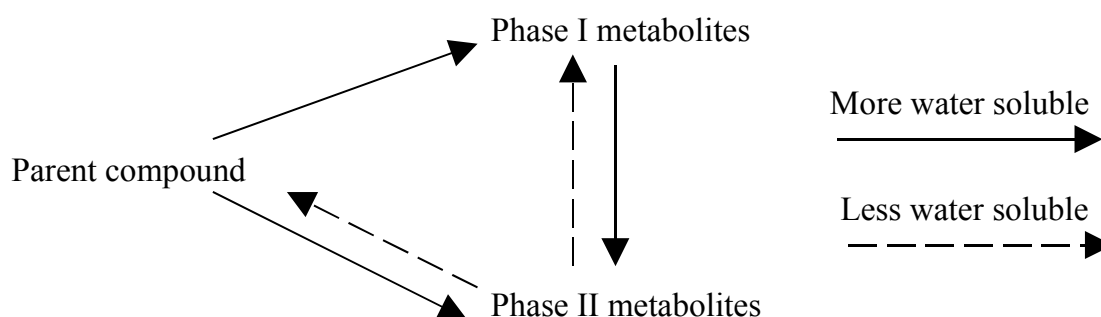
Figure 2-1 : Metabolisation of parent compound into phase 1 and phase II metabolites

2.1.3 Sources of antibiotics in the environment

A large number of antibiotics are used in human and veterinary medicine, reaching the environment through their manufactures, use and disposal. Figure 2-2 illustrated the major source of antibiotics into the environment with possible fate in different compartment of environment. After intake human antibiotics are absorbed and metabolized in the body and

execrated via urine and/or feces, then mainly enter STPs. Beside this route, direct input into environment also possible via combined sewage overflow and dispose of surplus drugs. So STPs effluent is consider as the major source of PhACs in environment. Sources of antibiotic contamination in our environment are more than just consumers expelling unabsorbed medications through excretion into septic systems and wastewater treatment plants. Effluent from pharmaceutical manufacturing plants contains antibiotics. Landfills, though considered to be contained, can also be sources. Sewage and wastewater from hospitals and veterinary clinics are also huge contributors to this problem (Rhodes *et al.,* 2000). Some of the largest sources of antibiotics in the waterways are animal farms, crop production, and fish farms (Wiggins *et al.,* 1999). In animal production, antibiotics are commonly used at sub therapeutic levels in animal feeds as growth promoters. About 24 million pounds of antibiotics are fed to farmed animals every year (Halling-Sorensen *et al.,* 1998). Pathways created by animal farming range from waste run-off, to manure being used as fertilizer, to other animals (e.g. birds, rodents) eating or transporting the treated food. In the world, around 110 tons of antibiotics are used in animal and crop production each year (Halling-Sorensen *et al.,* 1998). Effluents from water treatment facilities are deposited directly into surface water at outfall stations. Leachate from septic systems and landfills is released into the unsaturated zone, but depending on soil conditions it may seep into groundwater or spread laterally until it meets a stream or other surface water. There has not been a lot of published research about the transport processes of antibiotics. The mobility of antibiotics is anticipated to be similar to that of pesticides because many possess the same physio-chemical properties (Halling-Sorensen *et al.,* 1998). This suggests that antibiotic mobility can be modeled after known pesticide mobility models. From pesticide research, it is well known that after application, pesticides are capable of seeping into the ground to be transported into groundwater or surface waters (Jones *et al.,* 2001).
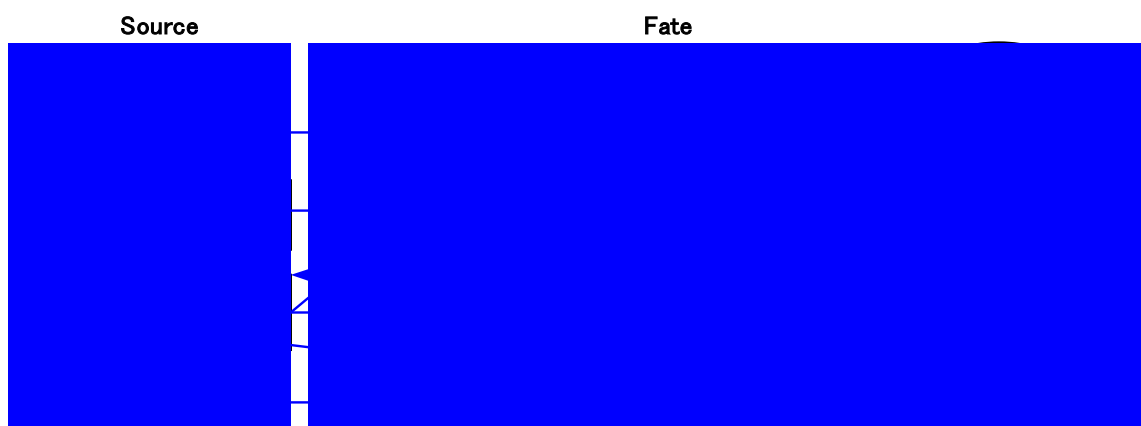
Figure 2-2: pathway of antibiotics in the Environment

2.1.4 Factor affecting antibiotics removal from wastewater in STPs

The fate of micropollutants during wastewater treatment depends on physico-chemical properties of the compound and operational parameters (biomass concentration, sludge retention time, hydraulic retention time, temperature and pH) of STPs. In the literature, sorption and biodegradation are reported to be two of the most important removal processes of micropollutants from wastewater and both processes are correlated with the availability of the substrate to the degrading microorganisms (Clara *et al.*, 2004; Clara *et al.*, 2005; Joss *et al.*, 2005).

2.1.4.1 Bioavailability

As biodegradation is the primary removal pathway for organics in the activated sludge process, the degree of bioavailability of a micro-pollutant is important (Vinken *et al.* 2004; Burgess *et al.* 2005). In STPs, the accessibility of micropollutants to the population of the activated sludge can be defined in terms of external and internal bioavailability. External bioavailability rather defines the accessibility of the substance to microorganisms, while internal bioavailability is limited to the uptake of the compounds into the internal cell compartment. In general, bioavailability consists of the combination of physico-chemical aspects related to phase distribution and mass transfer, and of physiological aspects related to microorganisms such as the permeability of their membranes, the presence of active uptake systems, their enzymatic equipment and ability to excrete enzymes and biosurfactants (Wallberg *et al.*, 2001; Cavret and Feidt 2005; Ehlers and Loibner 2006). Higher bioavailability and thus potential for biological degradation of pollutants depend mostly on the solubility of these compounds in aqueous medium.

2.1.4.2 Sorption

Sorption mainly occurs via absorption and adsorption mechanisms. Absorption involves hydrophobic interactions of the aliphatic and aromatic groups of compounds with the lipophilic cell membrane of some microorganisms and the fat fractions of the sludge. Adsorption takes place due to electrostatic interactions of positively charged groups (e.g., amino groups) with the negative charges at the surface of the microorganism's membrane.

2.1.4.3 Biodegradation

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Biodegradation defines the reaction processes mediated by microbial activity (biotic reaction). In aerobic processes, microorganisms can transform organic molecules via the succession of oxidation reactions to simpler products for instance other organic molecules or mineralized to CO_2 (Siegrist *et al*. 2004; van der Meer 2006). The degradation rates are strongly dependent upon environmental conditions, such as the redox potential of the systems and the microbial populations present. The acclimatization of microorganisms to the substrate requires time and the affinity of the bacterial enzymes for the micropollutant in the activated sludge influences the pollutant transformation or decomposition (Spain *et al*. 1980; Matsumura 1989).

2.1.4.4 Abiotic degradation and volatilization

The removal of micropollutants by volatilization during the activated sludge process depends on vapour pressure (Henry's constant) and octanol water partition coefficient (*Kow*) of the analysed micropollutant, and becomes significant when the Henry's law constant (H) ranges from 10^{-2} to 10^{-3} (Stangroom *et al*., 1989). At very low H/Kow ratio, the compound tends to be retained by particles (Galassi *et al,.* 1997; Roger 1996). The rate of volatilization is also affected by gas flow rate and therefore, high efficiency submerged aeration systems such as fine bubble diffusers should be used to minimize volatilization rates in wastewater treatment plants (Stangroom *et al*. 1989).

2.1.5 Removal mechanism of antibiotics in biological STPs
2.1.5.1 Background

The aim of a sewage treatment plants (STPs) is to remove suspended solids and easily boidegradable materia (C/N/P) from the wastewater. STPs that function on a biological principle are the most widespread. The typical treatment plants have the following treatment stages: screens, sand trap/fat separator: primary sedimentation tank, biological reactor, secondary sedimentation tank, sand filtration and chlorination (Figure 2-3).
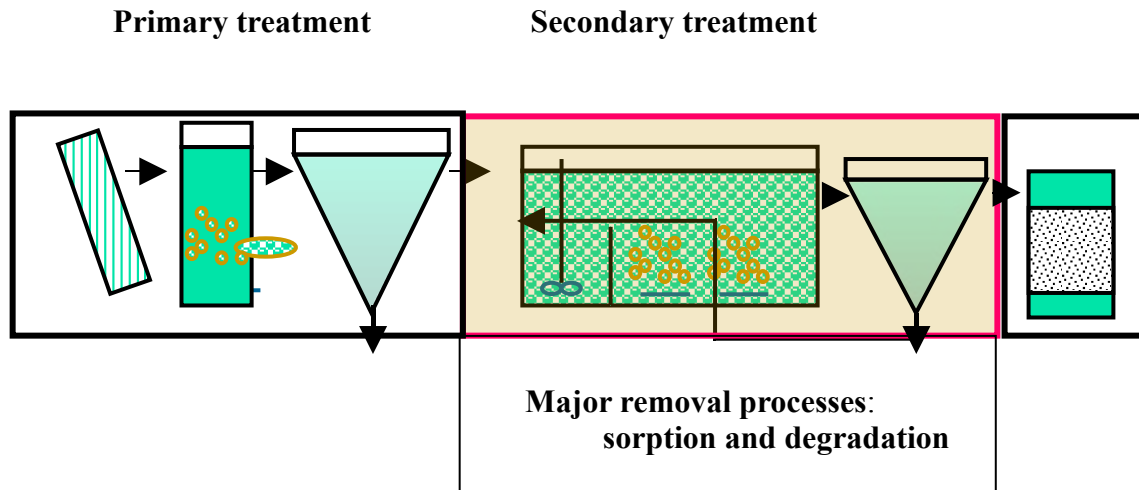
Figure 2-3: Unit operation of a conventional activated sludge system.

In a sewage treatment plant, a pharmaceutical compound and its metabolites can follow one of three patterns in behavior:

- Hydrophilic (often formed by metabolism, e.g. clofibric acid) and persistent substances, remain in the aqueous phase and may pass though the STP and therefore reach the aquatic environment.

- Degradable substance, transformed into smaller molecular entities or into carbon dioxide and water. During the treatments of the STP, chemical and biological degradation could occur extensively. Aerobic degradation by microorganisms could lead to biotransformation of the drug substance and its metabolites, with a conversion into breakdown products and some mineralisation could occur (conversion into CO_2 and H_2O). Hydrolysis and photolysis, two main chemical degradation processes, could lead to chemical transformation of the drug. Some pharmaceutical substances are susceptible to these chemical and/or biological degradation processes while others are not.

- Part of the lipophilic and not readily degradable substances will be settled in the tanks of the STPs and retained in the sludge. Hydrophobic compounds are concentrated (by several orders of magnitude) in the sludge compared with the sewage from which the sludge was derived. When the sludge is used as fertilizer and dispersed on fields, pollutants or drugs, or their biologically active metabolites, may threaten the ground water (depending on their mobility in the soil system) and hence affect terrestrial and aquatic organisms. For this reason, the agriculture use of sewage sludge is no more allowed in Switzerland (ordinance on substances).

2.1.6.2 Behavior in STPs

In primary and secondary treatment, sorption and biodegradation are major removal process respectively.

2.1.6.1 Sorption

In general, the more hydrophobic a chemical is, the greater the amount   that will accumulate in the solid phase (e.g., sludge). The   following guide to the significance of sorption can be used.

- Log Kow <2.5 Low sorption potential
- Log Kow >2.5 but <4.0 Medium sorption potential
- Log Kow >4.0 High sorption potential

The sorption is usually modeled with a simplified linear equation (1)

$$Kd = \frac{X}{Xss * S} \text{--------- (1)}$$

Kd is the sorption constant (L/g), which is defined as the partitioning of a compound between the sludge and the water phase. S (g/L) represents the concentration of suspended solids in the activated sludge tank, X and Xss (g/L) is the sludge and dissolved concentration of the substance respectively.
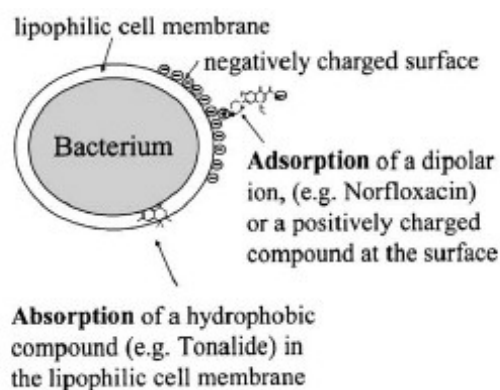
Figure 2-4: sorption mechanism of norfloxacin by Activated sludge (Siegrist et. Al., 2004)

Kd value of activated sludge of some common antibiotics was estimated from biological wastewater treatment process as well as batch experiment. For example, antibiotics: azithromycin, 0.38±0.09 L/gm; clarithromycin, 0.26±0.095 L/gm; erythromycin,0.165 L/gm.

In general there is a relation between *Kow* and *Kd* when the molecule is uncharged. But charged molecules may interact with charged moieties in the sludge (Schwarzenbach et al., 2003). As an example, charged molecules such as the fluoroquinolone antibacterial ciprofloxacin and norfloxacin (Figure2-4) exhibit high sludge–wastewater partition coefficients (log*Kd* = 4) in activated sludge reactors despite their negative log K*ow* = -1).

2.1.6.2 Biodegradation

From the published data it was observed that degradation of PhACs follows the pseudo first order reaction kinetics (equation 2).

$$\frac{dC}{dt} :$$

---------------- (2)

where C (mg/l)is total concentration, Kbio (L/gSS/d) is first order biodegradation constant, Xss is biomass concentration (g/l) and Cs is soluble concentration (mg/l).

Further the following subdivision of compound according to their degradability is proposed （Joss et. al., 2006）:

- Kbio<0.1 : no substantial removal by degradation(<20%)
- 0.1<Kbio<10: partial removal (i.e. between 20% and 90%).
- Kbio>10: more than 90% removal by biological degradation;

And as antibiotics are not presence in high concentration, so they are generally not the substrate for microorganism, as a result is was assumed that they are degraded cometabolically during degradation of substrate for energy. Figure 2-5 illustrated a conceptual removal mechanism in bioreactors
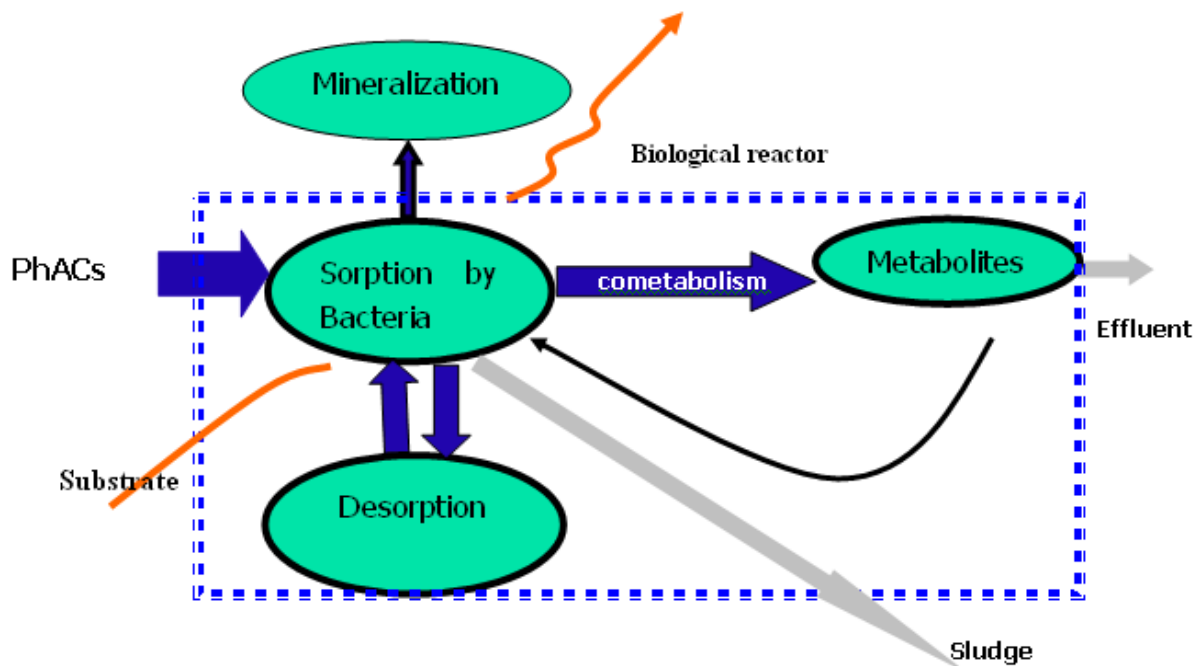
Figure 2-5: Sorption and degradation of PhACs by activated sludge

Certain PhACs have been shown to be removed more efficiently by reducing the sludge loading rate (SLR) and/or increasing the hydraulic retention time (HRT).Both these factors are ultimately determined by the sludge age (*SRT*) of the plant. Increasing sludge age results in a reduction of the SLR and an increase in HRT. This enables populations of slower growing bacteria to develop and also serves to increase the potential for the acclimatization of the population to the compounds encountered. This change in the bacterial population with time means any chemicals in the sewage are exposed to a greater array of bacteria and bacterial enzymes, increasing the likelihood that they will be degraded to less harmful compounds; however, recalcitrant polar organics may still pass through (Table 2-1). STPs employing nitrification and denitrification also exhibit significantly   lower concentrations of PhACs in effluent such as ibuprofen and naproxen .This is probably a consequence of the diverse bacterial compositions   within a nitrifying and denitrifying system. Nitrification is a highly oxygenated process, while denitrification requires anoxic and anaerobic   conditions. These differences give rise to a sequence of differing bacterial   populations, which may act synergistically and result in a greater degree   of degradation being achieved. For example, a compound may be partially   biodegraded during nitrification, with the resulting product then degraded   fully in the denitrified system.   Utilization of nitrification–denitrification and increasing the sludge age   of the majority of sewage treatment plants would (along with most

other   options) be likely to involve a number of associated environmental costs in   terms of resource and energy consumption, which would need to be balanced   against the potential benefits of a reduced pharmaceutical load in the   effluent. However, most modern treatment facilities already have these systems   in place (often in conjunction with biological phosphorus removal) to   control nutrient release. Therefore the cost would be offset somewhat by existing   legal requirements. It may be that moves to limit nutrients to receiving waters have also reduced pharmaceutical and other related contaminants. However, there may be scope to optimize pharmaceutical removal at little extra cost.

2.1.7 Fate, Degradation Pathways and Persistence of Antibiotics in the Environment

The environmental fate and degradation of antibiotics in waterways has been investigated much more in Europe and Canada than in the United States. Sorption and mobility studies may give an indication of the potential for biodegradation or persistence of antibiotics in the environment. Substances with high sorption to minerals or organic material in soils or manure are likely to have slow degradation rates, as they are unavailable for degradation by microorganisms (Jensen 2001). Unfortunately, research has shown that the physio-chemical characteristic of individual antibiotics does not always correlate with their affinity for sorption. Oxytetracycline (OTC), a commonly used antibiotic in both terrestrial and aquatic animal farming, has a K_d value of more than 1000 making it highly immobile in soil. However, the antibiotics metronidazole and olanquindox, which are used interchangeably with OTC in farming activities, are fully recoverable in leachate (Raboelle and Spliid 2000).

Table 2-1 Relation between SRT and Biodegradation of PhACs in STPs(Siegrist et al.,2004).

In aerobic soils many antibiotics used in agriculture degrade relatively quickly, with half-lives ranging from 22-80 days, into non-degradable metabolites (e.g. ceftiofur sodium, monccin and sarafloxacin hydrochloride) (Velagaleti *et al.* 1984). Soil sterilization inhibits the degradation of these substances which has led researchers to believe that micro-organisms may be responsible for their breakdown (Jensen 2001). Still, other antibiotics respond differently to breakdown. Researchers have found that drug metabolites of chlorotetracycline excreted by medicated livestock (e.g. as glucuronides) are decomposed by bacterial action in liquid manure and reconverted into active drugs (Warman and Thomas 1981).

2.1.8 Effects of antibiotics on the Environment

The results of antibiotics entering our waterways are not widely known; this is because it has only been over the last few years that people have started to become concerned about the potential effects. Another reason is that the concentrations of antibiotics found in waters are usually quite low, in the low parts per billion ranges, and there have not been reliable analytical methods to measure these low concentrations (Koplin 2002). Though individual antibiotic concentrations are low, there are so many different antibiotics that when combined they could lead to serious health and environmental problems. Little is known about the potential interactive effects that may occur from these complex mixtures, let alone the metabolites that

can be formed as they break down (Koplin 2002).

Some of the major concerns are that entire tropic levels of bacteria will be wiped out in some ecosystems or that multiple drug resistant bacteria will flourish and make its way into the food chain. Unfortunately, both of these concerns have been realized.

2.1.8.1 Effects on Biota

When evaluating the effects of antibiotics on microbial communities it is important to keep in mind that target organisms vary between antibiotics. Antibiotics may have a broad spectrum of activity or be active against one family of bacteria (e.g. gram-negative or gram-positive). Indigenous communities of bacterial and fungal populations are very complex and they have the important task of cycling nutrients. Some processes are driven by just a few species, where others, such as decomposition of organic matter, are driven by teamwork between many types of microorganisms. Proper cycling of nutrients is critical for quality soils and essential for maintaining sustainable use of agricultural lands. Nitrogen is one of the most important nutrients for agricultural systems, and its cycling is driven by only two genera of gram-negative bacteria (e.g. *Nitrosomonas* and *Nitrobacter*) (Jensen 2001). Gram-negative and wide spectrum antibiotics, such as sulfonamides and tetracycline could seriously inhibit nutrient cycling if concentrations reached high enough levels. This result has been observed in laboratory studies, but no field studies have found antibiotic concentrations at levels that would seriously disrupt the nitrification process (Jensen 2001). Oxolinic acid, which is commonly used in the fish farming industry, has been shown in laboratory experiments to be extremely toxic to *Daphnia magna*, a common freshwater crustacean; reproductive abilities were completely destroyed by levels of this antibiotic at one order of magnitude lower than acute toxic levels (Wollenberger *et al.* 2000). This may result in serious disruption of tropic levels in these areas since *Daphnia* are a major food source in freshwater systems. Calaniod copepods (*Temora turbinata*) have been shown to have decreased adult size, abnormal growth patterns and reduced egg production when exposed to OTC at concentrations above 1ppm (Halling-Sorensen *et al.* 1997). OTC also severely affects plants; studies done with pinto beans (*Phaseolus vulgaris*) showed significant depression of dry weights and root structure when watered with a solution of 10 mg/ kg (Batchelder 1982). It is unlikely that concentrations of antibiotics would be found in high enough concentrations on farms where manure from medicated animals was spread to disrupt bacterial colonies. However, if bacterial populations were altered as the result of antibiotic contamination the feeding of microbivore species like mites and nematodes, who are strongly linked to their bacterial food source, would be

significantly impacted and this trend could continue up the food chain (Beare *et al.* 1992).

2.1.8.2 Bacterial Resistance

2.1.8.2.1 Bacterial Resistance in the environment

Bacteria resistant to antibiotics have been found in the aquatic environment (Kummerer, 2004; Kim and Aga, 2007; Schluter et al., 2007; Watkinson et al., 2007; Caplin et al., 2008; Vanneste et al., 2008) and in soil (Schmidt and Rombke, 2008). Whether resistance may develop in sewage treatment plants (STPs) is currently under discussion. In biofilms the bacterial density is very high, both in the aerobic and anaerobic septic tanks of STPs, in drinking water tubes, and in sediments. Biofilms are not a taxonomic barrier to the horizontal transfer of genetic material. A prerequisite for a direct transfer of resistance is that the bacteria are able to survive, or that the genetic material is at least stable enough for transfer to the new environment, e.g. from the human body to surface water, which is colder and much poorer in nutrients. Therefore, the question is whether the input of antibiotics into the environment is an important source in the emergence of resistant bacteria in the environment, i.e. is the concentration of the antibiotic and the bacterial density high enough, is the exposure long enough to promote resistance or to select resistant bacteria, or is the transfer of resistance from already resistant bacteria following improper use of antibiotics much more important than the input of the antibiotic compounds themselves? The link between the presence of antimicrobials and the favoring of resistant bacteria as well as the transfer of resistance at concentrations as low as those found for antimicrobials in the environment is not yet established. Often, the data used to assess the environmental effects of antibiotics are not adequate to establish how long bacteria maintain antibacterial resistance in the absence of continued selective pressure for that resistance. Knowledge of sub-inhibitory concentrations of antimicrobials and their effects on environmental bacteria is scarce and contradictory, especially with respect to resistance. However, there is evidence that antibiotic resistance is already present in natural environments and that it can be exchanged between bacteria for at least a decade (Davison, 1999). Schluter et al. (2007) concluded that animal, human and plant pathogens and other bacteria isolated from different habitats (among them waste water treatment plants) share a common pool of resistance determinants that can easily be exchanged. The transfer as well as the new combination of resistance genes is most likely to occur in compartments with high bacterial density, i.e. bio-films. Some results indicate that the transfer of resistance and the selection of resistant bacteria are not favored at antibiotic concentrations as high as those found in hospital effluents or the aquatic environment (Ohlsen et al., 1998, 2003). An important source for the

resistance material found in hospital effluents, municipal sewageand STPs is the input of bacteria that have already become resistant through the use of antibiotics in medical treatment. There are reports that the widespread use of biocides such as triclosan and quaternary ammonium compounds used in hospitals and homes could select for antibiotic-resistant bacteria (Hingst et al., 1995; Russel, 2000). For example, triclosan has been shown to select for low level antibiotic resistance in *Escherichia coli* (McMurryet al., 1998), and high-level ciprofloxacin resistance in triclosansensitive *P. aeruginosa* mutants (Chuanchuen et al., 2001). However, in general, the presence of resistant bacteria and genetic material correlated with resistance do not correspond with the concentrations and activity spectrum of compounds found in the environment. For example, b-lactams have been detected in the environment at only very low concentrations and they are easily hydrolyzed at ambient temperature (Helland et al., submitted for publication; Langin et al., 2009), whereas resistant bacteria and genetic material encoding resistance against certain b-lactams have been found in STPs. Additionally, resistance against vancomycin has been found in European sewage and waters, even though only small quantities of vancomycin are used in Europe. The results of Al-Ahmad et al. (in press) and Wiethan et al. (2000) suggest that bacteria which have already become resistant through the application of antibiotics will not necessarily have a selective advantage in sewage treatment. Concentrations of antibiotics and disinfectants are normally some orders of magnitude lower in the free water phase of the environment than in therapeutic use (Lorian, 2005).The concentration of antibiotics may be much higher if the active ompounds are persistent and accumulate e.g. by sorption to solid surfaces in certain environmental compartments such as sewage sludge, sediments or soil. In these cases, the role of antimicrobial concentration could differ to that in water. It is not known how strong the antibiotics are sorbed and under what circumstances they are still biologically available and active after sorption. These findings indicate that the input of bacteria that are already resistant into the environment may be more important for the presence of resistant bacteria in the environment than the active compounds themselves.

2.1.8.2.2 Bacterial Resistance: Role of sewage and sewage treatment plants

The concentrations of antibiotics in municipal sewage and in sewage treatment plants (STPs) are typically lower by of factor of 100 compared to hospital effluent (see above). Resistant and multi-resistant bacteria such as *E. coli, P. aeruginosa, Acinetobacter, pseudomonads, Enterobacteriaceae,* and phylogenetically distant bacteria, such as members of a- and b-Proteobacteria, are present in municipal sewage as well as in the aeration tanks and

the anaerobic digestion process of STPs. Resistance against b-lactams, quinolones, tetracycline and sulfamathoxazole/trimethoprim and other sulphonamides has been found in waste water and sewage sludge all over the world using classical means such as cultivation and resistance testing as well as the detection of resistance encoding genes (Kummerer, 2004; Schluter et al., 2007). It has not yet been shown that permanent exposure to antibiotics in sewage systems promotes the development of antibiotic resistance and selective effects on bacterial communities. A multi-resistant *Acinetobacter* strain which is known to be able to survive in sewage was introduced to a laboratory-scale sewage treatment plant (LSSTP) containing a mix of antibiotics at a concentration up to 100-fold above that expected in the aquatic environment in Germany and reflecting nation-wide antibiotic use (Al-Ahmad et al., in press). The strain was resistant to seven of the antibiotics present. Despite the antibiotics present and the resistance pattern, this bacterial strain was neither detectable in the LSSTP by classical microbiological methods, nor by chemotaxonomy. Furthermore, 2 weeks after introduction of the bacterium into the treatment plant, the genetic material responsible for the multi-resistance could no longer be detected. These results suggest that the continuous input of resistant bacteria due to the application of antibiotics is by far much more important than the input of antibiotics. However, this topic requires further consideration and investigation. Resistant bacteria are eliminated quite well from sewage in STPs. Up to 99% of *Campylobacter* spp. was eliminated from sewage water through treatment in a STP. During the waste water cleaning process in a number of municipal STPs, the number of selected resistant pathogens has been reduced up to 99% (Duong et al., 2008) which seems to be a sufficient level of elimination. The input of resistant bacteria into three different STPs and their elimination was monitored (Wiethan et al., 2001). One of the STPs received effluent from several hospitals in a large community (650,000 population equivalents). The other two were located in the countryside, one of them receiving communal sewage and effluent from two convalescent homes (7500 population equivalents), the other one received only municipal sewage (14500 population equivalent). The STPs were different in process engineering. Elimination rates were 95–99% for E. coli, *Pseudomonas spp.* and *Enterococcus spp.* For resistant bacteria elimination levels were 93.5–100%. And there was no difference detected between resistant and nonresistant bacteria. These results apply for both winter and spring. A correlation between input i.e. size and number of hospitals passing waste water and the STPs was not found (Wiethan et al., 2001). However, factors like dilution effects, survival of antibiotic resistance genes (ARGs), and additional regrowth caused by agriculture or natural background load, which can be higher than human input (Edge and Hill, 2005), must also be taken into account. Another question is,

how far waste water disinfection is part of a hygiene illusion. Moreover, a spread of bacteria and resistance does not seem to be expected in the process of the generation of drinking water from surface water because of corresponding reduction potentials caused by preparation techniques (Feuerpfeil et al.,1999). In a study conducted by Engemann et al. (2006) it was found that light exposure should be maximized in receiving waters in order to maximize the loss rate for the resistance genes *tet(O), tet(W), tet(M), tet(Q)* after release. Results for other ARGs are missing. As ARGs are similar in chemical nature it can be expected that all ARGs are sensitive to light.

2.1.8.2.3 Antibiotics resistance in surface water

Bacteria that are resistant to antibiotics are present in surface water. A correlation between resistant bacteria in rivers and urban water input has been found, as have antibiotic resistance genes (ARGs) (Kummerer, 2004; Edge and Hill, 2005; Salmore et al., 2006; Watkinson et al., 2007). Vanneste and co-workers (2008) isolated plant pathogenic bacteria were isolated that were resistant to copper and/or streptomycin from waterways even in areas where no agriculture or horticulture is present and waterways are not used for crop irrigation. These results suggest that natural waterways could be a source of genes that confer streptomycin resistance. Antimicrobial resistance has also been found in marine bacteria (Neela et al., 2007) and bacteria living in estuaries or coastal waters polluted with sewage water (Kummerer, 2004; Kimiran-Erdem et al., 2007). Even in remote places such as the Arctic Sea, E. coli isolates originating from Arctic birds carry antimicrobial drug resistance. These results show that resistance genes can even be found in a region where no selection pressure exists (Sjolund et al., 2008). In a study using ciprofloxacin and ceftazidime it was concluded that the average concentrations of ciprofloxacin and ceftazidime actually found in surface water will clearly be below concentrations able to change bacterial populations. This was monitored by classical microbiological methods such as Gram staining, aminopeptidase and catalase tests as well as employment of metabolic fingerprints using the Biolog system (Wiethan et al., 2001). However, some methodical restrictions have to be taken into account in this study. Peak et al. (2007) determined the abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. The abundance of six tetracycline resistance genes *tet(O), tet(Q), tet(W), tet(M), tet(B) and tet(L),* were quantified over time in wastewater lagoons at concentrated animal feeding operations (CAFO) to assess how feedlot operation affects resistance genes in downstream surface waters. Resistance gene levels were highly seasonal with abundances being 10–100 times greater in the autumn ersus the summer.

These results demonstrate that antibiotic use strategy strongly affects both the abundance and seasonal distribution of resistance genes in associated lagoons, which has implications for water. Leaking swine waste storage pits and the land-application of swine manure can result in the dispersion of resistant bacteria to water sources. In a study by Sapkota et al. (2007) the median concentrations of enterococci, fecal coliforms, and *E. coli* were 4- to 33-fold higher in surface water and groundwater samples collected up- and down-gradient from a swine facility. Fecal bacteria counts were also elevated, indicating the animals, i.e. antibiotic application, as the source. Higher minimal inhibitory concentrations for four antibiotics in enterococci isolated from down-gradient versus up-gradient surface water and groundwater were observed. The following trend was observed with respect to the concentrations of ARGs for sulphonamides and tetracyclines by Pruden et al. (2006): dairy lagoon water > irrigation ditch water > urban/agriculturally impacted river sediments, except for sul II, a sulphonamide ARG, which was absent in ditch water. It was noted that tetracycline ARGs *tet(W)* and *tet(O)* were also present in treated drinking water and recycled wastewater, suggesting that these are potential pathways for the spread of ARGs to and from humans. The antibiotic resistance (AR) patterns of 462 *E. coli* isolates from wastewater, surface water, and oysters were determined by Watkinson et al. (2007). The rates of AR and multiple-AR among isolates from surface water sites adjacent to waste water treatment plant (WWTP) discharge sites were significantly higher (P < 0.05) than those among other isolates, whereas the rate of AR among isolates from oysters exposed to STP discharges was low (<10%).

2.1.8.2.4 Antibiotics resistance in drinking water

Antibiotic-resistant bacteria were detected in drinking water as early as the 1980s and later in the 1990s. These authors found that resistant bacteria identified using classical microbiological methods, i.e. standard plate counting, occurred within the distribution network of drinking water supply systems. They concluded that the treatment of raw water and its subsequent distribution selects for antibiotic-resistant bacteria. In agreement with these data, increased phenotypic resistance rates were also detected at drinking water sampling points (Kummerer, 2004; Scoaris et al., 2007). *Aeromonas* spp. was most often investigated. At present, there is insufficient information available to reach a final conclusion on the significance and impact of the presence of resistant bacteria in the environment which would allow for the assessment of the potential risks related, for instance, to human health and ecosystem functions. Currently, it is thought that the input of antibiotics in general as well as from hospitals seems to be of minor importance, at least in terms of resistance. Up to now,

antibiotics have not been detected in drinking water. However, is that true for all countries and places? As for health effects, antibiotics could contribute to allergic reactions if they would be present in drinking water. As this effect is primarily restricted to penicillins and especially to penicillin G, and as b-lactams are probably inactivated easily by the cleavage of the b-lactam ring, this should not be of utmost importance. The impact of antibiotics present in the aquatic environment on the frequency of resistance transfer is questionable. The information available to date suggests that the input of resistant bacteria into the environment from different sources seems to be the most important source of resistance in the environment. What has been is that it is critical to prevent the selection of resistant strains in the first place. Therefore, the prudent use of antibiotics and disinfectants will significantly reduce the risk for the general public and for the environment (Harrison and Lederberg, 1998; House of Lords, 1998; Gunnarsson and Wennmalm, 2008; Niquille and Bugnon, 2008). This not only means limiting the duration of selective pressure by reducing the treatment period and the continuous use of sub-therapeutical concentrations, but also includes controlling the dissemination of antibiotics being used, as well as prudent monitoring of resistance. A full environmental risk assessment cannot be performed on the basis of the data available. There is still a lack of fundamental data on the fate and effects of antibiotics and ARGs in the environment. The availability of such data is a prerequisite if proper risk assessment and risk management programs for both humans and the environment are to be undertaken. Drinking water should not contain any chemicals of anthropogenic origin for numerous reasons. Therefore, the prudent use of antibiotics and the restriction of their input into the aquatic environment are necessary. Starting with the life cycle of a medicinal product, three spheres of activity should be considered in which solutions to the problem addressed can be implemented (Kummerer,in press; http://www.start-project.de; Kummerer and Schramm, 2008): drug development (development of active pharmaceutical ingredients, that are optimized both for their efficacy in humans and their degradability in the environment), handling of drugs (changes in current prescription practices, utilization, and disposal patterns towards a greater degree of environmental compatibility) and technical emissions control in urban water management (optimization of wastewater disposal, wastewater treatment, and drinking water processing in the removal of pharmaceutical residues).

2.1.9 Antibiotics regulatory Issues: environmental perspective

Currently there are no regulations for the monitoring of any antibiotics in ground, surface, or drinking waters. This is because concentrations of antibiotics are generally low, in

the parts per billion ranges and are deemed by the Environmental Risk Assessment (ERA) to have no significant effect on the environment (Velagaleti and Gill 2001). Regulations that are in effect now relate to the disposal of unused or expired antibiotics under the Current Good Manufacturing Practice (CGMP) regulations set forth by the FDA (FDA 1998). This regulation calls for the incineration of all disposed of antibiotics by the manufacturer (FDA 1998). However, with more awareness of the effects of this type of pollution, the scientific community is beginning to recognize the importance of structuring plans to begin regulating as the need arises. The American Chemical Society holds yearly symposiums based on current scientific research; last years topic was: Pharmaceuticals and personal care products in the environment: scientific and regulatory issues. This topic is going to be a hot issue for years to come.

2.1.10 Position of this research

From the literature review it is understood that antibiotics are a common contaminant in sewage water and they are frequently detected. Antibiotics are partially removed in STPs. STPs with higher sludge retention time and biological nutrient facility have relatively higher antibiotics removal efficiency compare to others   but the mechanism is still hindered by the lack of information. From this point of view this study investigated a wide range of antibiotics behavior   in STPs and hypothesized that there could be a relation between nitrogen removal mechanism and antibiotics removal in STPs.   This study also investigated the occurrence and fate of antibiotics in STPs varied with technology and located in different geographical areas.

2.2 Pandemic influenza and antiviral use
2.2.1 Background

Recent outbreaks of highly pathogenic avian influenza in poultry in East Asia (H5N1), Canada (H7N3), and the Netherlands (H7N7), and their subsequent transmission to humans, have intensified concern over the emergence of a novel strain of influenza with pandemic potential. Three influenza pandemics occurred during the 20th century, with varying degrees of severity; outcomes ranged from the high levels of illness and death observed during the 1918 Spanish flu pandemic (estimates of deaths range from 20 to 100 million [Johnson et al., 2001]) to the much lower levels observed during the pandemics of 1957 and 1968 ($\approx$1 million deaths each (Nguyen-Van-Tam et al., 2003). While recognizing that the characteristics of future influenza pandemics are difficult to predict, the World Health Organization (WHO) has

recommended that nations prepare pandemic contingency plans (WHO 1999). Several have been drafted, and some have been published (Patriarca et al., 1997), although all are subject to continuous refinement. Surveillance, on both a local and global scale, will enable policy makers and practitioners to act during the early phases of a pandemic. However, the likely rapid global spread of a pandemic strain will limit the time available to implement appropriate mitigating strategies, and preemptive contingency planning is needed. A number of intervention strategies can reduce the impact of influenza pandemics. During interpandemic years, influenza vaccination is used to reduce deaths and disease. However, vaccine is unlikely to be available in time or in sufficient quantities for use during a pandemic (Webby et al., 2003, Fedson et al., 2003). Other, nontherapeutic, disease control options may be used, such as those used during the outbreak of severe acute respiratory syndrome. The neuraminidase inhibitors (NIs) reduce the period of symptomatic illness from both influenza A and B viruses (Stiver G 2003) and both are recommended for use in the United Kingdom for treatment of at-risk adults who are able to begin treatment within 48 hours of onset of symptoms. Oseltamivir is also recommended for the treatment of at-risk children >12 months of age. The development of antiviral resistance has been reported for NIs, particularly related to oseltamivir use in children, although current evidence suggests that resistant strains are pathogenically weakened. The use of NIs for treatment of pandemic influenza remains an option since they may improve individual disease outcomes and the effect of the disease in the population.

An influenza pandemic is likely to increase demands on healthcare providers, especially in hospitals. Except in Japan, current levels of NI use are low. Any strategy involving NI use would require stockpiles of these drugs. The potential use of antiviral agents for prophylaxis has been investigated elsewhere and may be of greatest use in the earliest phases of a pandemic to retard the spread of the virus. Earlier pandemic influenza modeling studies have also focused on the economic effect of vaccination (Meltzer et al., 1999) and the use of NI prophylaxis for disease control. We assessed the potential effect of using NIs for treatment on the estimated number of influenza-related hospitalizations likely to occur during a pandemic.

2.2.2 **Overview of Available Antiviral drugs**
There are two group of antiviral drugs for a widespread use during an influenza pandemic, M2 inhibitors amantadine and rimantadine (adamantanes) as well as neuraminidase inhibitors oseltamivir and zanamivir.

2.2.2.1 **Adamantane class (M2 inhibitors): Amantadine**

Amantadine inhibits the M2 membrane protein ion channel activity. This results in inhibition of the acidification of the virus interior which is required to promote fusion of the viral envelope with the endosome and for dissociation of the M1 matrix protein from the ribonucleoprotein complex (uncoating). Consequently, viral replication is blocked at an early stage of infection. Other effects may occur at later stages of the virus replication cycle. Amantadine is absorbed well with oral bioavailability, as reported in the published literature, of 62-93% in the young and 53-100% in the elderly. Amantadine is not metabolised and is excreted unchanged in urine. Thus, the plasma half-life of amantadine is considerably prolonged in patients with impaired renal function (justifying dose adjustment in case of renal impairment) and the half-life of amantadine in elderly men after multiple doses is almost double that of the young men. Average half-lives of 8.3 and 13 days have been recorded in patients on chronic haemodialysis. Amantadine inhibits human H1N1, H2N2 and H3N2 subtypes of influenza A. Amantadine (as well as rimantadine) does not inhibit influenza B. Antiviral activity has been demonstrated *in vitro* in cell culture at concentrations from 0.01 to 1.5 mg/ml (0.05-7.5 mM). Amantadine has been used for prophylaxis during the pandemics in 1968 caused by H3N2 and in 1977 caused by H1N1 influenza virus strain. Avian and equine subtypes of influenza A may also be sensitive. The H5N1 avian influenza strains exhibit different sensitivity to amantadine. While clade 1 H5N1 viruses (from Vietnam, Thailand and Cambodia) were shown to carry M2 inhibitor resistance mutations for amantadine, the more recent H5N1 strains from Indonesia, China and Eastern Europe appear to be sensitive to amantadine.

In human influenza viruses, resistance is due to point mutation in the M gene. It has been also observed that a co-infection in mice with a mixture of amantadine-resistant and amantadine-sensitive strains of influenza virus resulted in the transfer of amantadine-resistance to a sensitive strain by reassortment. It has been estimated that the overall frequency of phenotypic amantadine resistance for both H1N1 and H3N2 among influenza virus isolates in the UK 1968-1999 was 2.3%. Since then the situation has markedly deteriorated. The primary resistance has exceeded 10% in recent surveys and may be much higher in certain areas of the Far East. Influenza A viruses resistant to amantadine (rimantadine) appearwithin 2 to 5 days of treatment in about 30% of patients, illustrating the rapidity with which resistant virus can replace sensitive virus during treatment. Limited data show that drug-resistant virus was higher in treated children (up to 80%). This may be related to more intense and prolonged virus

replication in children, which could be due to the absence of pre-existing immunity. Amantadine (and rimantadine)-resistant viruses are able to transmit the disease. Amantadine-resistant viruses are cross-resistant to rimantadine and vice versa. Cross-resistance with the neuraminidase inhibitors does not occur.

2.2.2.2 Neuraminidase inhibitors: Oseltamivir phosphate-Tamiflu

Oseltamivir phosphate is an orally administered prodrug that, after absorption *in vivo,* is metabolised into oseltamivir carboxylate (OC), the clinically active metabolite. OC binds to highly conserved amino acid residues in the active site of neuraminidase, which is one of the two major surface glycoprotein antigens of influenza viruses. In influenza-infected host cells, newly formed viruses are transported to the cell membrane. The progeny viruses remain attached to the cell membrane until they are cleaved from the surface by the proteolytic activity of the viral neuraminidase. Prevention of this neuraminidase activity results in clustering of the newly formed viruses onto the surface of the host cells, thereby preventing further transmission of the viruses into adjacent cells. Prevention of the neuraminidase activity therefore effectively interrupts the virus life cycle and eventually aborts the influenza infection. Conversion of the prodrug to the active metabolite is predominantly due to hepatic esterase activity. No further metabolism of the parent or active molecules has been detected in man, and the *in vitro* studies indicated no interaction with cytochrome P450 isoenzymes. Oseltamivir carboxylate (the active compound) has a mean apparent elimination half-life of 6.3 h. Oseltamivir is eliminated mainly via kidneys in the form of the active drug. In the context of a pandemic threat, simultaneous administration of a neuraminidase inhibitor and an adamantane has been considered. *In vitro* studies suggest that the potential synergy between amantadine and oseltamivir may delay the development of resistant viral strains. A recent study examined the pharmacokinetics of oseltamivir and amantadine when given in combination. In this study, there was no indication that co-administration of either compound influenced the pharmacokinetics of the other. Therefore, the use of these two drugs in combination might be safe in a pandemic situation. However, clinical evidence for the efficacy is not yet available. Use of oseltamivir during 2009 influenza epidemic by novel H1N1 virus was successful. There are no data on the use of oseltamivir in a real pandemic situation. Very limited data are available on the use of oseltamivir in humans for treatment of influenza caused by avian influenza strains, notably the H5N1 strains. The data suggest that higher doses and longer duration of treatment may be needed than those used for seasonal influenza but the efficacy and safety of such an approach remains to be evaluated.

Oseltamivir is indicated for the prophylaxis of influenza in adults and children aged one year and older. Oseltamivir administered once daily has been shown to be effective both in seasonal prophylaxis among healthy persons and in post-exposure prophylaxis within families. In seasonal prophylaxis, the duration of medication has been up to 6 weeks, and in the post-exposure prophylaxis it has been 7-10 days. In seasonal prophylaxis among healthy adults, the protective efficacy of oseltamivir against laboratory-confirmed influenza was 74%, and against culture-proven influenza 87%. Even higher protection was observed in a study of 6-week seasonal prophylaxis among frail elderly subjects in residential home care setting; the protective efficacy against laboratory-confirmed influenza was 92%. In addition, the protective efficacy among elderly persons who had been vaccinated against influenza was 91%. In post-exposure prophylaxis in the family setting, the overall protective efficacy of oseltamivir among the contacts (aged >12 years) of an influenza-positive index case was 89%. In another post-exposure prophylaxis study that included also children aged 1 year or older, the protective efficacy was 68%.

2.2.3 Antiviral use during influenza pandemic

Influenza antiviral medicinal products and pandemic influenza vaccines have a complementary role in the management of an influenza pandemic. In contrast to pandemic vaccines, influenza antivirals can be used from the very early phase of the influenza pandemic. It is highly probable that a specific vaccine against the pandemic strain will not be widely available during the first months of the pandemic. The estimates of efficacy of the influenza antivirals in a pandemic situation are largely based on their use in treatment and prophylaxis of seasonal influenza epidemics. However, the epidemiological pattern, the disease severity and demographics of patients of the pandemic influenza may be different. These differences may have implications for the optimal posology of the antivirals. Nevertheless, the goals for the use of antivirals and vaccines should be the reduction of morbidity and mortality due to the influenza infection. The usefulness of antivirals for treatment of influenza is restricted by the need to initiate treatment within two days of the onset of clinical symptoms. Even then, the treatment effect is modest and uncertain against the pandemic strains. Early treatment of a large number of patients in a pandemic situation is a major logistical challenge that needs to be carefully considered in the national pandemic plans. For the time being, no studies have been able to demonstrate convincingly that the antivirals will reduce serious complications, hospitalisations or mortality, which is considered as the most important goals of anti-influenza therapy. This is also reflected in the difficulties in demonstrating an effect on serious

complications during the relatively mild seasonal epidemics.

Thus, successful use of antivirals during a pandemic cannot be based on treatment of symptomatic disease alone because of their limited efficacy, risk of viral resistance and the availability of the products (size of the stocks and logistics of early treatment). According to currently available clinical data and on results of modelling studies, it appears that targeted prophylaxis of influenza will be a feasible approach to the use of influenza antivirals during an influenza pandemic:

For certain groups of individuals who are essential for the key functions of the society (e.g. health care workers, decision makers, police, firemen etc.), long-term prophylaxis should be considered in the very early phase of the pandemic and until a vaccine becomes available.

For the general population, prompt treatment of index cases combined with prophylactic use by their close contacts (post-exposure prophylaxis) is potentially a useful approach to mitigate the effects of the pandemic and to slow down the progression of the pandemic at the time when an effective vaccine is not available. In certain circumstances of the early phase of the pandemic, containment of the disease may be possible. However, this approach requires a well-functioning pandemic plan, including rapid diagnostics and rapid distribution of the antivirals to the target population.

A wider long-term prophylactic use of antivirals will have a major impact on the size of the necessary stocks as well as on the distribution system. The feasibility of this approach remains uncertain. Factors which might be taken into account in the choice of antiviral product(s) may be based on the pharmacological properties of the available agents, possibilities to use them in different groups of individuals, as well as on practical aspects related to the stockpiling and distribution. In spite of the uncertainty, the recommendation takes notice of the current epidemiological situation, i.e. the widespread infection of domestic and wild birds by the H5N1 strain of influenza virus ("bird flu") – a potential ancestor of a pandemic strain.

2.2.4 Position of this research

Influenza pandemics are unpredictable but recurring events that can have severe consequences on societies worldwide from our life to natural resource. At present most of the nations have their pandemic influenza action plan which mainly focus on direct health related issue but there is less or no attention on protection of water ecosystem or even secure our drinking water supply sources. Therefore, investigation and fate of antiviral drugs in our existing protection system (STPs, fate in the environment, dilution) during seasonal influenza epidemic is necessary to provide a surrogate for better understanding and increase our

knowledge on these emerging drugs. Prediction of environmental concentration during a future pandemic and risk assessment is also necessary. Protection of water resource and reduction of environmental health risk from the technological point of view is also an important issue need to consider.

2.3 Conclusion

The main source of human antibiotics and veterinary antibiotics are STPs effluent and agricultural runoff, respectively. Antibiotics are ubiquitous in the environment: wastewater, river water, ground eater and even in drinking water. Antibiotics are removed partially in STPs and in the environment. Both sorption and biodegradation are expected to be responsible for antibiotics removal, but the biodegradation mechanism and the role of microbial communities on their degradation is still hindered by lack of information. Antibiotics are of important pharmaceuticals need to keep more attention due to their role in spread antibiotics resistance among pathogenic microorganism and as they are design to be highly bioactive.

Due to emergence of pandemic influenza virus, antiviral drugs are taking much more public attention and research interest due to their widespread use during pandemic. Still now, antiviral drugs, especially anti-influenza drug Tamiflu®, Relenza®, Amantadine, were not detected in the environment. For the first time, this study detected antiviral drugs in the environment and their removal in STPs.

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CHAPTER III

DEVELOPMENT OF ANALYTICAL METHODS FOR ANTIBIOTICS AND ANTIVIRAL DRUGS IN WASTEWATER

3.1 Developments of analytical methods for antibiotics in wastewater

3.1.1 Introduction

Antibiotics have been used for several decades in both human and veterinary medicine for their antibacterial properties and as growth promoters. Day by day antibiotics consumption is increasing in both industrialized and developing countries. The widespread use of antibiotics has led to a growing interest in their fate after consumption and excretion. These compounds are often partially metabolized in the organism and are excreted as the parent compound or as metabolites in urine and face before being discharged, in the case of human medicines, into wastewaters (Hirsch *et al.*, 1999). Soluble compounds are poorly removed by wastewater treatment plants (WWTPs) (Ternes, 1998; Daughton and Ternes, 1999; Sacher *et al.*, 2001) and may reach surface waters, where some compounds were detected at concentrations up to a few μg /L (Hirsch *et al.*, 1999; Lindsey *et al.*, 2001; Sacher *et al.*, 2001; Batt *et al.*, 2006).The occurrence of antibiotics in aquatic environments is of ecotoxicological concern because of potential ecosystem alteration. Prolonged exposure to low doses of antibiotics leads to the selective proliferation of resistant bacteria, which could transfer the resistance genes to other bacterial species (Levy *et al.*, 1997). Contamination of the aquatic environments by antibiotics and other pharmaceutical compounds has been reported in recent studies through discharge from domestic sewer systems and agricultural runoff. A nationwide survey conducted by the United States Geological Survey (USGS), Toxic Substances Hydrology Program-reported the presence of human and veterinary drugs in 80% of the streams sampled (Kolpin *et al.*, 2002). Recent advancement in analytical chemistry, liquid chromatography-mass spectrometry (LC/MS) and LC/MS/MS have experienced an impressive progress, both in terms of technology development and application. LC/MS/MS is indicated as the technique of choice to assay pharmaceuticals and their metabolites, and is especially suitable for environmental analysis because of its selectivity. Liquid chromatography coupled to mass spectrometry, or tandem mass spectrometry (LC–MS/MS) is extensively used to   monitor   antibiotics in the environment due to the high sensitivity and specificity. In previous studies, the Hydrophilic-Lipophilic

Balance (HLB) cartridge has been employed to extract trace antibiotics from wastewater samples (Göbel *et al*., 2004, Göbel *et al.* 2007, Okuda *et al.,* 2008 Ghosh *et al.* 2009).

In this study, I developed a sensitive and specific method for simultaneously analyzing twenty antibiotics in wastewater using solid phase extraction (on HLB cartridge) following LC–MS/MS analysis. The twenty target antibiotics (Table3-1), including the first to the fourth generation, were those used in human and veterinary therapy that might be detected in the environment.
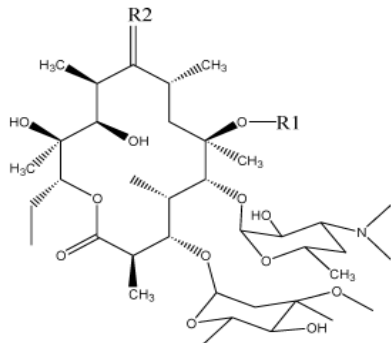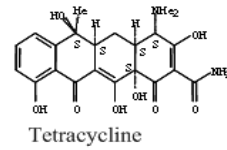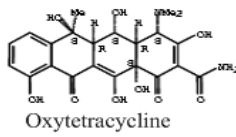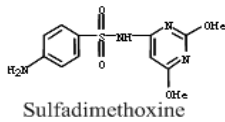
3.1.2 Methodology

3.1.2.1 Chemical and reagents

Ampicillin, azithromycin, ciprofloxacin, clarithromycin, enrofloxacin, levofloxacin, lincomycin, nalidixic acid, norfloxacin, novobiocin, oxytetracycline, roxithromycin, salinomycin, sulfadimethoxine, sulfadimizine, sulfamerazine, sulfamethoxazole, sulfamonomethoxine, tetracycline and trimethoprim were purchased from Wako pure chemical company Ltd. (Osaka, Japan) and Fluka Japan . All antibiotics were of analytical grade (purity > 95%). Formic acid (HPLC grade) and methanol (LC/MS grade) were also purchased from Wako. Individual standard solutions at 1mg/ml were prepared by weighing and dissolving in methanol. All working standards were prepared before analysis.

3.1.2.2 Sample collection and preparation

Twenty four hour composite samples of primary effluent after primary sedimentation, the secondary effluent after secondary sedimentation were collected from a STP located in Otsu, Shiga, Japan. Each 1L of sample was collected in glass bottle from primary influent and secondary effluent, and then immediately acidified (pH 3) by ascorbic acid at sampling location to reduce microbial activity, further degradation with chlorine and enhance trapping of the antibiotics on the solid-phase extraction (SPE) cartridge. Sample volume for primary effluent was 100ml and for secondary effluent it was 200mL. Finally primary effluent sample volume was adjusted to 200mL by water. After arriving at laboratory samples were filtered (GF/B, Whatman, UK) and each sample was divided in two parts, then Na_2EDTA at 1 g/L was added. One part of the sample was spiked by 50ng mixed standards and another was considered as blank sample. Recoveries were calculated by comparing spiked and blank sample. Solid-phase extraction was performe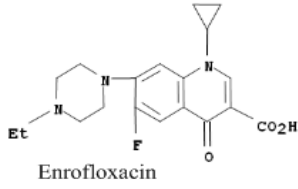d on 6-mL Oasis HLB sorbent cartridges (200 mg; Waters, MA, USA) using a Waters, Sep-Pak concentrator SPC-10. The sorbent material is a copolymer of two monomers, *N*-vinylpyrrolidone and divinylbenzol.

Ampicillin

Azithromycin

Ciprofloxacin

Enrofloxacin

Levofloxacin

Lincomycin

n-Pr

Nalidixic acid

Novobiocin

Norfloxacin

Sulfamethozazole

Salinomycin

Sulfadimidine

Sulfamonomethoxine

Sulfamerazine

Sulfadimethoxine

Oxytetracycline

Tetracycline

	R_1	R_2
Clarithromycin	CH_3	
Roxithromycin	H	

Trimethoprim
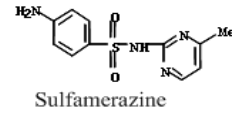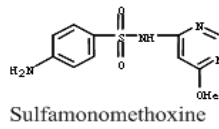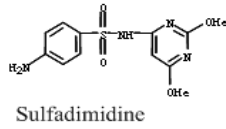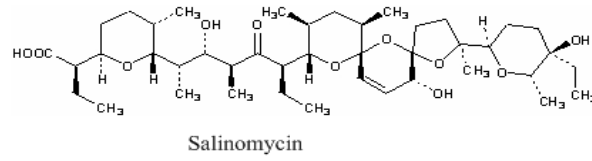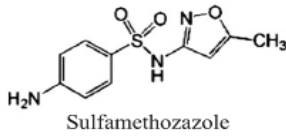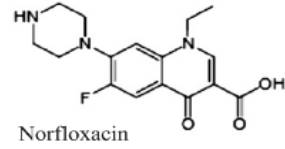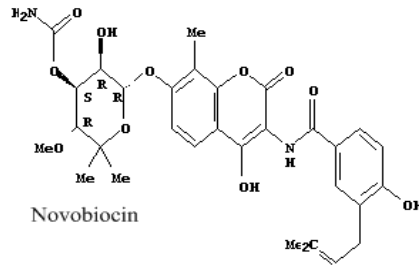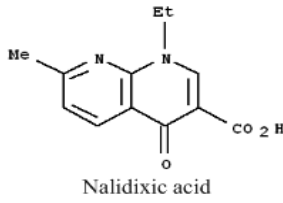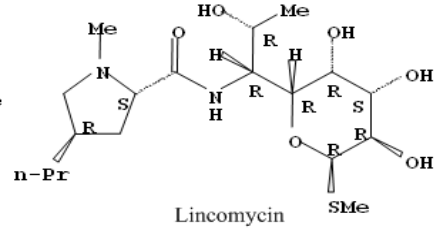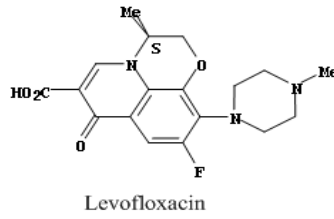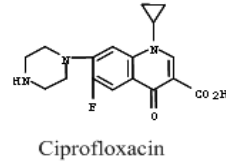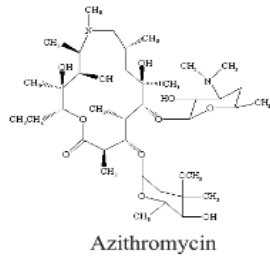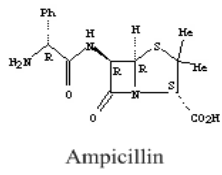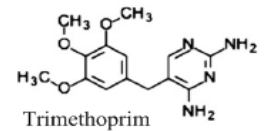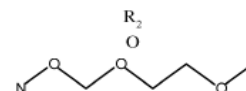
Figure 3-1 chemical structure of selected antibiotics.

The cartridges were preconditioned with 6mL methanol and 6mL water (pH ~3.5). The wastewater samples were percolated through the cartridges at a flow rate of 5 mL/min. After percolation, the cartridges were washed with 2 mL of water-methanol (95:5) and the eluent was discarded. Subsequently, the cartridges were dried completely by air for 2 h. The analytes were then

Sample Collection (1L)
(add ascorbir acid)

↓

Filtration with GF/B (1.0μm)

Sample volume 200mL   Sample volume 200mL

↓   ↓

Add 1g/LNa2EDTA + 1g/L NaCL   Add 1g/LNa2EDTA + 1g/L NaCL

↓

Spike antibiotic standards
(50 ng)

S   Solid Phase Extraction (SPE)   Solid Phase Extraction (SPE)   B
P   (Oasis HLB 6cc, 200mg Waters;   (Oasis HLB 6cc, 200mg Waters;   L
I   precondition with 4mL MeOH   precondition with 4mL MeOH   A
K   6ml Milli-Q water; Flow rate 5mL/min) 6ml Milli-Q water; Flow rate 5mL/min) N
E   K

        ◄── Washing(5% 2mlMeOH;pH 4) ──►

S   SPE cartridge dry at room temperatue   SPE cartridge dry at room temperatue   S
A   by air on vacume manifold for 2h   by air on vacume manifold for 2h   A
M   M
P   Elutade Antibiotics from SPE cartridge   Elutade Antibiotics from SPE cartridge P
L   by 6mL MeOH   by 6mL MeOH   L
E   E

        Evaporate MeOH by nitroge gas purge   Evaporate MeOH by nitroge gas purge
        to dryness   to dryness

        Re-construction to final volume 1mL   Re-construction to final volume 1mL
        (0.01% formic acid: MeOH - 9:1)   (0.01% formic acid: MeOH - 9:1)

LC-MS/MS analysis

Figure 3-2 Sample preparation for antibiotics analysis

eluted with 6mL of methanol into 10-mL graduated glass vessels. Methanol was evaporated under a gentle nitrogen stream at 37⁰C to dryness and reconstituted with acidified milli-Q water (0.01% formic acid):Methanol solution (9:1) to final volume of 1mL (*i.e.* concentration factor of 200). Final extracts were stored in 2mL glass vial (Waters) and analyzed within 6h.
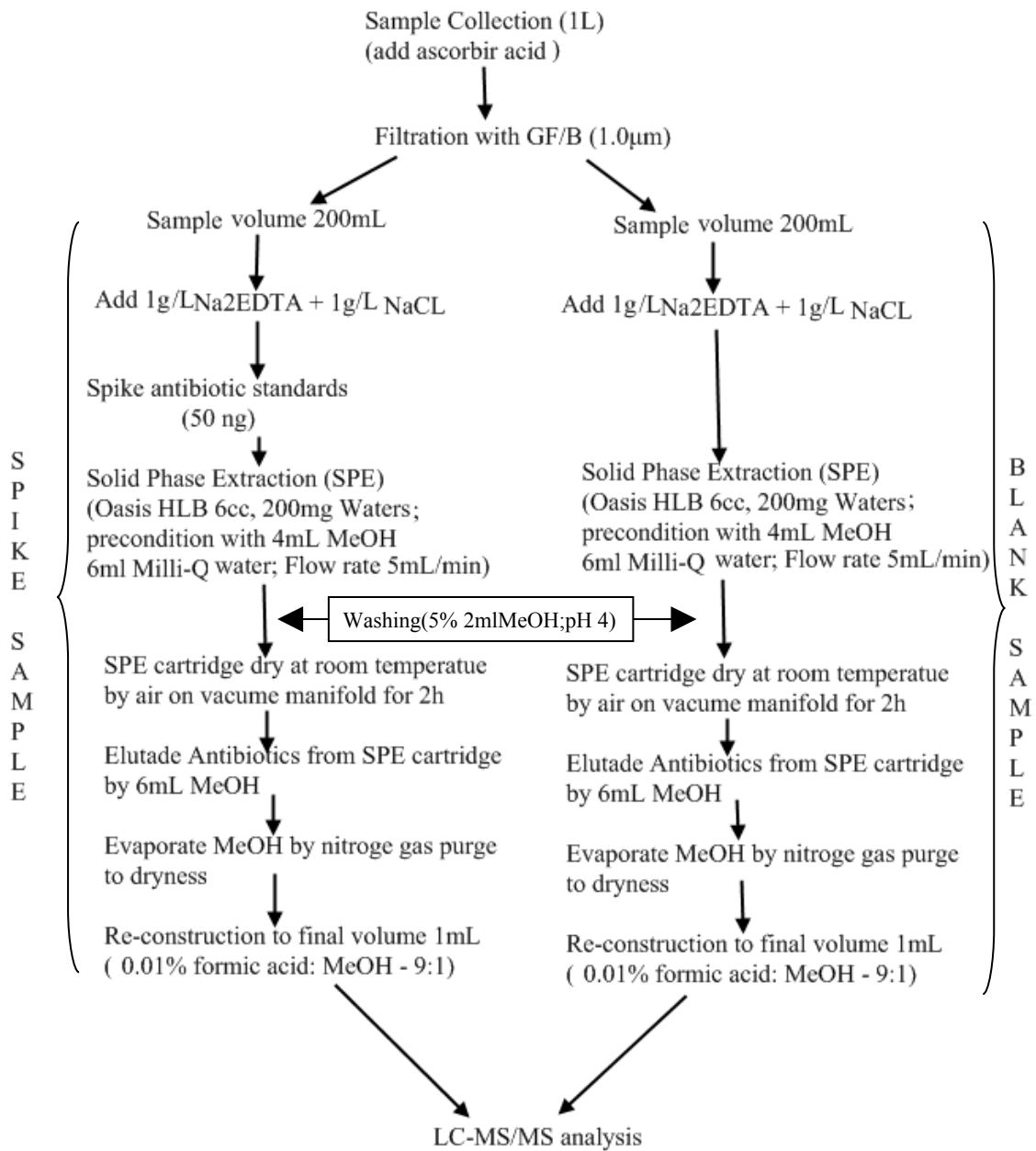
3.1.2.3 Liquid chromatography–tandem mass spectrometry

Chromatographic separation of the antibiotics were achieved with a Waters Acquity Ultra Performance™ liquid chromatography (UPLC™) separation module with a binary pump system equipped with UPLC BEH C18 column (100×2.1 mm , 1.7 μm particle size). Optimum separation was achieved with a binary gradient consisting of 0.01% formic acid (v/v) in water (solvent A) and methanol (solvent B) at a flow rate of 0.35 ml/min. The gradient elution setting was: 0–2min: 90% A; 2–8 min: 90–75% A; 8–14 min: 75–45% A; 14–16 min: 45% A; 16-19 min: 45-5% A; 19-21min: 5-90% (return to initial conditions); 21–23min: equilibration of the column. The column temperature was kept at 60°C and injected sample volume was 10 μL. The UPLC system was coupled to a Quattro Micro ™ API mass spectrometer with the Electro spray ionization (ESI) source (Waters company Ltd.).

Liquid chromatography
    UPLC AQUITY, Waters
    Column: UPLC BHE C18 100×2.1 mm , 1.7 μm particle size
    Flow rate : 0.35mL/min
    Injection volume: **10 μL**
    Binary solvent pump
    Mobile phase: 0.1%FA (A) and MeOH (B)

    Gradient:
    0–2min: 90% A;
    2–8 min: 90–75% A;
    8–14 min: 75–45% A; 1
    4–16 min: 45% A;
    16-19 min: 45-5% A;
    19-21min: 5-90% ;
    21–23min: equilibration of the column
MS/MS

Quattro Micro ™ API
Ionization : Electro spray ionization (ESI)
Electrospray source block: 120 °C
Desolvatation temperature: 400 °C
Capillary voltages: 0.5 kV;
cone and desolvatation gas flow 50 and 900 L/h respectively

The instrument control, data acquisition and quantification were performed by Mass Lynx 4.1 software

Figure 3-3 UPLC-MS/MS operating condition for antibiotics analysis

Table 3-1 Target antibiotics list and optimized UPLC–ESI–MS/MS conditions for the analysis of antibiotics residues by MRM in positive and negative ion mode

Antibiotics (ESI mode)	Precursor ion (m/z)	Product ion (m/z)	Cone Voltage(V)	Collision Energy(eV)	Retention Time (min.)	LOD (ng/L)
Sulfonamides						15
Sulfadimethoxine(+)	311	155.9	30	20	4.40	23
Sulfadimidine(+)	279	185.9	25	15	4.9	14
Sulfamerazine(+)	265.2	155.9	25	18	3.2	13
Sulfamethoxazole(+)	254	155.9	25	15	5.8	16
Sulfamonomethoxine(+)	285	155.9	25	15	3.97	18
Macrolides						
Azithromycin(+)	749.5	591.4	40	25	10.9	12
Clarithromycin(+)	748.9	157.9	30	20	14.8	6
Roxithromycin(+)	837.7	679.4	25	20	15.0	18
Quinolones						
Ciprofloxacin(+)	332.2	231	25	35	5.4	12
Enrofloxacin(+)	360.2	245.2	30	26	6.1	17
Levofloxacin(+)	362.1	318	30	20	4.8	18
Nalidixic acid(+)	233.3	215.1	35	14	3.6	13
Norfloxacin(+)	320.2	276.2	25	18	4.9	13
Beta lactam						
Ampicillin(+)	350.3	105.8	16	20	6.2	9.8
Tetracyclines						
Tetracycline(+)	445.1	409.9	20	20	3.8	30
Oxytetracycline (+)	461.1	425.9	20	20	5.5	25
Others						
Lincomycin (+)	407.5	126.1	30	22	4.47	12
Novobiocin (-)	611.3	205.2	50	50	3.2	19
Salinomycin (+)	749.6	241.2	60	35	19.5	19
Trimethoprim(+)	291.4	230.2	35	20	4.42	2

During quantification optimization, each antibiotic was individually infused as a standard solution into the initial mobile phase (50%solventA: 50%solventB) directly into the mass spectrometer at a concentration of 5 mg/L. During the infusion, the parameters (*e.g.* cone voltage, collision energy, ionization mode) were optimized for each compound in order to obtain the maximum sensitivity with the highest amount of product ions available and the

most sensitive Multiple Reaction Monitoring (MRM) transitions were determined for each antibiotic. Table 3-1 shows the MS/MS parameter optimized for transitions selected in the multiresidue quantitative method. The parameters of the mass spectrometer were as follows: electrospray source block and desolvatation temperature: 120 and 400°C respectively; capillary voltages: 0.5 kV; cone and desolvatation gas flow 50 and 900 L/h respectively. The instrument control, data acquisition and quantification were performed by Mass Lynx 4.1 software.

3.1.2.4 Validation of the analytical procedures

For the method validation, 24h composite samples from a STP (Shiga, Japan) were taken. Breakthroughs were determined by extracting spiked wastewater samples (duplicate analyses) using two stacked cartridges. A breakthrough on the first cartridge triggered enrichment on the consecutive cartridge, which was then eluted separately. For the primary effluent and secondary effluent sample with a spiked mixed analyte of 500ng was used and extracted. Complete elution of the cartridges was verified by eluting cartridges of spiked samples for a second time with 3 mL of acetone as a stronger solvent. The acetone extract was then treated as a separate sample. Instrumental limits of detection (LODs) and limits of quantification (LOQs) were calculated on the basis of standard deviation of the repeated measurement ($n = 5$) of a standard mixture (100 pg on column). The LOD is defined as 3 times and the LOQ as 10 times the standard deviation. If the resulting value for the LOQ was below the linear range, the lower limit of the linear range was set as LOQ. Sample-based LOD and LOQ were defined as concentrations in a sample matrix resulting in peak areas with signal-to-noise ratios (S/N) of 3 and 10, respectively. Since samples typically contained analytes in higher amounts, the concentration corresponding to the defined S/N was determined by downscaling, using the measured concentration and the assigned S/N of the peaks assuming a linear correlation through zero. Instrumental precision of the measurement was assessed using an average of five independent injections of 100 pg on column of a standard mixture. The precision of the entire method was determined using three replicates of each matrix investigated, spiked with 50 ng of analyte prior to extraction. It is indicated by the relative standard deviation of the measured concentrations of native plus spiked analyte. For recovery studies over the entire procedure, wastewater samples (duplicate analyses) were spiked prior to extraction with standard and with 25 and 50 ng of analytes, respectively. The calculated amount of antibiotics minus the amount already present before spiking was then divided by the spiked concentration.

3.1.2.5 Identification and Quantification

For each antibiotic, the highest transitions of the precursor and daughter ion were monitored. Together with the retention times, they were used to ensure correct peak assignment and to evaluate peak purity. An external calibration curve, plotting peak area against concentration, was obtained by diluting standards in a solution containing 0.01% formic acid in water and MeOH of 9:1 ratio respectively. A standard curve was acquired at the beginning and at the end of a measurement series. At least five concentration points in the appropriate concentration range were used for quantification. Concentrations in the samples were calculated by comparing the peak area ratios of the analytes in blank sample and their spike standards in the SPE extracts. These results were corrected with the corresponding recovery rates obtained in the same matrix and sample batch to provide accurate amounts. For routine determination, duplicate analyses of all samples were performed. Procedural blanks, consisting of Milli-Q water, were analyzed with each set of 2 extractions as a control for laboratory contamination. Additional instrumental blanks using Milli-Q water were checked with each calibration curve in order to uncover potential analytical interferences.

3.1.3 Result and discussion
3.1.3.1 Method Development

The crucial parameters for enrichment, separation, and detection of the analytes were identified and optimized. The pH of the sample proved to be the most influential variable during sample extraction. A critical impact on the retention of the analytes on the cartridge material was observed, especially for sulfonamides caused by their amino groups. The enrichment tests between pH 2 and 6 revealed, as expected, highest recoveries at pH 4 for the sulfonamides, while the recovery of the macrolides and trimethoprim showed no strong pH dependence. This behavior can be explained by the charge state of the sulfonamides at the particular pH values. The interaction with the cartridge material is strongest for analytes in uncharged forms occurring at a pH of 4 in the case of the sulfonamides. The dilution of the primary effluent samples prior to enrichment additionally increased signal intensity provided by the mass spectrometer in most cases by a factor of 2. For the macrolides and trimethoprim, no significant improvement was observed. The higher the sample volume the more matrixes will be introduced into the mass spectrometer within one run. On the other hand, a high enrichment factor is desirable to achieve the low limits of detection, which are necessary for the environmental analysis of antibiotics. The sample volume was optimized by cartridge

breakthrough study. The matrix effect is a notable problem in LC/MS/MS analysis due to the co-eluting interference. In this study, I compared the chromatograms of target antibiotics in the same sample extracts with and without washing (5% MeOH; 2mL) after concentration on cartridge. It was found that the signal/noise (S/N) ratios for most of target antibiotics were greatly improved by the washing procedure.

3.1.3.2 Method Validation

The developed method was validated for primary effluents and secondary effluents. For breakthrough studies, samples representing unnaturally high concentrations and high loads of sample matrix were enriched on two stacked cartridges. No quantifiable amounts of the analytes could be detected on the second cartridge for both primary and secondary effluent sample matrixes. When testing for complete elution, no quantifiable amounts of analytes could be measured in the acetone eluates of already eluted cartridges. Thus, the analytes are quantitatively enriched by one cartridge and exhaustively eluted by the procedure described above. For the standard curves, good linearity was observed with correlation factors typically above 0.99. The instrumental LOD ranges between 2 and 30 ng/L (Table 3-1). Since the LOD and LOQ in an individual sample can be higher or lower than the average LOD and LOQ, all concentrations resulting from peaks with S/N greater than or equal to 3 and 10, respectively, are considered valid results. The instrumental precision of the method was addressed and the following relative standard deviations were obtained: the retention time ranged between 0.06 and 0.35% and the peak area between 1.3 and 9.2%. The precision of the entire method (reproducibility) is indicated by the standard deviation of multiple analyses and ranged between 3 and 15%. Accuracies of the method were determined by recovery studies over the entire procedure. The resulting recoveries obtained in all matrixes investigated were generally above 70% and bellow 130%. Recoveries, and thereby LODs and LOQs, of the analytes vary between samples, mainly due to varying matrix effects. The values for the combined measurement uncertainty vary between 2 and 18% with the analyte and the matrix investigated.

3.1.3.3 Wastewater Applications

The developed method was successfully applied to the analyses of wastewater samples from a STP located in Shiga prefecture, Japan. In the STP, mechanically treated

wastewater (primary effluent) passes through conventional activated sludge treatment, followed by secondary settling (secondary effluent). Chapter IV shows the results obtained from duplicate analyses of 24-h composite samples of the primary and secondary, effluents in different STPs.

3.2 Developments of analytical methods for antiviral drugs in wastewater

3.2.1 Introduction

Influenza virus infections continues to cause significant morbidity and mortality worldwide and placing a considerable economic burden on individuals, families, businesses and healthcare providers. Seasonal influenza epidemics cause tens of millions of respiratory illnesses and 250,000 to 500,000 deaths worldwide each year (WHO 2003). During the 20th century, influenza pandemics caused millions of deaths, social disruption and profound economic losses. The outbreak of Spanish flu in 1918 was the most major one, causing estimated 40 million deaths worldwide including 390,000 in Japan (MHLW 2005). Influenza pandemics occur when a new strain of the influenza virus is transmitted to humans from another animal species. Species that are thought to be important in the emergence of new human strains are ducks, chickens and pigs. Recent emergences of highly pathogenic avian influenza virus (H5N1) and report on influenza virus resistance against available antiviral drugs are of great concern. The available options for the control of influenza are limited and vaccination serve as the primary defense against influenza but due to the rapid change in viral antigenic determinants, annual vaccination is required and is not always effective. At present, two groups of antiviral drug have become available for the treatment of influenza infections are the neuraminidase inhibitors (*e.g.* Tamiflu®) and the M2 ion channel inhibitors (*e.g.* Amantadine). Oseltamivir phosphate (OP) (Figure 3-4) which is marketed as Tamiflu®, is recommended by World Health Organization (WHO) for both treatment and prophylaxis of influenza, and considered an important first-line defense in the event of a future influenza pandemic. OP is a prodrug which is rapidly and extensively hydrolyzed *in vivo* to its active metabolite oseltamivir carboxylate (OC) (Figure 3-4), a potent and selective inhibitor of influenza A and B virus neuraminidase. OC is excreted (over 80% of oral dose) unchanged (Sweetman, S.C., 2007). Amantadine was approved by the U.S. Food and Drug Administration in October 1966 as a prophylactic agent against Asian influenza and eventually received approval for the treatment of Influenzavirus A in adults. In 1969 the drug

was also discovered by accident to help reduce symptoms of parkinson disease and drug-induced extrapyramidal syndromes. Recent development of neuraminidase inhibitor suppressed the use of amantadine in developed countries but still widely use in developing countries

Recently, Tamiflu is widely used in Japan for influenza infection. Like many other pharmaceutically active compounds, STPs discharge will be the main source of OC (as OC excreted unchanged from body via urine and face) in surface water environment, if this drug is not remove significantly in the STPs. The most subtype of influenza A viruses are circulate in waterfowls where they remain, multiplies and excreted in large number through droppings (Olsen *et al.* 2006). Interestingly, waterfowls stay close to the treated wastewater discharge points, where temperature is relatively higher than surrounding and have enough available foods, especially in winter-the time of common seasonal influenza. Therefore, widespread use of the antiviral OP to fight seasonal influenza in humans could actually lead to the development of OC resistant strains of the viruses in wild birds (Fick *et al.* 2007 and Singer *et al.* 2007). Beside virus resistance, a mass administration of OP during future pandemic condition is also an important issue for drinking water safety and ecological health risk.

In this study I developed a method for OC and AMN detection in STPs discharge and in river water using solid phase extraction, followed by liquid chromatography tandem mass spectrometry (SPE-LC/MS/MS).

3.2.2 Methods

3.2.2.1 Chemicals and reagents

Oseltamivir carboxylate (OC) and oseltamivir carboxylate label with deuterium (D3-OC) were provided by F. Hoffmann-La Roche Ltd, Pharma Research, and Basel, Switzerland. Acetonitrile (LC/MS grade), methanol (LC/MS grade), formic acid (99.9%, for MS), acetic acid, sodium chloride and ammonium hydroxide were purchased from WAKO, Japan. Ultra pure water was generated with a Milli-Q Integral water purification system (Millipore, MA). Individual standard solution of OC and D3-OC at 1mg/10ml was prepared by weighing and dissolving in methanol. All working standards were prepared during experiment and analysis. Amantadine (AMN) was bought from Sigma-Aldrich (Taufkirchen Germany). Standard solution of AMN was prepared in ethanol at 1mg/ml.

AMN
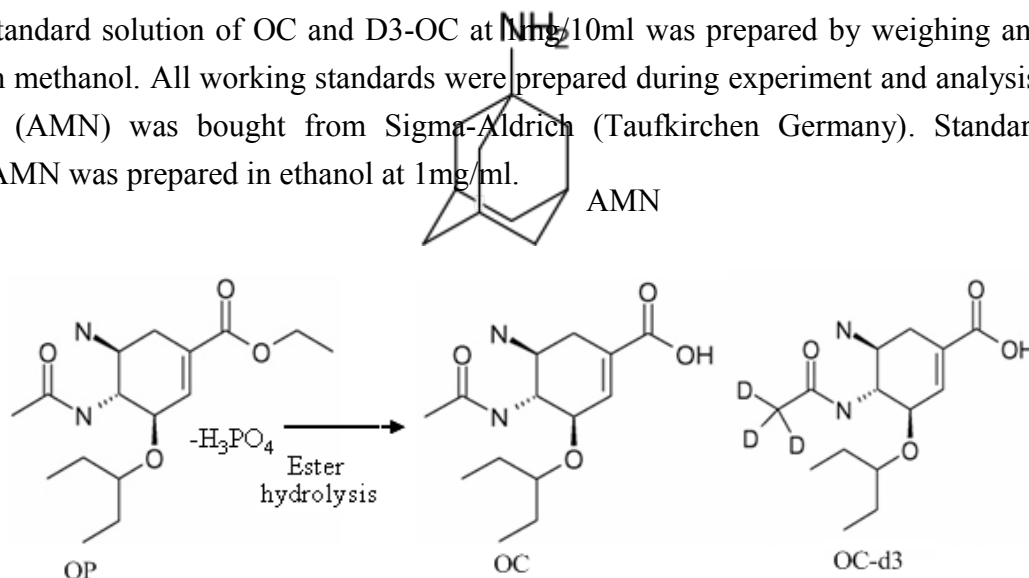
OP   OC   D3-OC

Figure 3-4 Chemical structure of selected amantadine (AMN), oseltamivir phosphate (OP), oseltamivir carboxylate (OC) and tri-deuterium surrogate standard of OC (D3-OC).

3.2.2.2 Sample Collection and Preparation

Grab samples of the STP discharge and the river water were collected into glass bottles. Ascorbic acid (1g/L) was previously placed in the bottle for acidifying the samples, quenched chlorine/ozone and to enhance analyte sorption on solid phase extraction cartridge. All the samples were filtered (GF/B, pore size 1μm) as soon as possible but no later than 5 h after sampling. The filtered samples were immediately extracted or kept at -20 °C in half-filled amber glass bottles in horizontal position until extraction. The sample volumes were 200mL for primary effluent, 300 mL for final effluent and 300mL for river water. Sample volume was selected after cartridge breakthrough study (same as antibiotics). After filtration sodium chloride (1 g/L) was added and sample pH was adjusted to 4 with sulfuric acid if needed, and the surrogate standard D3-OC (50 ng) was added. Solid-phase extraction was performed on 6-mL Oasis HLB sorbent cartridges (200 mg: Waters, Corp., Milford, MA) using concentrator. The cartridges were preconditioned with 3ml of methanol and 3ml of milli-Q water (pH 4). All samples were passed through the cartridges at a flow rate of 5 mL/min. After concentration, the cartridges were dried completely by air on vacuum manifold for 2 h. The analyte were then eluted from the cartridge with 6 mL of methanol containing 2% ammonia and passed through Sep-Pak® Plus NH2 (350mg: waters Corp., Milford, MA) cartridge (for clean up) into 10-mL graduated glass vessels and dried by a gentle flow of nitrogen at 37^{0}C temperature. The sample final volume was adjusted to 0.5 mL with water containing 20% acetonitrile (concentration factor of 600).

3.2.2.3 Liquid chromatography–tandem mass spectrometry

Chromatographic separation of OC and D3-OC were achieved with a Waters Acquity Ultra Performance™ liquid chromatography (UPLC™) separation module with a binary pump system equipped with UPLC BEH C18 column (100×2.1mm, 1.7 μm particle size). Optimum separation was achieved with a binary gradient consisting of 0.1% formic acid (v/v) in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.35 ml/min. The gradient elution setting was: 0 to 2 min: 10% B; 2 to 4 min: 10 to 75% B; 4 to 4.30 min: 75 to 90% B; 4.30 to 5.30 min: 90% B; 5.30 to 5.80 min: 90% to 10% B; 5.80 to 8.0 min: 10% B (equilibration of the column). The column temperature was kept at 60°C and injected sample volume was 10 μL. The UPLC system was coupled to a Quattro Micro ™ API mass spectrometer with the electrospray ionization (ESI) (Waters company Ltd.).During quanoptimization OC, D3-OCand AMN was individually infused as a standard solution into the initial mobile phase (50% solvent A: 50% solvent B) directly into the mass spectrometer at a concentration of 1mg/L. During the infusion, the parameters (cone voltage, collision energy, ionization mode) were optimized for each compound in order to obtain the maximum sensitivity with the highest amount of product ions available and the most sensitive Multiple Reaction Monitoring (MRM) transitions were determined. Supplementary documents shows the MS/MS parameter optimized for transitions selected in the MRM method. The parameters of the mass spectrometer were as follows: electrospray source block and desolvatation temperature: 120°C and 400°C respectively; capillary voltages: 2.5 kV; cone and desolvatation gas flow 50 and 900 L/h respectively. The instrument control, data acquisition and quantification were performed by Mass Lynx 4.1 software.

3.2.2.4 Method Validation

OC, D3-OC and AMN were analyzed with MRM at the highest precursor ion/product ion transitions. Positive identification of OC was based on UPLC retention time compared with that of D3-OCsurrogate standard. Seven point's calibration curves were constructed for quantifications, ranging between 0.5 ng/mL and 400 ng/mL. The accuracy of the overall analytical   procedure were evaluated with blank river and STP discharge water samples i.e. sample free of OC (collected in summer -non influenza season), spiked at 100ng/ L in quintuplicate and analyzed in quintuplicate, finally compared to a direct injection of a standard   mixture at the same concentration. Blank samples were previously analyzed   to confirm the absence of any significant peak at the selected   transition. Reproducibility was

assessed by five times injections of OC free sewage discharge and river water spike at 50ng/L.The method was considered accurate if recoveries were in the 70–140% range, and precision was satisfactory if the relative standard deviation was lower than 15%. Instrumental limits of detection (LOD) and limits of quantification (LOQ) were calculated on the basis of standard deviation of the repeated measurement (n =5) of a standard solution (100 pg on column).The LOD is defined as three times and the LOQ as ten times the standard deviation. In sample OC an AMN was consider to be quantifiable when the signal of the peak was ten times higher than the background noise.

3.2.3 Results and discussion
3.2.3.1 LC-MS/MS parameter

The optimal LC/MS/MS conditions are important for the unequivocal identification of antiviral drugs at very low levels in environmental samples. Since the ESI is largely dependent on the solvent conditions, the mobile phase composition is usually investigated. In this study, a acetonitrile/water containing formic acid was used since this mobile phase composition produced better separation and a 2–4-fold increase in the signal intensity, as compared to the methanol/water containing formic acid for OC.

Using a 1.7μm particle size UPLC column, very sharp peaks were obtained, with peak widths of 10–15 s. This means that the UPLC system has high sensitivity and separation. The target antiviral drugs were analyzed by MS/MS in the MRM mode. The cone voltage and collision energy were optimized for each compounds by injecting standard solutions into the mass spectrometer to generate the most abundant MRM transitions for the purpose of specification and quantification (Table 3-2). In their mass spectra, all the precursor ions were protonated molecular ions ([M +H]+) (Figure3-5 and Figure 3-6).

3.2.3.2 Solid phase extraction

The pH of the sample proved to be the most influential variable during sample enrichment on solid phase extraction cartridge. The effect of pH on the extraction efficiency in pure water for the cartridge was studied by adjusting pH value of the sample at 4.0, 7.0 and 11.0. Increase in pH led to a reduction in the extraction efficiency of OC and AMN. In the light of the result we selected pH 4.0. For breakthrough studies, river and STP influent and effluent samples (100,
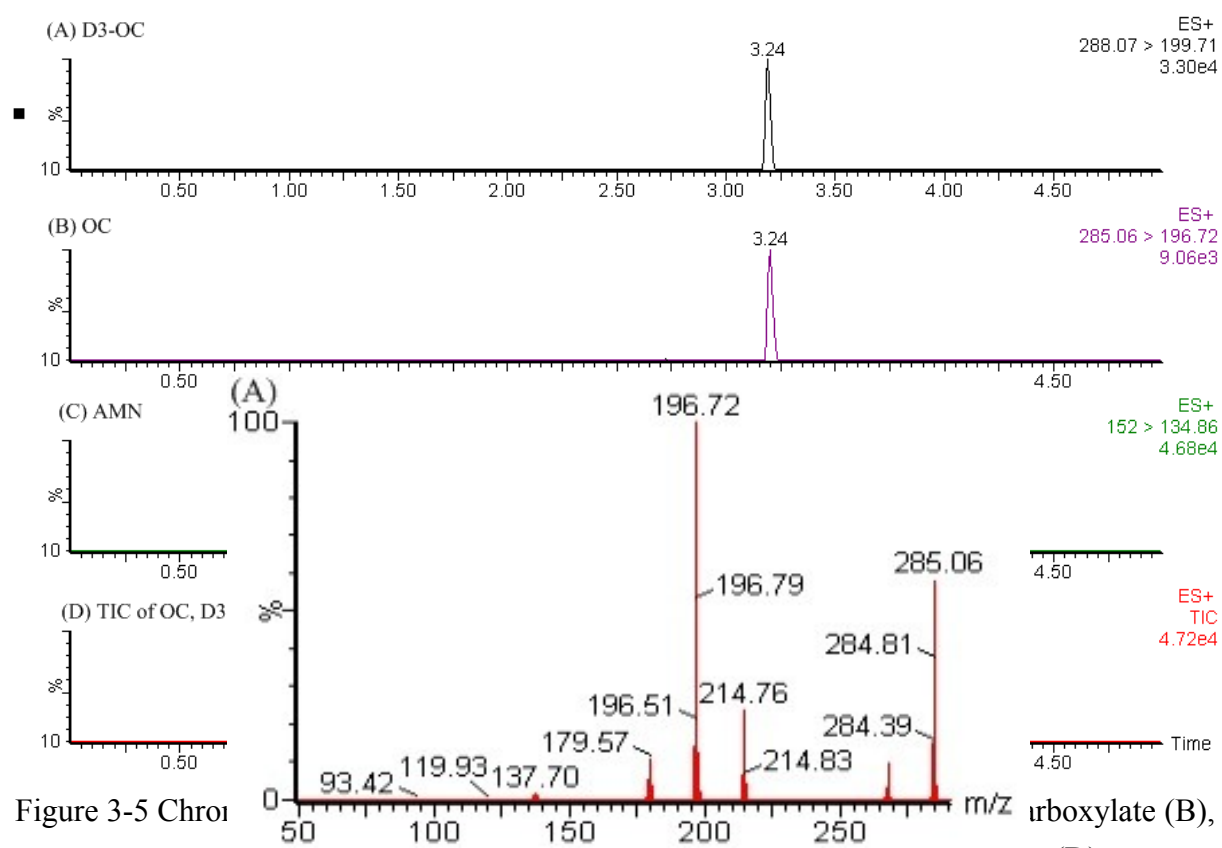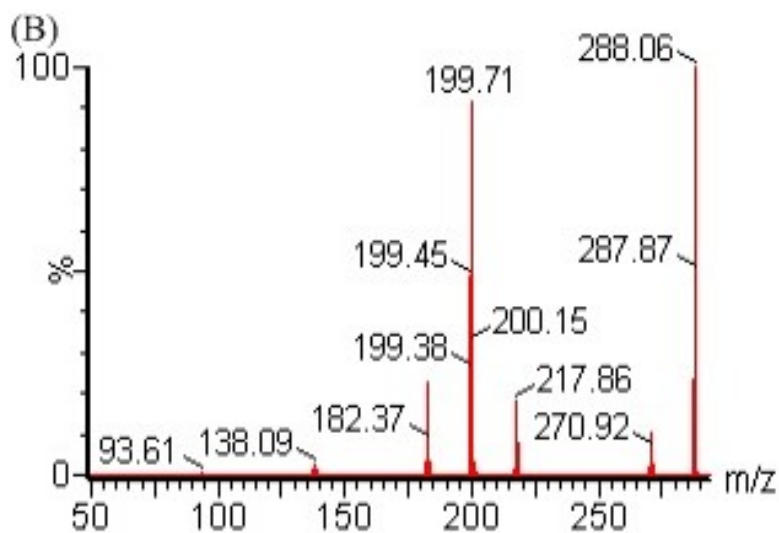
(A) D3-OC

3.24

%

10

0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50

(B) OC

3.24

%

10

0.50 4.50

(C) AMN

%

10

0.50 4.50

(D) TIC of OC, D3

%

10

0.50 4.50

Time

(A)

100

196.72

196.79

285.06

284.81

196.51 214.76

179.57 284.39

93.42 119.93 137.70 214.83

0

50 100 150 200 250 m/z

Figure 3-5 Chroi ... irboxylate (B), amantadine (C), surrogate standard of OC (B), Total ion chromatogram (D)

(B)

100

288.06

199.71

199.45 287.87

200.15

199.38

182.37 217.86

93.61 138.09 270.92

0

50 100 150 200 250 m/z

300, 500 and ... and sample matrix
was enriched ... r primary effluent
and 300ml fo ... ch were maximum
volumes with ... surrogate standard
D3-OC was ... their similarity in
physico/chem ... d by one cartridge
and exhaustiv ... Plus NH2 cartridge
for clean up ... ess and instrument
sensitivity sig

(C)

100

151.91

134.84

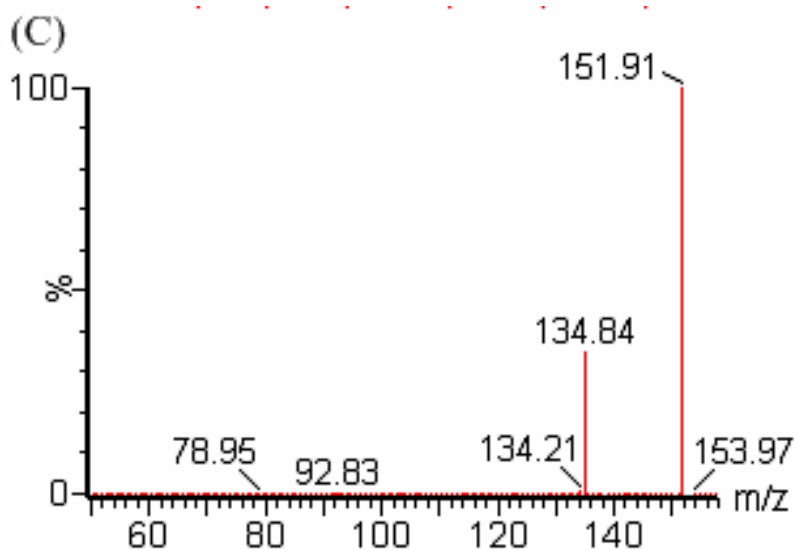78.95 92.83 134.21 153.97

0

60 80 100 120 140 m/z

Figure 3-6 Mass spectral fragmentation of Oseltamivir carboxylate(A), Oseltamivir carboxylate surrogate standard (B) and amantadine (C)

Table 3-2 MS/MS parameter, methods precision, accuracy and limit of detection of oseltamivir carboxylate and amantadine

Analyte	CV (V)	CE (eV)	PI (m/z)	DI (m/z)	RT (min)	Precision RSD (%); N=5	Accuracy Recovery ± SD(%); N = 5	LOD ng/L
OC	20	10	285.1	196.7	3.24	9.5	Primary effluent :128 ± 11 Secondary effluent :120 ± 7 River water : 110 ± 8	6
D3-OC	20	10	288.1	199.7	3.24	-	-	-
AMN			152	134.86	2.91	4.2	Primary effluent :72 ± 8 Secondary effluent :81 ± 6 River water : 92±5.2	4

Oseltamivir carboxylate mass fragmentation

Amantadine mass fragmentation
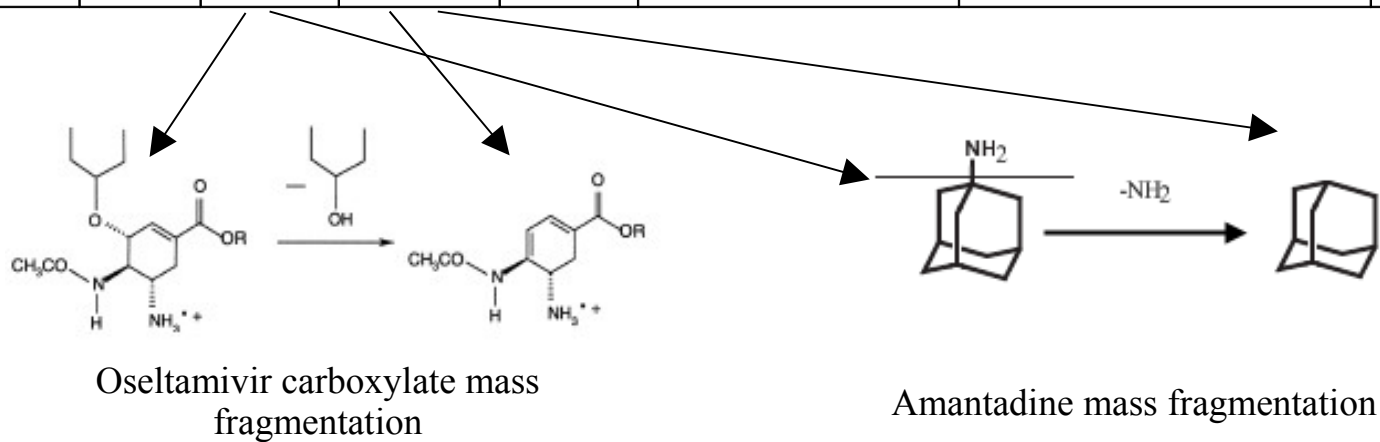
3.2.3.3 Validation and application.

For the standard curves, linearity was observed with correlation factors typically above 0.99 between 5pg to 4000pg on column for OC and 2pg to 5000pg for AMN. The precision of the entire method (reproducibility) was ranged between 4 and 9.5 % for OC and 4.5% for AMN. The resulting recoveries of OC obtained in primary effluent, secondary effluent and river water were 128 ± 11,120 ± 7 and 110 ± 8 % respectively. The extraction yield in the sample was determined by peak area ratios of OC/D3-OC and successfully applied (Figure 3-7). The method maximum values of LOD and LOQ were 3.6 ng/L and 12 ng/L respectively. The representative chromatogram of OC and D3-OCat LOQ is in Figure 3-7. The developed method was successfully applied to the analyses of river water and sewage treatment plants discharge (Figure 3-5).
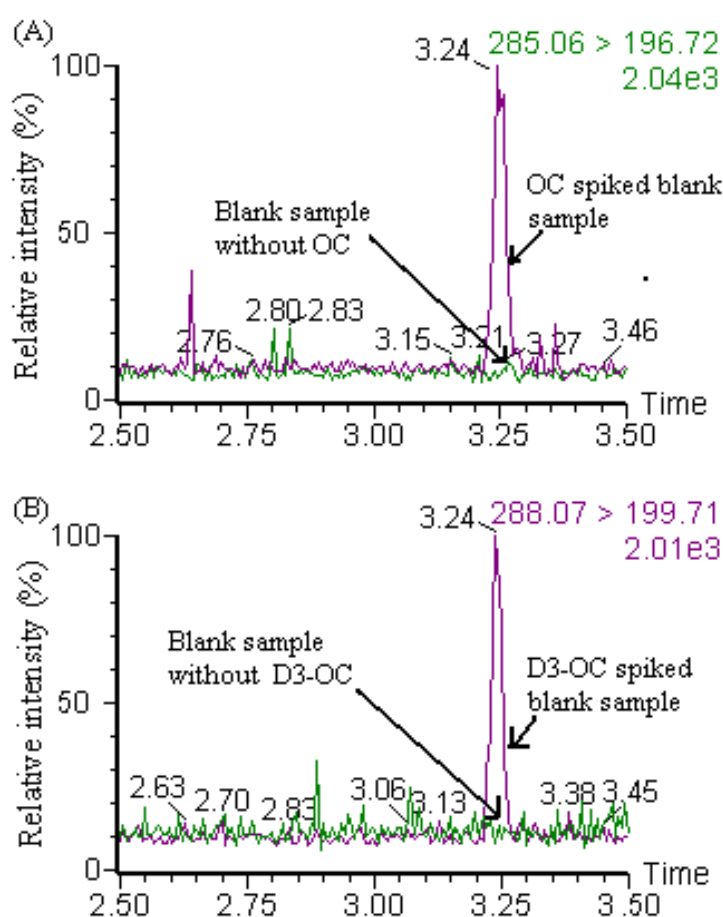

Figure 3-7. The representative chromatogram of OC and D3-OC at LOQ spiked in blank sample

3.2.4 Conclusions

3.2.4.1 Development of analytical methods for antibiotics

Solid-phase extraction Oasis HLB cartridges coupled with liquid chromatography and tandem mass spectrometry were successfully applied for the determination of selected antibiotics in wastewater. As a result of this method's applicability to wastewater samples spanning the whole treatment process, it can be used to investigate the fate of these compounds through the various steps of wastewater treatment. The resulting information can be used to evaluate the performance of wastewater treatment procedures and to highlight options for the optimization of WWTPs with the aim of minimizing the input of antibiotics into ambient receiving waters. The presented method provides the necessary basis for a comprehensive study on antibiotics in wastewater treatment including alternative wastewater technologies and advanced tertiary treatment such as membrane filtration, ozonation or advance oxidation process. With this method, we therefore present a powerful tool to fully assess the fate and occurrence of a wide range of antibiotics in wastewater treatment plants.

3.2.4.1 Development of analytical methods for antiviral drugs

For the first time a method for oseltamivir carboxylate and amantadine in wastewater and river water was developed. Recent emergence of highly pathogenic influenza and widespread use of antiviral drugs during seasonal epidemic or pandemic is an emerging issue for aquatic environment protection and possibility of antiviral resistance in wild water fowls. This method will therefore provide a powerful tool to assess the behaviors in wastewater treatment plants and receiving water.

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Göbel, A., McArdell, C. S., Joss, A., Siegrist, H. Giger, W.,2007). Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. *Science of the Total Environment*, **372**(2–3), 361–71.

CHAPTER IV

OCCURRENCE AND REMOVAL OF ANTIBIOTICS IN DIFFERENT SEWAGE TREATMENT PLANTS LOCATED IN DIFFERENT GEOGRAPHICAL AREA

4.1 Occurrence and removal of antibiotics in sewage treatment plants in Japan and their effects on bacterial ammonia oxidation

4.1.1 Introduction

Tons of pharmacologically active compounds (PhACs) are consumed yearly in human and veterinary medicine for diagnosis, treatment and prevention of diseases. Since PhACs are used for our benefit, they were rarely viewed as potential environmental pollutants. The first report on PhACs in water environment was published in the late 1970s (Hignite *et al*., 1977). Recent studies have shown a wide contamination of water environment by PhACs and their main source in the environment was effluent from sewage treatment plants (STPs) (Halling-Sorensen *et al*., 1998; Daughton and Ternes, 1999). This issue has been steadily gaining attention in recent years both within the academic community and among the general public. The recent advancement of more reliable and sensitive analytical techniques that detailed research in this area has become possible. Among PhACs, antibiotics are of important concern due to their role in growing antibiotic resistance among pathogenic bacteria. Besides resistance issue, antibiotics may upset sensitive natural ecosystem as they are designed to be highly bioactive. Clinically-important antibiotics are virtually ubiquitous contaminants in sewage water and STPs are the first defense line against the release of these compounds in the environment. Different surveys analyzing pharmaceuticals compounds including antibiotics in wastewater have been carried out in the US (Batt *et al*., 2006), Europe (Gobel *et al*., 2004, Golet *et al*., 2002) and Asia (Gulkowskaa *et al*., 2008; Yasojima *et al*., 2005). Moreover, only a few studies reported the fate and dynamics of these emerging contaminants in STPs and there is still lack of information on the occurrence and fate issue of these emerging contaminants among STPs varying in operation and treatment technologies. Presence of antibiotics at substantial concentration in influent of centralized or decentralized STPs may cause adverse effects to sensitive biological processes such as nitrification, which is still hindered by the lack

of information. Inhibition of nitrification under uncontrolled conditions may lead to accumulation of ammonia and/or nitrite, nitrate in the effluent which are regulated.

The objectives of this chapter (Chapter IV) were to compare the occurrence and fate of antibiotics (azithromycin, ciprofloxacin, clarithromycin, enrofloxacin, levofloxacin, lincomycin, nalidixic acid, norfloxacin, roxithromycin, salinomycin, sulfadimethoxine, sulfadimizine, sulfamerazine, sulfamethoxazole, sulfamonomethoxine, tetracycline and trimethoprim) at four full-scale STPs in

Table 4-1 A summary of the sewage treatment plants characteristics and operating conditions

STP ID	Average flow (m³/day)	Population served	Treatment processes		HRT (h)	SRT (days)	Primary	Secondary	Tertiary
STP1	576,265	775,500	Section 1	A2O	Chlorination	12.0	19.0	Yes		
			Section 2	AO	Chlorination	11.5	16.0		Yes	
			Section 3	CAS	Chlorination	9.5	18.0			
STP2	9,500	33,900		AOᵃ	SF	14.0	13.0		Yes	
STP3	50,000	236,000		AOᵃ	SF	11	17.0		Yes	
STP4	57,000	99,000	Section 1	CAS	Chlorination	5.5	18.0	Yes		
			Section 2	AOᵇ	Chlorination	2.8	14.0		Yes	

HRT: Hydraulic retention time; SRT: Sludge retention time; CAS: Conventional activated sludge; A2O: Anaerobic/Anoxic/Aerobic; AO: Anoxic/Aerobic; SF: Sand filtration; AOᵃ: coagulant based Anoxic/Aerobic using coagulant; AOᵇ: Anoxic/Aerobic using biological carrier

Japan, as well as to elucidate the effects of antibiotics (sulfamethoxazole, lincomycin, enrofloxacin, clarithromycin and trimethoprim; these are representative from broad antibiotics group investigated in this study) as individual and in mixed condition on nitrifier (a relatively sensitive microbial community in activated sludge) in a batch experiment through bacterial ammonia oxidation measurement. The selected STPs employed a wide variety of treatment processes (Table 4-1).

4.1.2 Methodology

4.1.2.1 Chemical and reagents

Azithromycin, ciprofloxacin, clarithromycin, enrofloxacin, levofloxacin, lincomycin, nalidixic acid, norfloxacin, roxithromycin, salinomycin, sulfadimethoxine, sulfadimizine,

sulfamerazine, sulfamethoxazole, sulfamonomethoxine, tetracycline and trimethoprim were purchased from Wako Company Ltd. (Osaka, Japan). All the antibiotics were of analytical grade (purity > 95%). Individual standard solutions at 1 mg/mL were prepared in methanol. All working standards were prepared before analysis.

4.1.2.2 Sample collection and preparation

Samples were collected in winter from four STPs located in Kyoto and Shiga prefecture, Japan. Brief descriptions of STPs are listed in Table 4-1. Each 1L of sample was collected in glass bottle from primary influent and secondary effluent, and then immediately acidified (pH 3) by ascorbic acid at sampling location to reduce microbial activity, further degradation with chlorine and enhance trapping of the antibiotics on the solid-phase extraction (SPE) cartridge. After arriving at laboratory samples were filtered (GF/B, Whatman, UK) and each sample was divided in two parts of 200ml, then Na_2EDTA at 1 g/L was added. One part of the 200ml was spiked by 50 μL mixed standards of 1mg/L and another 200ml was considered as blank sample. Recoveries were calculated by comparing spiked and blank sample. Oasis hydrophilic–lipophilic balance (HLB) extraction cartridges (6cc, 200 mg; Waters) were used for solid phase extraction. Cartridges were pre-conditioned with 3ml methanol, followed by 3mL of Milli-Q water. Samples (each 200ml) were passed through the cartridge at a flow rate of 5mL/min using concentrator and then vacuum dried for 90 min. Elution was carried out with 6 mL of methanol in 10ml glass vial. Methanol was evaporated under a gentle nitrogen stream at 37^0C to dryness and reconstituted with acidified milli-Q water (0.01% formic acid): methanol solution (9:1) to final volume of 1mL (*i.e.* concentration factor of 200).

4.1.2.3 Liquid chromatography–tandem mass spectrometry

Analysis of the antibiotics was conducted by method described in Chapter III. Briefly, chromatographic separation of the antibiotics were achieved with a Waters Acquity Ultra Performance™ liquid chromatography (UPLC™) separation module with a binary pump system equipped with UPLC BEH C18 column (100× 2.1 mm , 1.7 μm particle size). Optimum separation was achieved with a binary gradient consisting of 0.01% formic acid (v/v) in water (solvent A) and methanol (solvent B) at a flow rate of 0.35 ml/min. The gradient elution setting was: 0 to 2min: 90% A; 2 to 8 min: 90 to 75% A; 8 to 14 min: 75 to 45% A; 14 to 16 min: 45% A; 16 to19 min: 45 to 5% A; 19 to 21min: 5 to 90% (return to initial conditions); 21 to 23min: equilibration of the column. The column temperature was kept at 60 °C and injected sample

volume was 10 μL. The UPLC system was coupled to a Quattro Micro ™ API mass spectrometer with the electrospray ionization (ESI) source. LOQs in the sewage water and sludge samples were defined as signal to noise (S/N) ratios of 10 or higher. LOQ for each compound in sewage were from 1 to 10 ng/L

4.1.2.4 Cultivation of nitrifying activated sludge (NAS) and nitrification inhibition experiment

Seed activated sludge was collected from a sewage treatment plant and cultivated at 30^0C by a fill-and-draw operation with 12h HRT and 60 day SRT in a glass-constructed 3L reactor in laboratory to get desired nitrifying activated sludge (NAS). In each cycle, 2L supernatant was withdrawn after settling for 30 min and same volume of fresh mineral salts medium was added. Substrate to support autotrophic nitrifier was composed of 25mM $(NH_4)_2SO_4$; 43mM K_2HPO_4 ; 0.2 mM $CaCl_2$; 0.73mM $MgSO_4$; 0.01mM $FeSO_4.7H_2O$; 0.007mM $CuSO_4.5H_2O$; 0.017mM EDTA and 0.4% $NaHCO_3$. The pH was controlled 7.5 to 8.0 by automatic addition of 30% $NaHCO_3$. DO was maintained between 1.0 and 1.5 mg/L. Nitrification inhibition in the presence of antibiotics was determined by oxygen uptake rate (OUR) measurements, at certain time intervals (10 to 15min.). The mixed liquor was taken from the parent reactor, centrifuged and then diluted with fresh medium (same as cultivation of NAS) into a capped glass vessel (respiration chamber) with a working volume of 200 mL and MLSS of 250mg/L. The temperature of the vessel was constant at 25^0C during the measurement period. Complete mixing of the respiration chamber was ensured by a magnetic stirrer. A decrease in DO in the vessel due to the substrate oxidation during the test period were measured by a DO probe (Mettler-Toledo InPro® 6800 GmbH, Switzerland). Inhibition was quantified in terms of the reduction in OUR with respect to the control using the equation 1.

$$(\%)inhibition = \frac{OUR_{control} - OUR_{antibiotic}}{OUR_{control}} \times 100 \text{ ------- (1)}$$

$OUR_{control}$ is OUR in the absence of antibiotics and $OUR_{antibiotic}$ is OUR in the presence of antibiotics.

4.1.3 Results and discussion

4.1.3.1 Occurrence of the antibiotics in the influent and effluent

All the antibiotics were virtually ubiquitous in influent and effluent but concentration

varied among STPs. The highest concentration recorded in influent was 4820, 1530 1134 and 1129 ng /L for clarithromycin in STP 4, STP2, STP3 and STP1 respectively (Figure 4-1; Table 4-2). Among quinolones, levofloxacin was detected in high concentration in influent followed by norfloxacin and ciprofloxacin . Clarithromycin, levofloxacin and azithromycin in influent at 883 ng/L, 981 ng/L and 371 ng/L respectively, were reported previously from Japan (Yasojima *et al*., 2005). In this study tetracycline (9 to 89ng/L), trimethoprim (26 to 111 ng/L) and sulfamethoxazole (159 to 176 ng/L) were detected in lower concentrations than data published from Europe, US and China. In Europe, sulfamethoxazole 230 to 570 ng/L, trimethoprim 220 to 440 ng/L and roxithromycin 10 to 40 ng/L was detected in influent (Gobel *et al*., 2007). A relatively higher concentration of trimethoprim was also detected in Sweden (1300 ng/L) (Lindberg *et al*., 2005). Similarly higher concentration of tetracycline (1300 ng/L), norfloxacin (460 ng/L) and trimethoprim (320 ng/L) in influents were measured in China (Gulkowskaa *et al*., 2008). In influent, only clarithromycin shared 41%, 55%, 48% and 58% among selected antibiotics detected in STP1, STP2, STP3 and STP4 (Figure 4-2), respectively, followed by levofloxacin (11 to 19%), azithromycin (6 to 12%), norfloxacin (6 to 10%) and sulfamethoxazole (6 to 8%). Similar to influent, antibiotics concentration varied in effluents among STPs. However the composition profiles of individual antibiotics in the effluent samples (Figure 4-3) were relatively similar to their corresponding influent samples. All macrolides and quinolones were detected in effluent samples. Clarithromycin was detected in high concentration (396 to 800 ng/L) followed by azithromycin (39 to 740 ng/L), levofloxacin (57 to 561 ng/L) and roxithromycin (14 to 275 ng/L).Among sulfonamides, only sulfamethoxazole was detected in all effluent sample ranging between 67 to 161ng/L. Tetracycline (3 to 39ng/L) and trimethoprim (24 to 72ng/L) were detected in lower concentration in effluent which corresponding to their influent concentration. Ciprofloxacin, levofloxacin, norfloxacin and sulfamethoxazole were detected in lower concentration in effluent from A20 and AO process compare to CAS process

Table 4-2 Antibiotics concentration in influent (Inf.) and their removal efficiency (Rev. %) during secondary treatment at the four STPs.

	STP 1				STP 2		STP 3			STP 4	
	Inf. (ng/L)	Rev. %			Inf. (ng/L)	Rev. %	Inf. (ng/L)	Rev. %	Rev. %	Rev. %	
		A2O	AO	CAS						CAS	AO
Azithromycin	160	41	34	39	160	75	279	86	1347	45	72
Ciprofloxacin	231	83	77	72	55	84	37	60	195	73	71
Clarithromycin	1129	53	55	50	1570	57	1134	65	4820	83	88
Enrofloxacin	85	72	74	70	7	51	11	65	23	38	44
Levofloxacin	532	90	77	62	451	87	255	40	587	77	78
Lincomycin	37	52	55	57	6	-39	40	29	24	33	-1
Nalidixic Acid	40	91	75	100	7	100	27	-	60	58	53
Norfloxacin	155	81	76	75	275	97	157	90	468	90	88
Roxithromycin	96	24	26	39	28	50	83	59	209	-32	52
Salinomycin	ND		-	-	ND	-	ND	-	60	95	91
Sulfadimethoxine	57	76	91	100	9	59	7	100	71	62	38
Sulfadimizine	ND	-	-	-	ND	-	ND	-	15	44	43
Sulfamerazine	ND	-	-	-	ND	-	ND	-	15	44	41
Sulfamethoxazole	159	56	40	39	177	62	184	12	176	26	9
Sulfamonomethoxine	ND	-	-	-	ND	-	ND	49	19	66	58
Tetracycline	65	40	61	40	9	60	16	78	89	72	73
Trimethoprim	26	-82	-46	-88	106	74	89	73	111	35	63

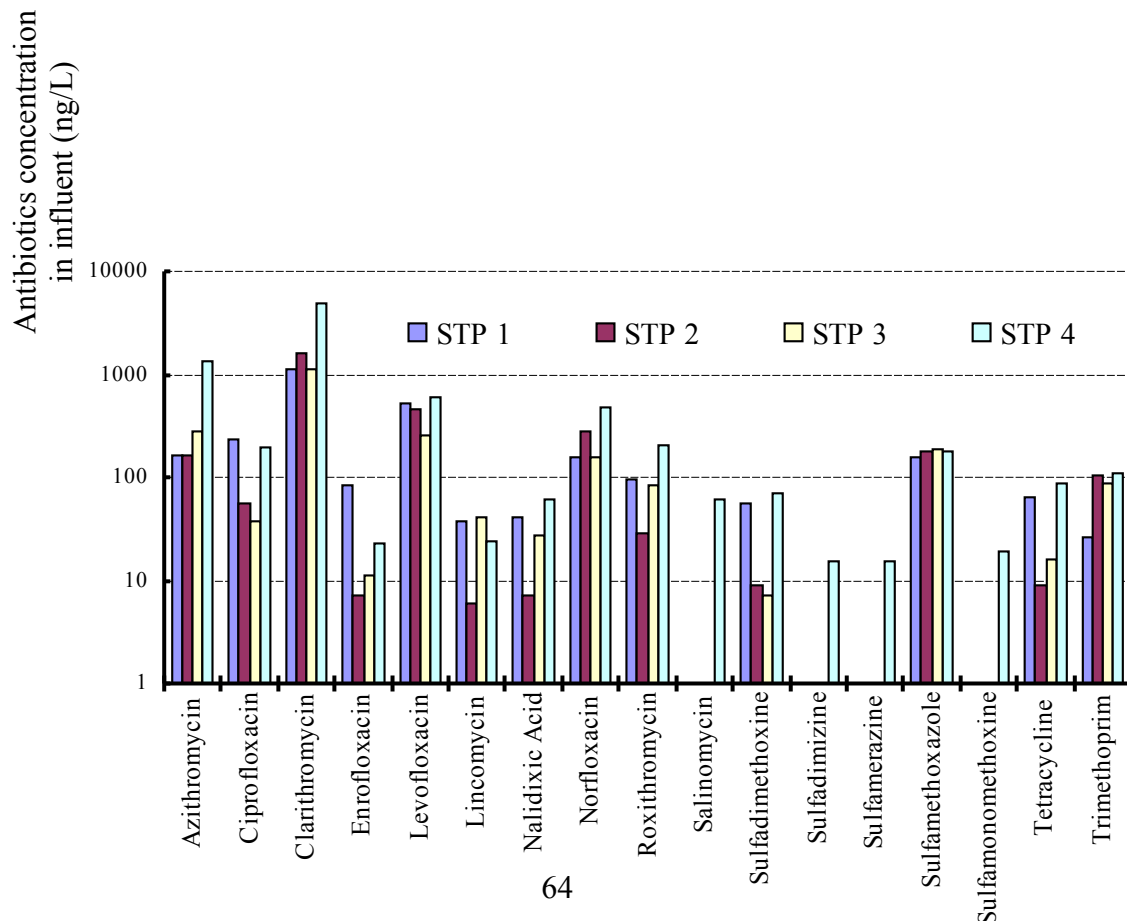ND: not detected (bellow limit of detection or S/N ration less than 10)

64

Figure 4-1 Antibiotics concentration in influent: STP1, STP2 and STP3, and STP4

effluent, although influent, HRT and SRT were relatively similar in the mention process (Table 1). Alike STP1, clarithromycin was detected in higher concentration in CAS process (800 ng/L) effluent of STP4 compare to AO process (583 ng/L) effluent. Azithromycin in CAS process effluent of STP4 was two fold higher than AO process. In contrast, ciprofloxacin and levofloxacin were in same level both AO and CAS process effluent of STP4. Higher concentration of sulfamethoxazole (700 ng/L) was also detected form New York, USA (Batt *et al*., 2007).   Trimethoprim was measured in higher concentration in effluents from Sweden (1300 ng/L) (Lindberg *et al*., 2005) and New York, USA (2500 ng/L) (Batt *et al*., 2007). Norfloxacin was detected 350-370 ng/L at Shenzhen Nan Shan, China (Gulkowska *et al*., 2008).
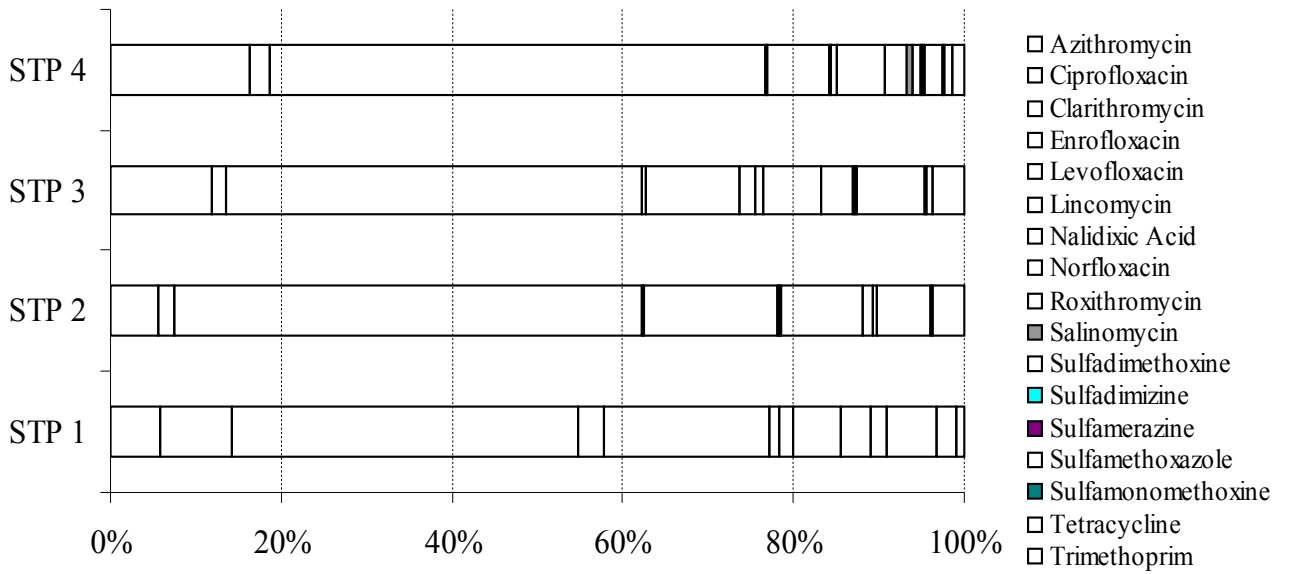
Figure 4-2 Composition profile of the selected antibiotics in influent from four STPs.
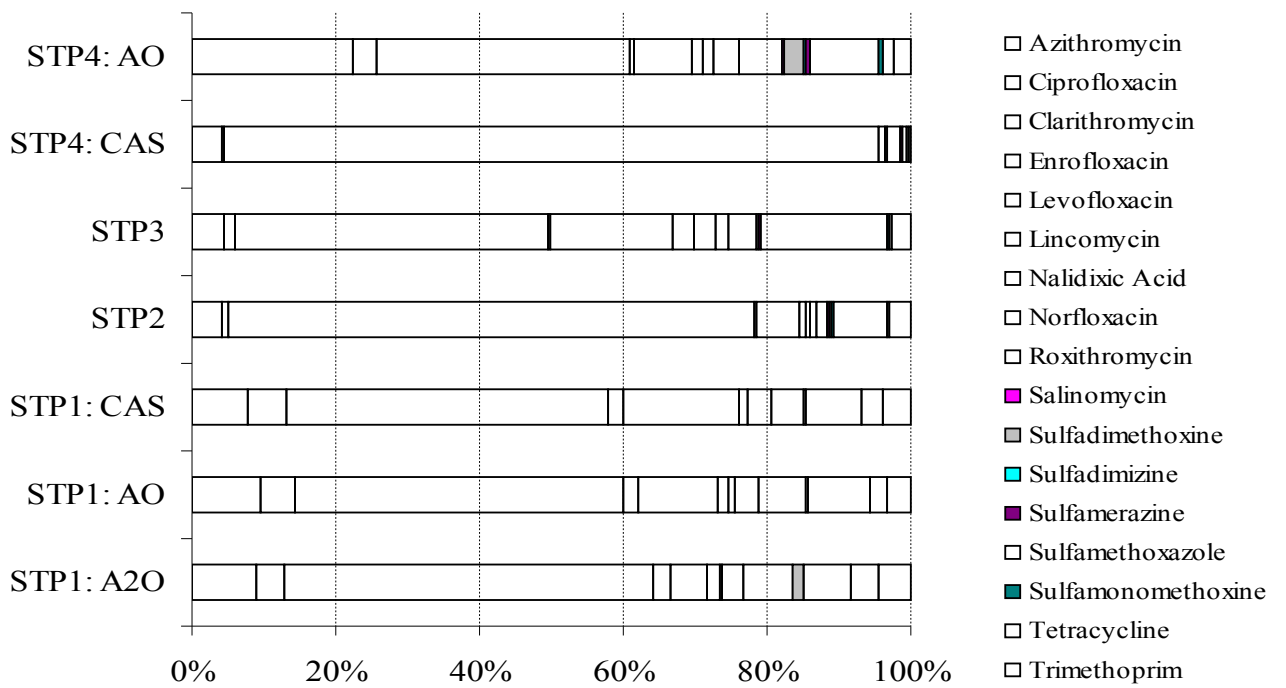

Figure 4-3 Composition profiles of the selected antibiotics in effluent from four STPs.

4.1.3.2 Elimination of the selected antibiotics in the treatment processes

Antibiotics removal efficiencies in the STPs were varied among compounds and STPs (Figure 4-4). The observed eliminations combined the reduction due to sorption and transformation processes. For both macrolides and quinolones compounds, a vary inconsistence picture is obtained with elimination rate ranging -32% to 88%. Among macrolides, clarithromycin (50 to 88%) and azithromycin (34 to 86%) showed relatively higher removal efficiency than roxithromycin (-32 to 59%). This removal rates relatively higher than the value reported from Switzerland, 45%, 25% and 60% for roxithromycin, clarithromycin and azithromycin, respectively (Gobel *et al*., 2007). Additionally, removal efficiency observed for these compounds in the A2O and AO process in this study tends to be higher than CAS (Table 2) and independent to HRT and SRT (Table 1). Negative removal of roxithromycin in CAS process at STP4 in unclear. The presence of de-conjugable metabolites are unlikely for macrolides (Gobel *et al*., 2007) but they are probably partly enclosed in feces particles and released during biological treatment, since they are mainly excreted with bile and feces, which may determine their low removal rate. Quinolones antibiotics showed higher removal compare to macrolides. Nalidixic acid (53 to100%) exhibited higher removal efficiency followed by norfloxacin (75 to 95%), levofloxacin (40 to 90%), ciprofloxacin (60 to 83%) and enrofloxacin (38 to 74%). Similar to macrolides, for most of the quinolones antibiotics removal efficiency were high in A2O and AO process than CAS process. Higher removal of quinolone antibiotics is related to sorption which is governed by electrostatic interaction between positive change compounds with negative charge bacteria. Quinolone antibiotics, ciprofloxacin and ofloxacin were eliminated in average of 84% and 83%, respectively, from Finland (Veino *et al*., 2007). Higher removal of quinolones also reported from Europe; ciprofloxacin 87% and norfloxacin 92% (Golet *et al*., 2003). In this study sulfamethoxazole (9 to 56%) exhibited relatively moderate removal efficiency. Trimethoprim had a very inconsistence removal efficiency ranging between -88% to + 74%.
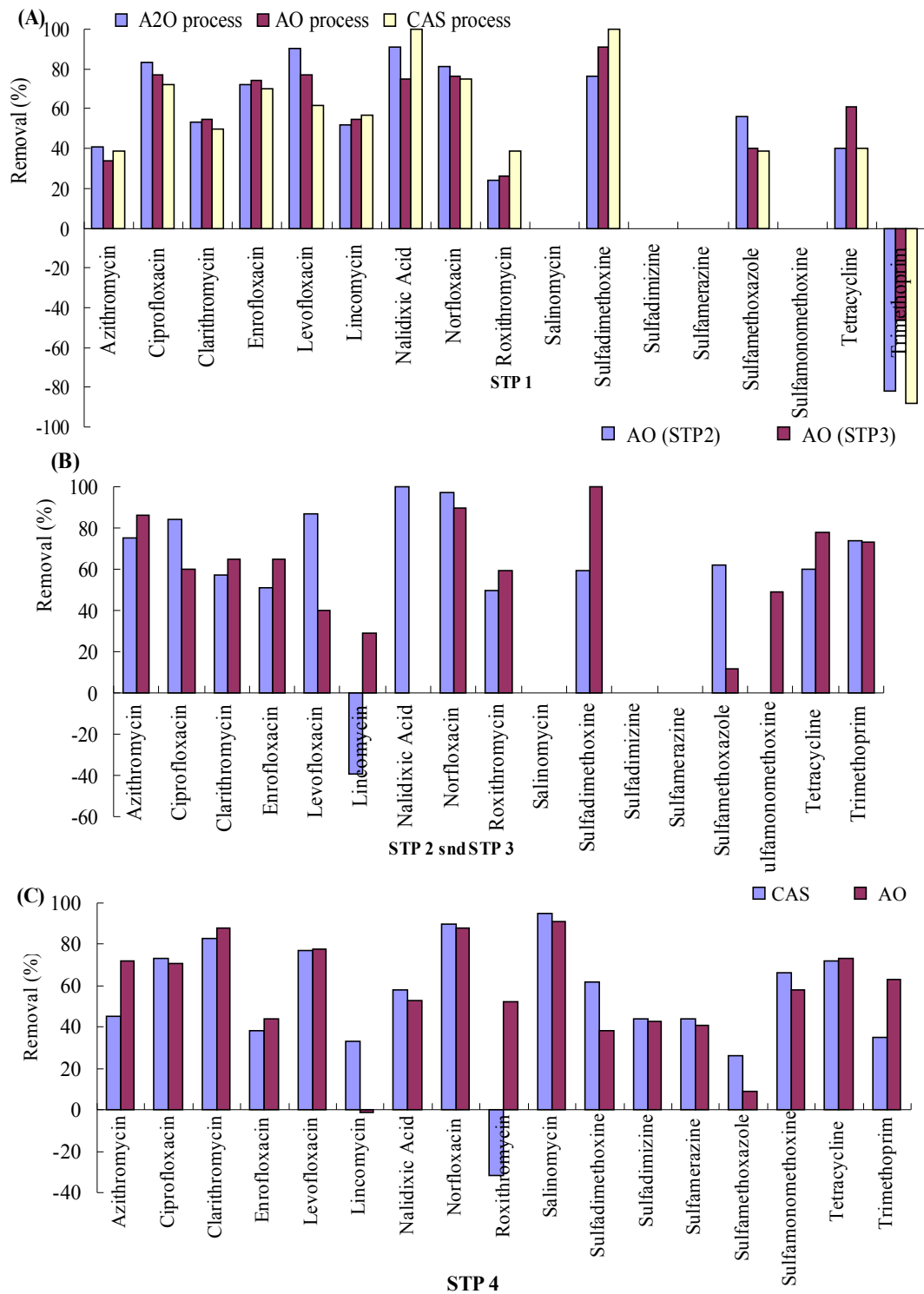
Figure 4-4 Antibiotics removal efficiencies during secondary treatment: (A) STP1,(B) STP2 andSTP3, and (C) STP4

4.1.3.3 Effects of antibiotics on bacterial ammonia oxidation

According to the study, antibiotics are ubiquitous in sewage water. Nitrification in STPs is a very sensitive process to many contaminants such as heavy metals. In this inhibition study we selected
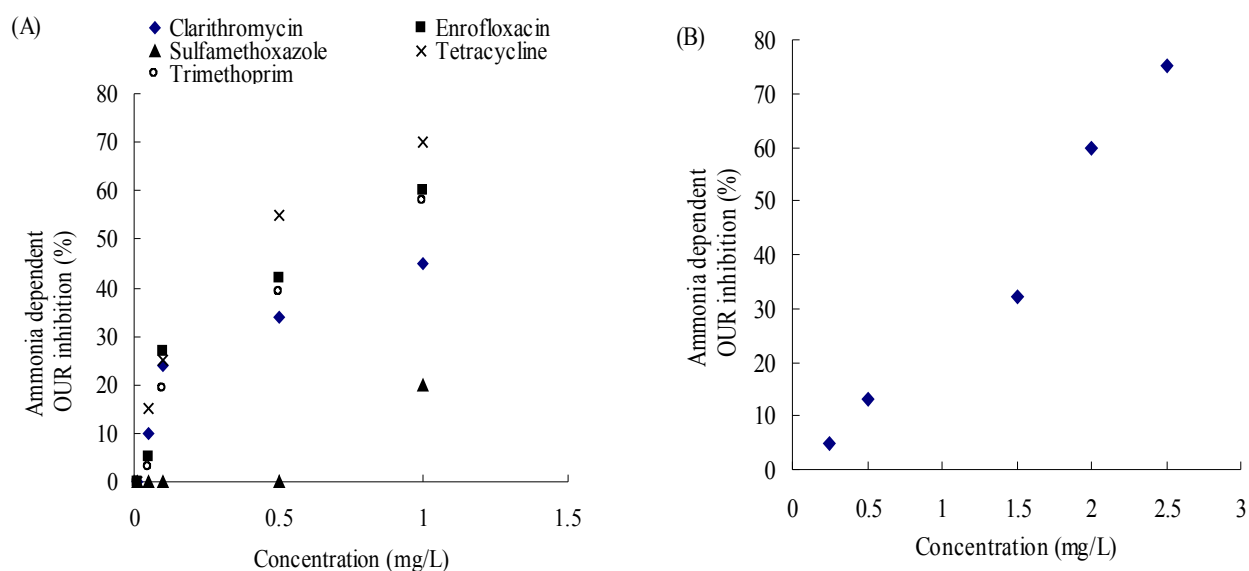

F**igure 4-5** Effect of the antibiotics (clarithromycin, enrofloxacin, sulfamethoxazole, tetracycline and trimethoprim) on bacterial ammonia oxidation, measured by ammonia dependent oxygen uptake rate. (A) as individual and (B) in mixed (five compound together) condition.

five antibiotics (clarithromycin, enrofloxacin, sulfamethoxazole, tetracycline and trimethoprim) representative from five wide antibiotics groups. It was observed that the individual antibiotics bellow 0.05 mg/L does not have any significant effect on bacterial ammonia oxidation (Fig. 3(A)).

Unexpected result was obtained for sulfamethoxazole; it did not show any inhibition effect bellow 1mg/L. All the antibiotics exhibited less than 15% inhibition at concentration 0.05 mg/L. At individual concentration 0.1 mg/L, the inhibition was between 20 to 30%. From this experiment it can be state that an individual antibiotic at present detected concentration in STPs does not have any effect on nitrification. In contrast, a totally different picture was observed when antibiotics were applied in mixed condition (Fig. 3 (B)). At 0.05 mg/L no individual antibiotics had inhibition over 15%, but at the same concentration in mixed (0.05 x 5 mg/L) condition it shows 25% inhibition. Inhibition 57% was observed at 0.3 mg/L and

over 80% at 0.5 mg/L, in mixed condition. From growth inhibition test of *Nitrosomonas europaea,* EC$_{50}$ of sulfadiazine, tetracycline and oxolinic acid was determined 17 mg/L, 4.0 mg/L and 1 mg/L (Halling-Sorensen, 2001).

4.1.4 Conclusions

All macrolides and quinolones were detected in influent and effluent at four STPs. Clarithromycin was detected in the highest concentration in influent (1129 to 4820 ng/L), followed by azithromycin (160 to 1347 ng/L), levofloxacin (255 to 587 ng/L) and norfloxacin (155 to 486 ng/L) and sulfamethoxazole (159 to 176ng/L). Clarithromycin alone shared around 50% load of the detected antibiotics in influent followed by levofloxacin (11 to 19%), azithromycin (6 to 12%), norfloxacin (6 to 10%) and sulfamethoxazole (6 to 8%). Among macrolides, clarithromycin (50 to 88%) and azithromycin (34 to 86%) showed relatively higher removal rate than roxithromycin (-32 to 59%). Quinolones antibiotics showed higher removal compare to macrolides. Most of the antibiotics removal efficiency was relatively high in A2O and AO based secondary treatment process than CAS process. At 0.05mg/L and bellow no individual antibiotics had inhibition of bacterial ammonia oxidation over 15%, but at the same concentration in mixed condition it shows 25% inhibition. Inhibition 57% was observed at 0.3 mg/L and over 80% at 0.5 mg/L in mixed condition. Antibiotics showed synergistic effect on ammonia dependent oxygen uptake rate by nitrifying activated sludge.

4.2 Occurrence and elimination of selected antibiotics in different biological sewage treatment technologies in Japan and China

4.2.1 Introduction

Different surveys analyzing pharmaceuticals compounds including antibiotics in wastewater and surface water have been carried out in the US (Batt *et al.*, 2006), Europe (Gobel *et al.*, 2004, Golet *et al.*, 2002) and Asia (Gulkowskaa *et a*l., 2008; Yasojima *et al.*, 2005). Moreover, only a few studies reported the fate and dynamics of these emerging contaminants in STPs, and there is still lack of information on the occurrence and fate issue of these emerging contaminants among STPs, varying in operation and treatment technologies at different geographic locations. Occurrence of sulfonamides macrolides and quinolones groups antibiotics were more documented than others, with variable removal efficiencies (Golet *et al.*, 2002; Lindberg *et al.*, 2005; Gobel *et al.*, 2007). Beta lactams antibiotics are widely used but few studies detected them in surface water sample, they seem to be hydrolysed fast (Hirsch *et al.*, 1999).

The objective of this study was to compare the occurrence and fate of the antibiotics at four different types of full-scale STPs; two from Japan (STP-A and STP-B) and two from China (STP-C and STP-D). The selected STPs represent a wide variety of sewage treatment technologies (Table 1). The target twenty compounds comprised a wide range of antibiotic groups; beta-lactams, sulfonamides, macrolides, quinolones, tetracycline and others (Table 2). The choices of these antibiotics were based upon their annual consumption, reported detection in surface water/wastewater and analytical capabilities.

4.2.2 Methodology
4.2.2 1 Chemical and reagents

Ampicillin, azithromycin, ciprofloxacin, clarithromycin, enrofloxacin, levofloxacin, lincomycin, nalidixic acid, norfloxacin, novobiocin, oxytetracycline, roxithromycin, salino-mycin, sulfadimethoxine, sulfadimizine, sulfamerazine, Sulfamethoxazole, sulfamonometho-xine, tetracycline and trimethoprim.

4.2.2.2 Sample collection and preparation

Samples were collected in November, 2007 from two STPs (STP-A: which is similar to STP-1 of previous study and STP-B: which is similar to STP-3 of previous study) located

in Japan, whereas sample from two STPs in Beijing, China, were collected in March, 2008. Brief descriptions of STPs are listed in Table 4-3.

Table 4-3. A summary of the sewage treatment plants characteristics and operating conditions

Plant ID	Location (City/Country)	Population served	Treatment processes				HRT (h)	SRT (days)
			Primary	Secondary		Tertiary		
STP-A	Kyoto/ Japan	775,500	Yes	Section 1	A2O	Chlorination	12.1	19
			Yes	Section 2	AO	Chlorination	11.6	16
			Yes	Section 3	CAS	Chlorination	9.4	18
STP-B	Shiga/ Japan	236,000	Yes		AO	SF	11	17
STP-C	Beijing/China	480,000	Yes		OD	-	17.4	16
STP-D	Beijing/China	810,000	Yes		A2O	UF + O_3	11.45	7

HRT: Hydraulic retention time; SRT: Sludge retention time ; CAS: Conventional activated sludge ; A2O: Anaerobic/Anoxic/Oxic; AO: Anoxic/Oxic; SF: Sand filtration; UF: Ultra filtration; O_3: Ozonation

For China samples, cartridges were kept in 4^0C after enrichment of sample and after return back to Japan elution step were performed. There was no effect of cartridge storage and travel on compounds (data not shown). Elution was carried out with 6 mL of Methanol in 10ml glass vial. Methanol was evaporated under a gentle nitrogen stream at 37^0C to dryness and reconstituted with acidified milli-Q water (0.01% formic acid)/Methanol solution (90/10) to final volume of 1mL (i.e. enrichment factor of 200) , sample preparation is same as previous section.
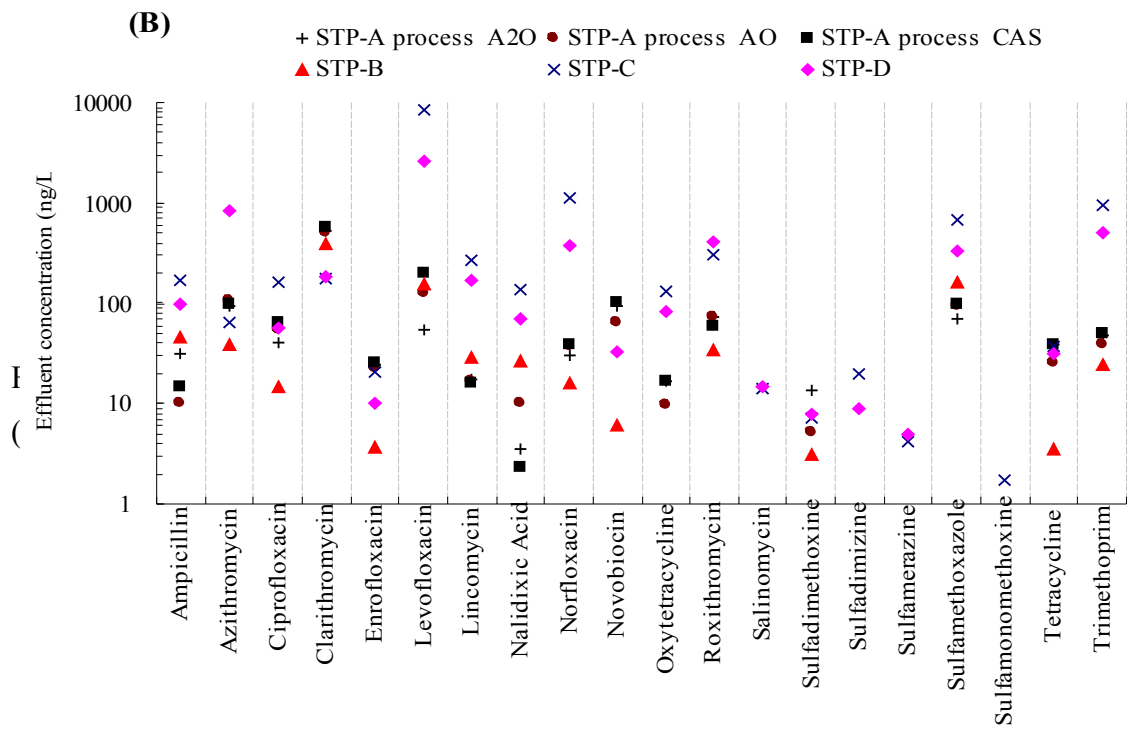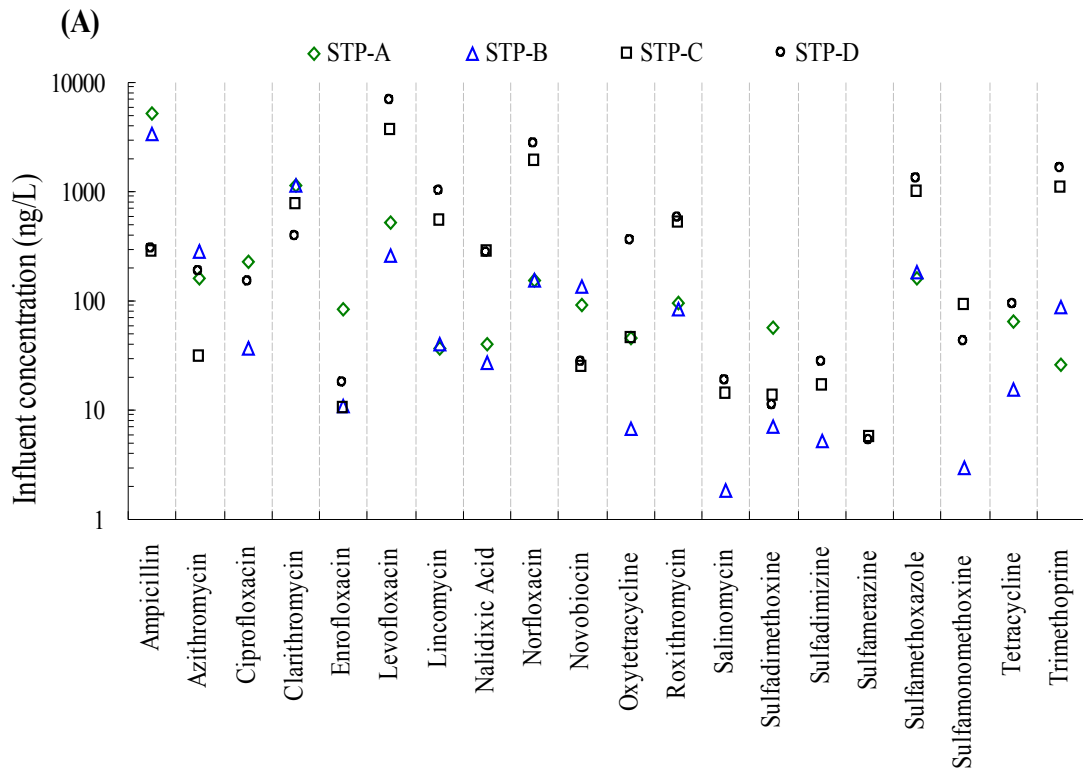
4.2.3 Results and discussion

4.2.3 1Occurrence of antibiotics in the STPs influent

The selected antibiotics concentrations in influent at four STPs are shown in Figure 4-6 Thirteen out of twenty selected antibiotics were detected in all influents. Concentration varied among location and STPs.

Sixteen antibiotics were detected in influent of STP-A at concentrations ranging from 26ng/L (trimethoprim) to 1129 ng/L (clarithromycin), whereas fourteen antibiotics were detected in STP-B influents ranging from 11 ng/L (enrofloxacin) to 1134ng/L (clarithromycin). Salinomycin, sulfadimizine, sulfamerazine and sulfamonomethoxine concentration were lower

than their methods detection limits in influents of both STP-A and STP-B. In addition oxytetracycline was not detected in STP-B influents. All selected macrolides and quinolones were detected in both STPs influent (STP-A and STP-B); clarithromycin occurred at the highest concentration (1129 ng/L and 1134 ng/L), followed by levofloxacin (532 ng/L and 255 ng/L), azithromycin (160 ng/L and 280 ng/L), norfloxacin (155 ng/L and 157 ng/L) and Ciprofloxacin (231 ng/L and 37 ng/L). Beta lactams ampicillin was detected in both STP-A and STP-B influents at concentration 340 ng/L and 260 ng/L respectively. Clarithromycin, levofloxacin and azithromycin in influent at 883 ng/L, 981 ng/L and 371 ng/L respectively were reported previously from Japan (Yasojima *et al.*, 2005).Except ciprofloxacin and tetracycline in STP-C, all target antibiotics were detected in STP-C and STP-D. Levofloxacin (6800 ng/L), norfloxacin (2775 ng/L), trimethoprim (1578 ng/) and sulfamethoxazole (1280 ng/L) were occurred at highest concentration in STP-D influent among all STPs. The occurrence pattern of antibiotics in STP-C followed STP-D with a little difference in concentration. Clarithromycin concentration (377-762 ng/L) in STPs influent in Beijing (STP-C and STP-D) was similar to Europe (300-600 ng/L) (Gobel *et al.*, 2004) but two orders lower than the concentration found in STPs in Kyoto (STP-A and STP-B). For azithromycin, the concentration was 160-279 ng/L in Kyoto and 31-180 ng/L in Beijing. In Europe, sulfamethoxazole 230-570 ng/L, trimethoprim 220-440 ng/L and roxithromycin 10-40 ng/L was detected in influent (Gobel *et al.*, 2007). Like Beijing, a relatively similar concentration of trimethoprim was also detected in Sweden (1300 ng/L) and New Mexico, USA (1400 ng/L) (Lindberg *et al.*, 2005; Brown *et al.*, 2006)

In contrast, a completely different pattern of antibiotics occurrences in influent were observed in Kyoto (STP-A and STP-B) and Beijing (STP-C and STP-D). Sulfonamide antibiotics; sulfadimizine, sulfamerazine and sulfamonomethoxine were detected only in influent from STPs in Beijing but not in Kyoto. Sulfamethoxazole and trimethoprim are often use together and were found in higher concentration in STP-C and STP–D (up to 1280ng/L and 1578ng/L). Sulfamethoxazole around 2800ng/L was reported from USA (Batt *et al.*,

(A)

◇ STP-A   △ STP-B   □ STP-C   ○ STP-D

(B)

+ STP-A process A2O   ● STP-A process AO   ■ STP-A process CAS
▲ STP-B   ✕ STP-C   ◆ STP-D

Japan

2007) which is two orders lower than Beijing. In this study Levofloxacin, norfloxacin, trimethoprim, and sulfamethoxazole were found in µg/l level in influent in Beijing whereas only clarithromycin were found in µg/l level in Kyoto.

4.2.3.2 Occurrence of antibiotics in the STPs secondary effluent

Similar to influent sample, concentration of the antibiotics varied among secondary effluent in the STPs (Fig. 2). Clarithromycin was detected in higher concentration in secondary effluent at STP-B (396   ng/L) and STP-A (536   ng/L, 511   ng/L and 561 ng/L in secondary effluent of A2O, AO and CAS process respectively). In STP-A, except nalidixic acid, all quinolones were detected in lower concentrations in secondary effluent of A2O and AO process than CAS process. Similarly, levofloxacin was detected in much lower concentration (54 ng/L) in A2O process effluent than CAS process effluent (203   ng/L). Levofloxacin (152 ng/L) and sulfamethoxazole (161ng/L) were detected over 100 ng/L in STP-B effluent (Fig. 2). Like influents, Salinomycin, sulfadimizine, sulfamerazine and sulfamonomethoxine were not detected in STP-A and STP-B secondary effluent. Interestingly nalidixic acid in STP-A secondary effluent, enrofloxacin and tetracycline in STP-B secondary effluent were below the methods detection limits. Comparatively higher level of   levofloxacin (2623 - 8628 ng/L), norfloxacin (370 - 1116 ng/L), azithromycin (65 - 847 ng/L), sulfamethoxazole (335 – 683 ng/ L) and trimethoprim (503 – 960 ng/l) were detected in STP-C and STP-D. Higher concentration of sulfamethoxazole (700 ng/L) was also detected form New York, USA (Batt *et al*., 2007). Trimethoprim was measured in higher concentration in effluents from Sweden (1300 ng/L) (Lindberg *et al*., 2005), New York, USA (2500 ng/L) (Batt *et al*., 2007) which are similar to STP-D effluent. Norfloxacin was detected 350-370 ng/L at Shenzhen Nan Shan, China (Gulkowska *et al*., 2008) which is similar to STP-D (370 ng/L) but around three times lower than STP-D (1116 ng/L). Azithromycin, levofloxacin, enrofloxacin and oxytetracycline were detected around two order magnitude higher in effluent than influent in STP-C. Similarly, azithromycin and novobiocin were detected in higher concentration in effluent than influent in STP-C. This can be explained by the presence of substances, *e.g.* human metabolites/conjugates which can subsequently be transformed to parents compound in biological treatment (Gobel *et al*., 2005; Gobel *et al*., 2007) and/or adsorption and desorption mechanism of compounds which may led to first sorption of the compounds in biological reactor and later desorption during return sludge mixing .

4.2.3.3 Elimination of antibiotics in the STPs

The removal efficiencies of the selected antibiotics in STPs in Kyoto (STP-A and STP-B) and Beijing (STP-C and STP-D) are presented in Figure 4-7. Antibiotics removal efficiencies in the STPs were calculated from the concentration difference in dissolved phase between influent and effluent samples, varied among compounds and STPs (Fig. 3). Removal efficiency varied due to specific treatment technology employed by individual STPs; the hydraulic and solid residence time at different STPs; and moreover physical and chemical properties of the antibiotics.

In general, macrolides antibiotics removal efficiencies (-109 to 77 %) were lower than other antibiotics in all the STPs. Varying results, including negative elimination was also observed for macrolides in literature (Gobel *et al.*, 2007). Macrolide antibiotics have been shown to be more persistent than some of the other antibiotics (Huang *et al.*,2001). Except levofloxacin (-135%) and enrofloxacin (-97 %) in STP-C, all quinolones antibiotics were removed moderately (40-90%) from STP-A, STP-B and STP-D. Levofloxacin, clarithromycin and azithromycin were removed 40%, 65% and 85% in STP-B, 60% , 50% and 38% in CAS process of STP-A, 90%, 53% and 41% in A2O process of STP-A, respectively. A moderate removal efficiency for levofloxacin (30-55%), clarithromycin (30-50%) and azithromycin (40-55%) were also observed from Japan (Yasojima *et al.*, 2005) These values are similar to ampicillin (40%) and trimethoprim (12%) removal in STP-C was lower than other STPs . Only clarithromycin (77%) and nalidixic acid (51%) were removed over 50% in STP-C. In STP-A and STP-B, ampicillin was removed over 90%. A higher removal of norfloxacin (78%) and tetracycline   (73%) was observed from STPs in southern China (Gulkowska *et al.*, 2008) which is similar to STP-D in Beijing, 86 and 66% respectively. Norfloxacin was removed around 40% in STP-C and there was no detection of tetracycline in influent form STP-C. Among sulfonamides antibiotics, only sulfamethoxazole was detected in sample in all STPs. Sulfamethoxazole was removed 56, 40 and 30% in A2O , AO and CAS process respectively in STP-A. On the other hand, the removal performance was 12 31 and 73% in STP-B, STP-C and STP-D. Sulfamethoxazole was removed in higher proportion in A2O process of STP-A and STP-D than AO, OD and CAS process. A similar removal of sulfamethoxazole (29-60%) was observed in CAS and fixed bed reactor in Switzerland in winter but negative removal in summer (Gobel *et al.*, 2007). Lower removal of antibiotics in STP-C
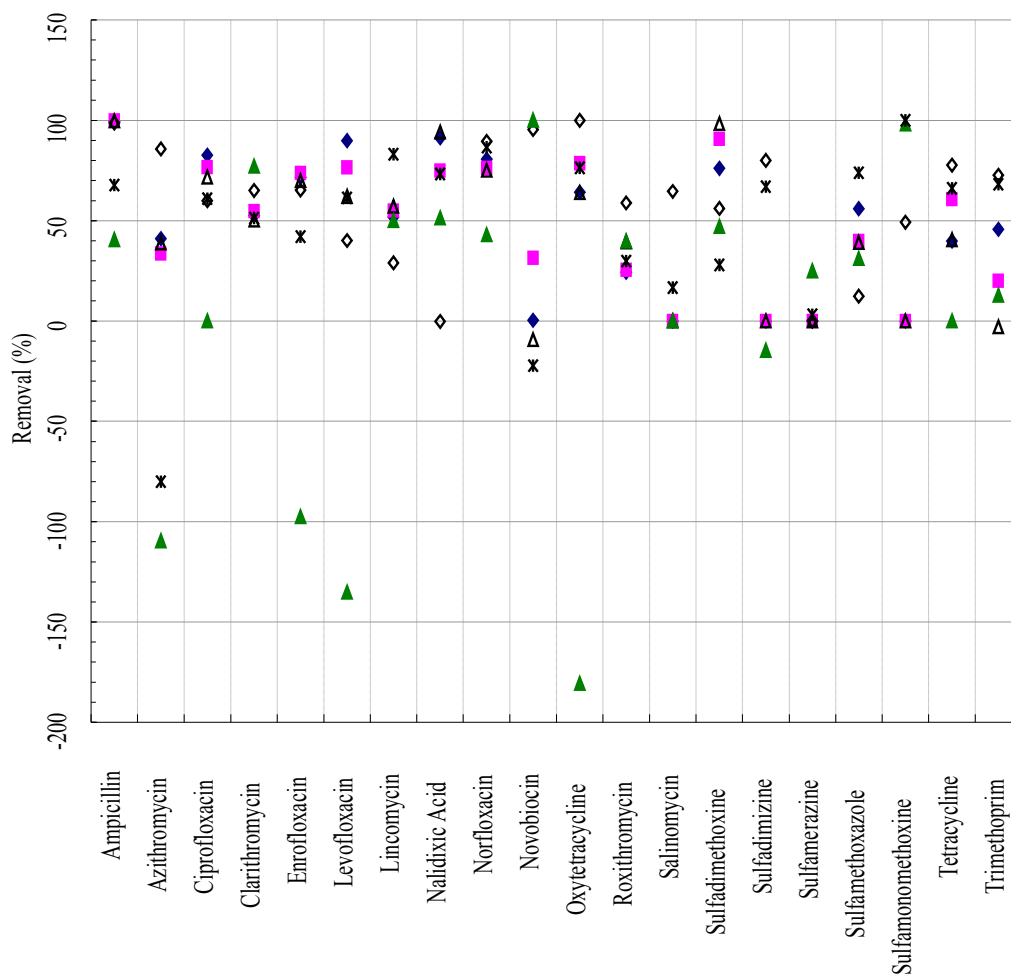
Fig. 4-7 Removal efficiencies of the antibiotics during primary + secondary treatments in STPs in Japan (STP-A and STP-B) and China (STP-C and STP-D).

could be related with its operation technology which is oxidation ditch (OD) based. In general, OD operated with relatively lower oxygen level than CAS, AO and A2O processes, and oxygen level is a limiting factor for biological activity in aerobic process. Trimethoprim was not removed in CAS based process in STP-A, whereas its removal efficiency was 20-70% in A2O (STP-A and STP-D) and AO (STP-A and STP-B) process. The reason for the inconsistent in removal efficiencies among these four STPs remains largely unknown, but

could be due to the differences in the daily loading of antibiotics. Longer HRT and SRT generally results in the higher removal of antibiotics in STPs (Batt *et al*., 2006, Clara *et. al.*, 2005). In this study, except STP-C (HRT 17.4h) all the STPs were operated relatively in similar HRT (9-12h). On the other hand STP-D had shorter SRT (7days) than others STPs (16-19days). Quinolones antibiotic are easily sorbed onto sludge due to electrostatic interaction between sludge and the compounds (Gobel *et al*., 2005). Lower quinolones antibiotics removal in CAS (HRT 9.4h) process compare to A2O (HRT 12.1h) and AO (HRT 11.6h) process in STP-A could a reason of low contact time.

4.2.3.4 Ultra filtration and ozonation as tertiary treatment for antibiotics removal

In STP-D, there was a wastewater reclamation facility connecting with secondary effluent. The facility was consisted of ultra filtration (UF) followed by ozonation. In UF there was no removal of the selected antibiotics (data not shown) in dissolved phase, but ozonation had significant impact on antibiotics removal from effluent. During ozonation tetracycline, ampicillin, lincomycin and oxytetracycline were removed over 95%, similarly trimethoprim, ciprofloxacin and norfloxacin were removed between 80-95% (Figure 4-8). Roxithromycin, levofloxacin and azithromycin had shown 70-80% removal. During ozonation, relatively lower removal efficiency (50-70%) was observed for enrofloxacin, clarithromycin, sulfadimizine, nalidixic acid and sulfamethoxazole (Figure 4-8). Salinomycin and sulfadimethoxine were not removed significantly (less than 30%) (Figure 4-8).
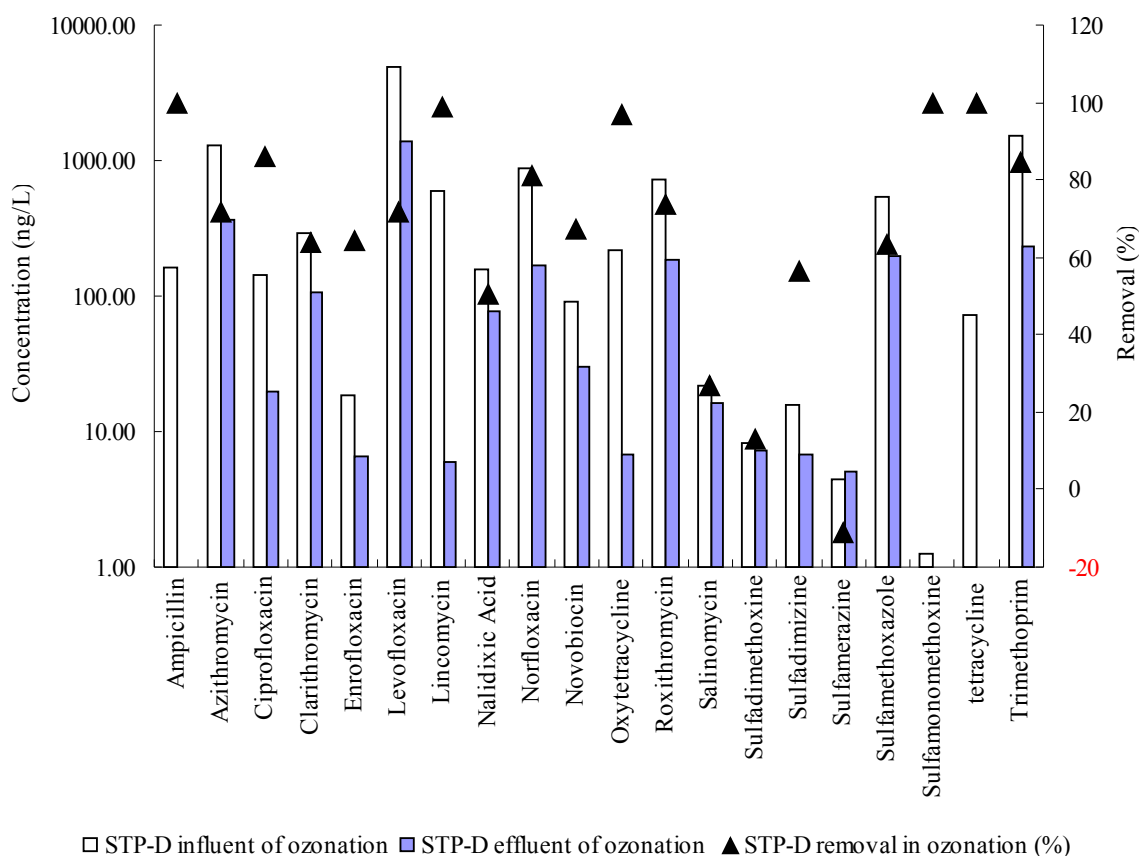
Fig. 4-8 Removal efficiencies of the antibiotics during ozonation at STP-D water reclamation line, in Beijing, China.

4.2.4 Conclusions

Thirteen out of twenty selected antibiotics were detected in all influents sample from STPs. Levofloxacin (6800 ng/L), norfloxacin (2775 ng/L), trimethoprim (1578 ng/L) sulfamethoxazole (1280 ng/L) and clarithromycin (1134 ng/L) were detected in higher concentration. The occurrences of antibiotics varied with geographical location and among STPs. Most of the antibiotics were detected in higher concentration in influent and secondary effluents from STPs in Beijing, China compare to Kyoto, Japan.

Similar to influent sample, levofloxacin (8628 ng/L), norfloxacin (1116 ng/L) trimethoprim (960 ng/L) sulfamethoxazole (683 ng/L) and clarithromycin (561 ng/L) were detected in higher concentration in effluent. Removal efficiency of the antibiotics varied with individual treatment technologies applied, in some extend A2O and AO based biological process was superior to CAS. Oxidation ditch based biological process was inferior to antibiotics removal. Macrolides antibiotics contributed higher proportion (around 50%) in total mass loading of the antibiotics in influent and effluent of STPs in Japan whereas it was

quinolones for STPs in China.

Ozonation as tertiary treatment of secondary effluent for wastewater reclamation provided significant elimination of antibiotics. Fifty present of the selected antibiotics were removed over eighty percent during ozonation. There was no elimination of antibiotics in dissolve phase during ultra filtration.

4.3 Summery

The occurrence and removal of antibiotics were investigated in this study. All macrolides and quinolones were detected in influent and effluent at four STPs. Clarithromycin was detected in the highest concentration in influent (1129 to 4820   ng/L), followed by azithromycin (160 to 1347   ng/L), levofloxacin (255 to 587   ng/L) and norfloxacin (155 to 486 ng/L) and sulfamethoxazole (159 to 176ng/L) in Japan. Clarithromycin alone shared around 50% load of the detected antibiotics in influent followed by levofloxacin (11 to 19%), azithromycin (6 to 12%), norfloxacin (6 to 10%) and sulfamethoxazole (6 to 8%). Most of the antibiotics removal efficiency was relatively high in A2O and AO based secondary treatment process than CAS process.

At 0.05mg/L and bellow no individual antibiotics had inhibition of bacterial ammonia oxidation over 15%, but at the same concentration in mixed condition it shows 25% inhibition. Inhibition 57% was observed at 0.3 mg/L and over 80% at 0.5 mg/L in mixed condition. Antibiotics showed synergistic effect on ammonia dependent oxygen uptake rate by nitrifying activated sludge.

Most of the antibiotics were detected in higher concentration in influent and secondary effluents from STPs in Beijing, China compare to Kyoto, Japan. Similar to influent sample, levofloxacin (8628 ng/L), norfloxacin (1116 ng/L) trimethoprim (960 ng/L) sulfamethoxazole (683 ng/L) and clarithromycin (561 ng/L) were detected in higher concentration in effluent. Removal efficiency of the antibiotics varied with individual treatment technologies applied, in some extend A2O and AO based biological process was superior to CAS and oxidation ditch. Oxidation ditch based biological process was inferior to antibiotics removal.

Ozonation as tertiary treatment of secondary effluent for wastewater reclamation provided significant elimination of antibiotics. Fifty present of the selected antibiotics were removed over eighty percent during ozonation. There was no elimination of antibiotics in dissolve phase during ultra filtration.

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CHAPTER V

ANTIBIOTICS BIODEGRADATION DURING NITRIFICATION: A NOVEL APPROACH TO REDUCE ANTIBIOTICS LOAD TO WATER ENVIRONMENT FROM NITROGEN ENRICHED WASTEWATER

5.1 Introduction

Since second quarter of the last century (the first antibiotic Penicillin was discovered by Sir Alexander Fleming in 1928), antibiotics have revolutionized medical services by serving as a miracle cure against bacterial infections. After administration, significant parts of the original antibiotics with possible metabolites are excreted with urine and feces, finally ending into municipal sewage treatment plants (STPs). Several studies have shown that antibiotics are present in effluent from STPs, surface water or even in drinking water which indicating these compounds are not removing significantly in conventional STPs with current operating conditions and STPs effluent are major source of antibiotics in surface water . Recently, antibiotic resistances in pathogenic bacteria in environmental samples were reported and raised the question on continuous exposure of antibiotics and possibilities of antibiotics resistance in natural environment. As a consequence, therapeutic options are become narrowing and discovery of new antibiotics is not keeping pace with the growing antibacterial resistance. Besides resistance in pathogenic bacteria, antibiotics may upset sensitive natural ecosystem as they are designed to be highly bioactive.

Recently there has been an increased effort to investigate the behaviors of pharmaceutically active compounds (PhACs) in STPs and a few studies have observed a higher removal of selected PhACs during longer solid retention time (SRT) (Clara et al., 2005 and Joss et al., 2006). Generally longer SRT is applied in STPs with biological nutrient removal process to facilitated slow growing bacteria, mainly nitrifier. Several studies have reported that nitrifying bacteria are capable of co-metabolizing a variety of organic mropollutants (Hyman et al., 1994, Keener and Arp, 1994, Batt et. al., 2006, Yi et al., 2006, vader 2000) that typically resist biodegradation. Therefore, we hypothesized that these nitrifying bacteria could play a key role in the biodegradation of antibiotics in sewage treatment system. The objective of this chapter V is to

identify the role of nitrifier on biotransformation of antibiotics during ammonia oxidation.

5.2 Methodology

5.2.1 Chemical and reagents

Sulfamerazine, sulfamethoxazole, enrofloxacin, clarithromycin and trimethoprim were purchased from Wako Company Ltd. (Osaka, Japan). All antibiotics were of analytical grade (purity > 95%). Formic acid (HPLC grade) and methanol (LC/MS grade) were also purchased from Wako. Individual standard solutions at 1mg/mL were prepared by weighing and dissolving in methanol. All working standards were prepared before analysis.

5.2.2 Cultivation of nitrifying activated sludge

Activated sludge was collected from a sewage treatment plant and cultivated at 30^0C by a fill-and-draw operation with 12h HRT and 40 day SRT in a glass-constructed 3L reactor in laboratory to get desired Nitrifying activated sludge (NAS). In each cycle, 2L supernatant was withdrawn after settling for 30 min and same volume of fresh mineral salts medium was added after drawn. Mineral salts medium was composed of 25mM $(NH_4)_2SO_4$; 43mM K_2HPO_4; 0.2 mM $CaCl_2$; 0.73mM $MgSO_4$; 0.01mM $FeSO_4.7H_2O$; 0.007mM $CuSO_4.5H_2O$; 0.017mM EDTA and 0.4% $NaHCO_3$. The pH was controlled between 7.5 to 8.0 by automatic addition of 30% $NaHCO_3$. DO was maintained between 1.0 and 1.5 mg/L. Data acquisition and process control were performed by linking DO sensor ((Mettler-Toledo InPro® 6800 GmbH, Switzerland), air flow controller, pH sensor (METTLER TOLEDO InPro® 3030, Switzerland) and dosing pump with EYELA process control system which include EPC 2000 ; BOX-D; FF3500 and system software ESS(Tokyo Rikakikai Co., LTD, Tokyo, Japan).

5.2.3 Sorption and biodegradation experiment by NAS
Sorption experiments

All the experiments were performed in two identical batch reactor (made of glass with water jacket to keep constant temperature) with a working volume of 2L and stirred at 200 rpm. NAS was harvested and washed twice with 10mM phosphate buffer trough centrifuged. Experimental facility was same as cultivation of nitrying activated sludge. NAS was mixed with filtered substrate (same composition as cultivation of NAS) to the reactors and the amount of

NAS in the reactor was adjusted to MLSS concentration of 350 mg/L. The pH was adjusted to 7.5 and 250 mg/L sodium azide was added to inhibit bacterial activity. All the tested antibiotics were added in mixture to obtain a final concentration of 5 μg/L each (this concentration is the maximal detected values in the centralized sewage treatment facility and in the surface environment). Every concentration level was sampled five times over a period of 12h (on the basis of a batch experiment, 12h was sufficient to reach the adsorption equilibrium). All experiments were performed in duplicate at 25^0C. From this experiments sorption coefficient *Kd* was calculate by equation (1) (Joss et. al., 2006).

$$K_d(L/g_{ss}) = \frac{S_{sorbed}}{X_{ss} \times C_s} = \frac{C_0 - C_s}{Xss \times C_s} \text{ ----------- (1)}$$

$K_d(L/g_{ss})=$ sorption coefficient; $S_{sorbed}(\mu g/L)=$ sorbed concentrations; $X_{ss}(g_{ss}/L)=$ biomass concentration; $C_s(\mu g/L)=$ concentrations after sorption equilibrium; $C_0(\mu g/L)=$ initial concentration

Biodegradation experiment

Same setup as sorption experiment but without sodium azide addition and DO in the reactors were controlled 1.5-2.0 mg/L using automatic air flow controller connected with DO sensor (like cultivation of NAS). There was no effect on NAS at 5 μg/L concentration of the tested antibiotics (confirmed by oxygen uptake rate, data not shown). Biomass concentration and nitrogen species (ammonia, nitrite and nitrate) were determined by following standard methods (APHA, 1992) .The biodegradation is assumed to follow a pseudo-first-order reaction rate law.

$$\frac{dC}{dt} =$$

$C(\mu g / L) = $ total concentration; $K_{bio}(L / g_{ss} / d) = $ biodegradation rate constant

As true degradation rate is influenced by the sorption, the degradation kinetics was derived for the observed dissolved concentrations considering sorption coefficient. From equation 1 and 2 , the reaction rate can be derived as (Joss et al., 2006):

$$\frac{dC_s}{dt} = r(\mu g / L / d) = \frac{-K_{bio}X_{ss}C_s}{1 + K_d X_{ss}} \text{---------------- (3) As } C = C_s + S_{sorbed} = C_s(1 + K_d X_{ss})$$

5.2.4 Nitrifier activity in biodegradation of the antibiotics in NAS

Allylthiourea was used as the inhibitor at to assess the antibiotics degradation activities of nitrifier in NAS i.e. in the absence of ammonia oxidation. NAS was separated by centrifugation and washed twice with 10mM phosphate buffer (pH 8) in order to remove background matrix and then added into the reactors containing substrate same as cultivation of NAS with MLSS of 350 mg/ L. The antibiotics mixture was added to achieve an initial concentration of 5µg/L and then allylthiourea at 7 mg/L (this concentration was the minimal to inhibit AMO activity of the NAS observed by ammonia dependent oxygen uptake rate, data not shown), the experiments were conducted at 25^{0}C with an initial pH of 8.0 with same setup and instrument control as biodegradation study.

5.2.5 Sample collection and preparation for antibiotics analysis

Samples were collected in amber glass bottle, filtered through syringe filter and acidified to pH 3-4 by 1M HCl to enhance trapping of the antibiotics on the solid-phase extraction (SPE) cartridge. Oasis hydrophilic– lipophilic balance (HLB) extraction cartridges (200 mg,6mL, Waters, Corp., Milford, MA) were used for solid phase extraction. Cartridges were pre-conditioned with 3ml methanol, followed by 3mL of Milli-Q water. For every sample there was duplicate, one was consider as blank and other one was spiked (3µg/L level) by standard and data were used for recovery calculation .Samples (10ml) were passed through the cartridge at a flow rate of 2 mL/min and then vacuum dried for 20 min. Elution was carried out with 6 mL of Methanol (2% ammonia in methanol) in 10ml glass vial . Methanol was evaporated under a gentle nitrogen stream to dryness, and reconstituted with acidified water/methanol (80/20, 0.01% formic acid) to reach a final volume of 1mL (i.e. enrichment factor of 10).

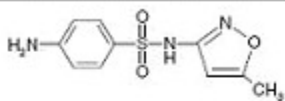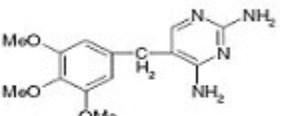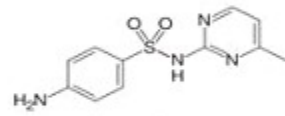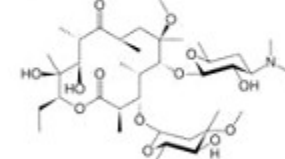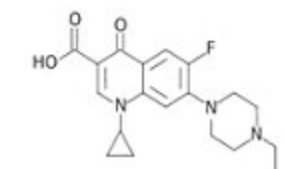## 5.2.6 Liquid chromatography tandem mass spectrometry

Chromatographic separation of the antibiotics were achieved with a Waters Acquity Ultra Performance™ liquid chromatography (UPLC™) separation module with a binary pump system equipped with UPLC BEH C18 column (100   × 2.1 mm , 1.7 μm particle size). Optimum separation was achieved with a binary gradient consisting of 0.01% formic acid (v/v) in water (solvent A) and methanol (solvent B) at a flow rate of 0.35 mL/min with column temperature at 60 °C. The gradient elution setting was: 0–1min: 15% B; 1–4 min: 15-20% B; 4-6 min: 20–60% B; 6–7 min: 60% B; 7-7.5min: 60-15% B; 7.5-10min: equilibration of the column. The injected sample volume was 10 μL. The UPLC system was coupled to a Quattro Micro ™ API mass spectrometer with the electrospray ionisation (ESI) source Z-spray™ (Waters company Ltd.). During quan optimization each analyte was individually infused as a standard solution into the initial mobile phase (50%solventA: 50%solventB) directly into the mass spectrometer at a concentration of 5 mg/L. The parameters (cone voltage, collision energy, ionization mode) were optimized for each compound in order to obtain the maximum sensitivity with the highest amount of product ions available and the most sensitive MRM (Multiple Reaction Monitoring) transitions were determined for each molecule (Table 1). ESI Positive mode was used and the parameters of the mass spectrometer were as follows: electrospray source block and desolvatation temperature: 120 and 400 °C respectively; capillary voltages: 0.5 kV; cone and desolvatation gas flow 50 and 900 L/h respectively. The instrument control, data acquisition and quantification were performed by Mass Lynx 4.1 software.

5.2.7 Validation of the analytical procedures

The antibiotics were analyzed with MRM, using the highest precursor ion/product ion transitions. Calibration curves were obtained by analyzing mixture standard solutions at six levels of concentration ranging between 0.5 μg/L and 100 μg/L, Precision and accuracy of the overall analytical procedure were evaluated with samples, spiked at the two following levels of concentration: 200 and 500 ppt, and compared   with a direct injection of a standard mixture, and reproducibility was assessed. The method was considered accurate if recoveries were in the 70–150% range, and precision was satisfactory if the RSD was lower than 10%. The limit of

quantification (LOQ) and limit of detection (LOD) were estimated as ten times and three times the signal of the highest peak generated by the background noise, respectively (Table 5-1).

Table 5-1 Monitored ions of the target analytes and their limit of quantification in synthetic wastewater

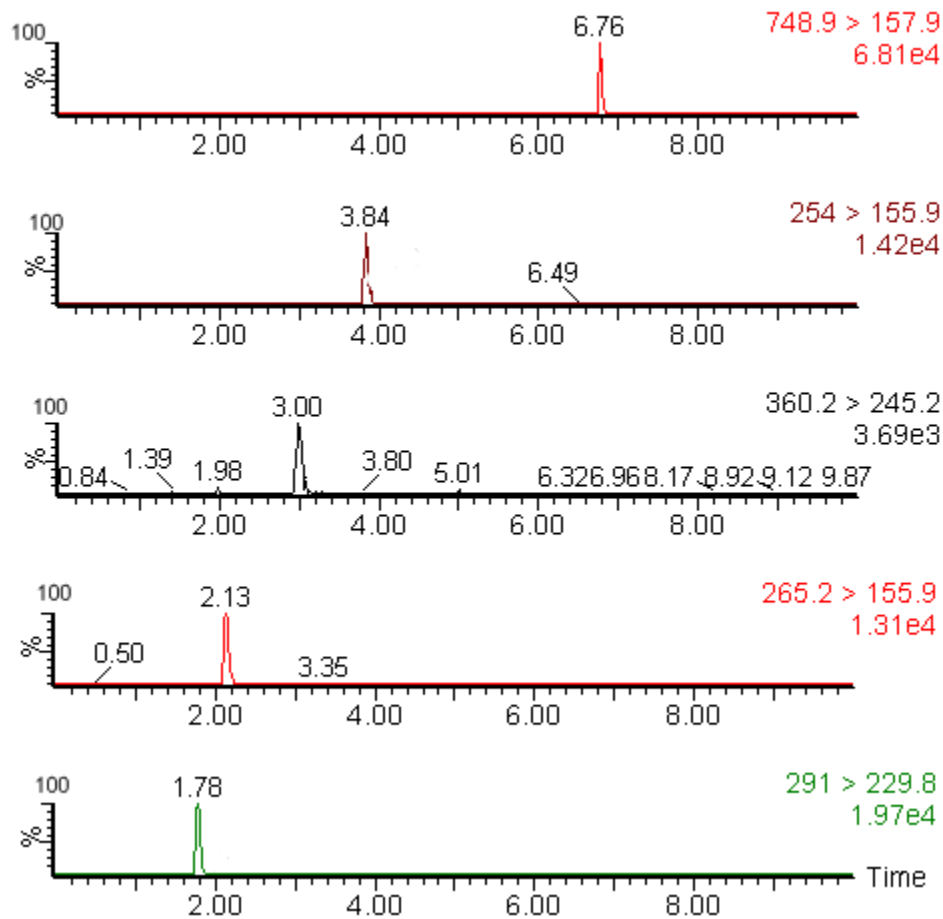| Compound (Group)	Structure	Precursor ion (m/z) / Products ion (m/z)	Retention time (RT in min)	Limit of quantification (LOQ ng/l)
Sulfamethoxazole (Sulfonamide)		254 > 155.9	3.84	9
Trimethoprim (Fluoroquinolone)		291 > 229.8	1.78	5
Sulfamerazine (Sulfonamide)		265.2 > 155.9	2.13	12
Clarithromycin (Macrolide)		748.9 > 157.9	6.76	7
Enrofloxacin (Fluoroquinolone)		360.2 > 245.2	3.00	12

Figure 5-1 Chromatographic separation of the antibiotics

5.3 Results and Discussion

5.3.1 The antibiotics sorption on nitrifying activated sludge (NAS)

In the sorption experiment sodium azide was used to inactivate the NAS. It was observed that sodium azide at 250 mg/L concentration was effective to inactivate the NAS without changing cell properties (confirmed by TOC measurement, data not shown). The influence of sodium azide addition in the tested antibiotics measurement was also monitored (figure 1.). Recoveries were between 83% and 117% which is similar to recoveries in fresh substrate (figure 1.).
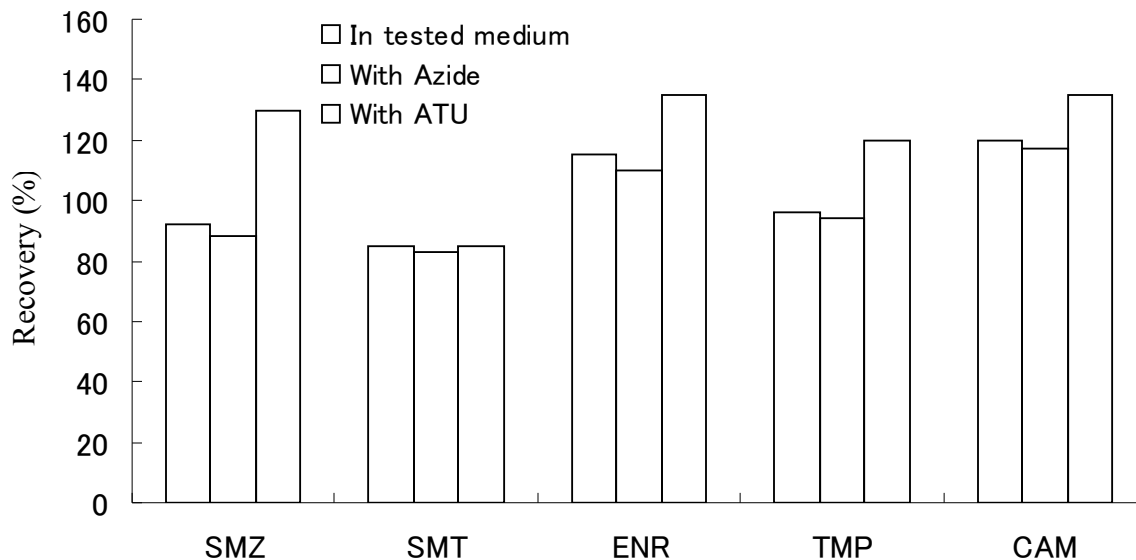
Figure 5-2. Recoveries of the antibiotics in the substrate, after addition of sodium azide (200 mg/ L) and allylthiourea (ATU , 5mg/L).   Sulfamerazine (SMZ), Sulfamethoxazole (SMT), Enrofloxacin   (ENR), trimethoprim(TMP) and Clarithromycin(CAM)

The results of the sorption experiment (i.e. sorption coefficient) and their relation with octanol-water partition coefficients are plotted   in figure 2. Generally the sorption tendency of chemicals is often correlated with their *Kow* values. As expected from the *log Kow* values, sulfamerazine, sulfamethoxazole, and trimethoprim showed very low sorption coefficient, which were 0.02, 0.025 and 0.04 L/gSS respectively. The contribution of sorption in overall removal study showed that compounds which have *Kd* value bellow 0.5 L/gSS are responsible for less than 10% removal through excess sludge (Joss et al., 2006).
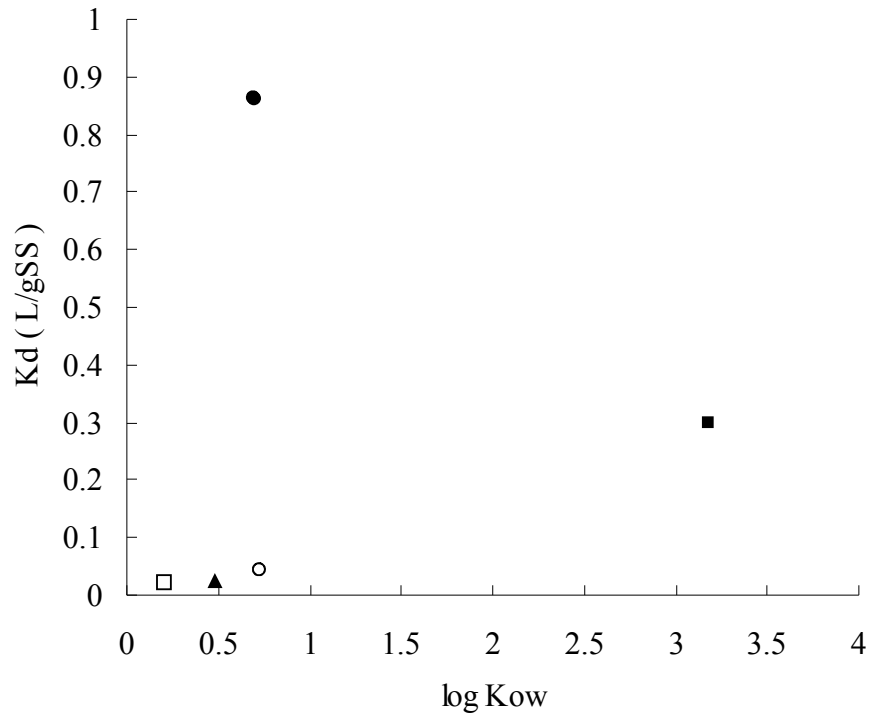
Figure 5-3 Relationship between determined sorption coefficient (*Kd*) with the octanol-water partition coefficient (logKow). Sulfamerazine (□), Sulfamethoxazole (▲), Enrofloxacin (●), Trimethoprim (○) and Clarithromycin (■).

Enrofloxacin has *logKow* value of 0.7, but it showed very high sorption tendency to sludge with sorption coefficient 0.86 L/gSS. This   can be explained by the effects of electrostatic interactions between the positively charged functional group of enrofloxacin and the negatively charged surfaces of the sludge as make up. Similar phenomena was also observed for ciprofloxacina and norfloxacin (Golet et al., 2003). Among the tested antibiotics clarithromycin has higher *Kow* value (3.18) with a sorption coefficient of 0.3 L/gSS, corresponding to around 5% removal with sludge.

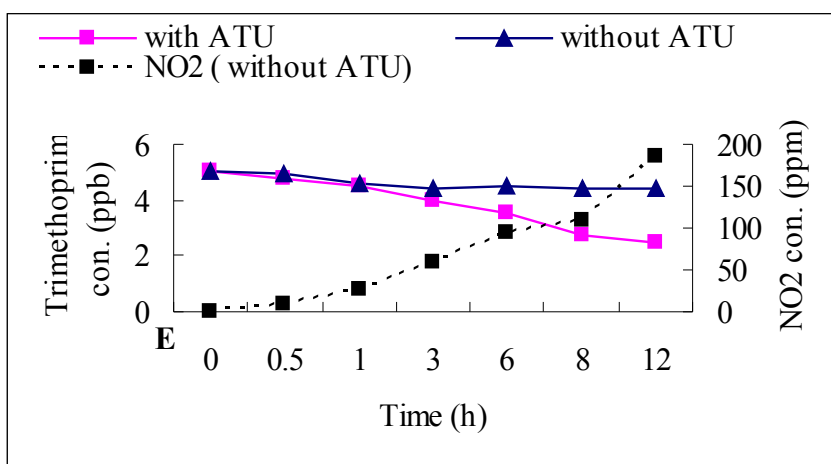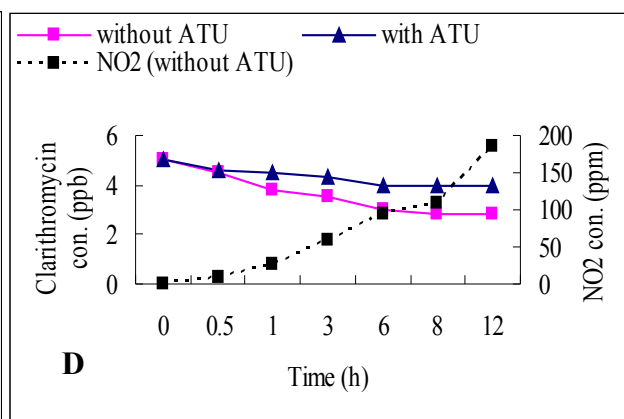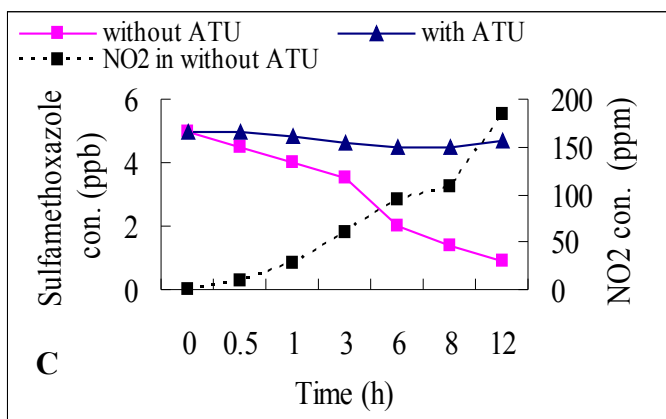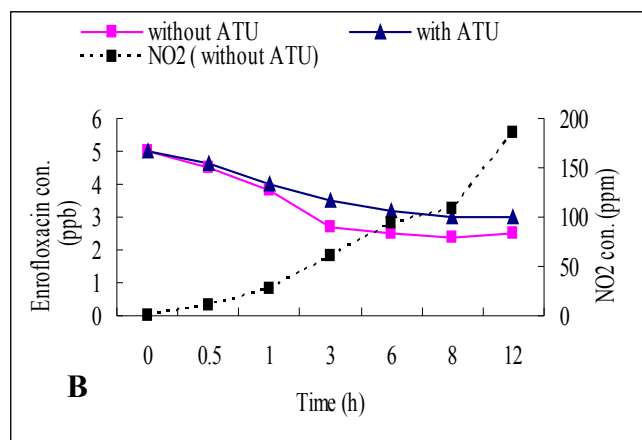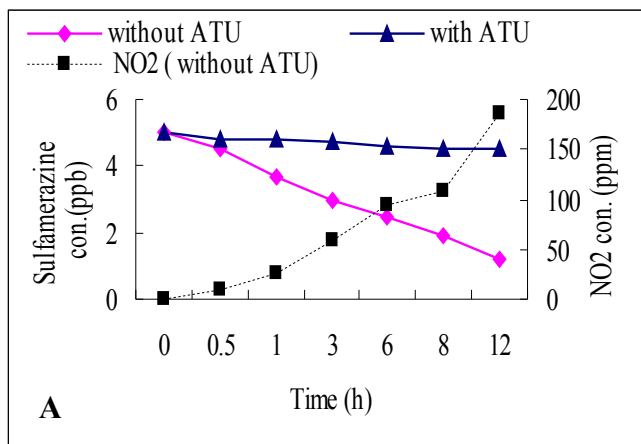Figure 5-4 . Biodegradation profile of tested antibiotics in the presence and absence of allylthiourea (inhibit transformation of ammonia to nitrite by nitrifier) by NAS. (A) Sulfamerazine, (B) Enrofloxacin, (C) Sulfamethoxazole, (D) Clarithromycin, and E) trimethoprim.

5.3.2 Biodegradation of the antibiotics by nitrifying activated sludge (NAS)

Nitrification is a two step process accomplished by the ammonia oxidizing bacteria and nitrite oxidizing bacteria. Several studies have demonstrated that ammonium monooxygenase (AMO) enzyme in the cells of nitrifying bacteria is capable of co-oxidizing many low molecular weight
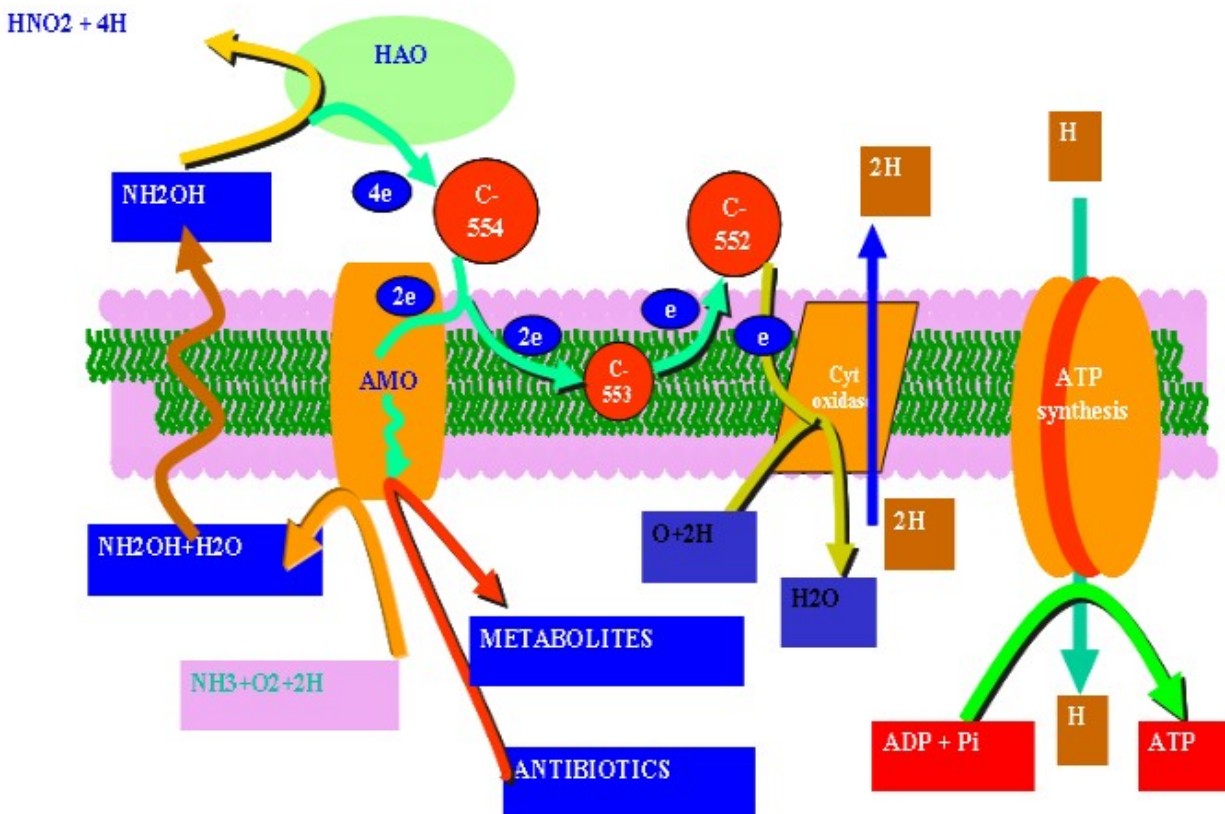
Figure 5-5 proposed removal mechanism of antibiotics during Nitrification 92

organic compounds. Examples of co-oxidations include several aromatics (Keener and Arp., 1994), halogenated hydrocarbons (Rashe et al., 1991), alkenes (Hyman et al., 1988) ethers and thioethers (Hyman et al., 1994). The substrate range of AMO also extends to several endocrine disrupting substances like 17 α-ethinyl estradiol (Yi and Harper., 2006, Shi et al., 2004, Yi and Harper., 2007). Enhanced biodegradation of iopmide and trimethoprim in nitrifying activated sludge was also observed (Batt et al., 2006). The biodegradation of sulfamerazine, sulfamethoxazole, enrofloxacin, trimethoprim and clarithromycin during batch experiment by

NAS is shown in figure 3. The degradation of the antibiotics followed pseudo first-order reaction kinetics with rate constants ($Kbio$) of 9.95 L/gSS/d, 7.6 L/gSS/d, 4.11 L/gSS/d, 3.12 L/gSS/d and 2.74 L/g/d for sulfamethoxazole sulfamerazine, trimethoprim clarithromycin and enrofloxacin respectively. This is the first time to explore the role of nitrifier on antibiotics degradations. The $Kbio$ values for N4-acetyl-sulfamethoxazole (sulfamethoxazole metabolites) and clarithromycin were observed <2 and 3.0-5.0 L/gSS/d respectively in MBR sludge (Joss et al., 2006). NAS showed higher degradation than MBR sludge. Among the five antibiotics sulfonamide antibiotics (sulfamethoxazole and sulfamerazine) degraded faster than others. The antibiotics removal efficiency by NAS were 82%, 76%, 60% , 50% and 44% for sulfamethoxazole, sulfamerazine , enrofloxacin trimethoprim and clarithromycin respectively. Enrofloxacin showed relatively low $Kbio$ value than trimethoprim and clarithromycin but higher overall removal efficiency. This could be explained by higher sorption tendency of enrofloxacin to sludge. Figure 4, shows the results of the batch experiment with simulated result (following equation 3) for sulfamethoxazole and sulfamerazine. The experiment shows that sulfamethoxazole, sulfamerazine, enrofloxacin, trimethoprim and clarithromycin are degradable by the NAS with different rates. These rates are relatively low for complete removal with current treatment strategies but higher than conventional activated sludge or even MBR sludge (Gobel et al., 2005 and Joss et al., 2006). Finally it can be stated that enhancing nitrification in existing sewage treatment system can reduce the antibiotics load to the water environment
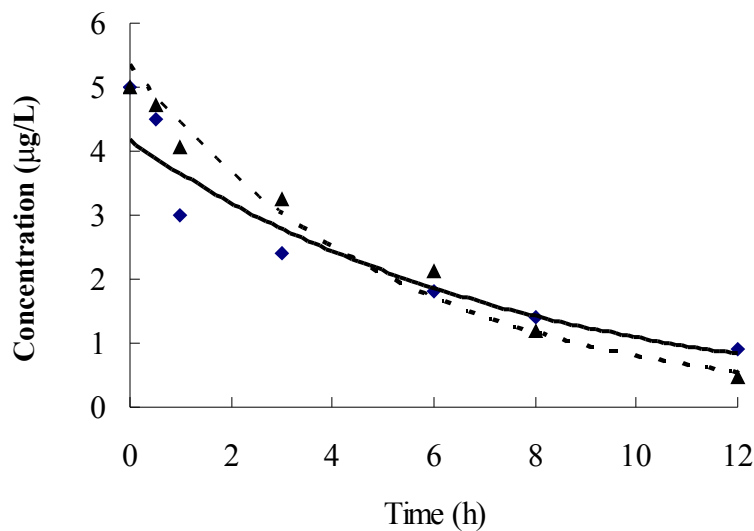

Figure 5-6 . Biodegradation of sulfamethoxazole (▲triangle with doted line) and

sulfamerazine (■diamond with solid line): the triangle and diamond points represent measured value whereas lines represent simulated result.

5.3.3 The role of AMO enzyme of nitrifier in NAS during degradation

Allylthiourea (ATU) was used as the inhibitor of ammonia oxidation, more specifically ammonia monooxygenase enzyme of   nitrifier in NAS   in batch experiments. Figure 5-7. represents the time changes in nitrite accumulation during the antibiotics degradation by NAS with/without
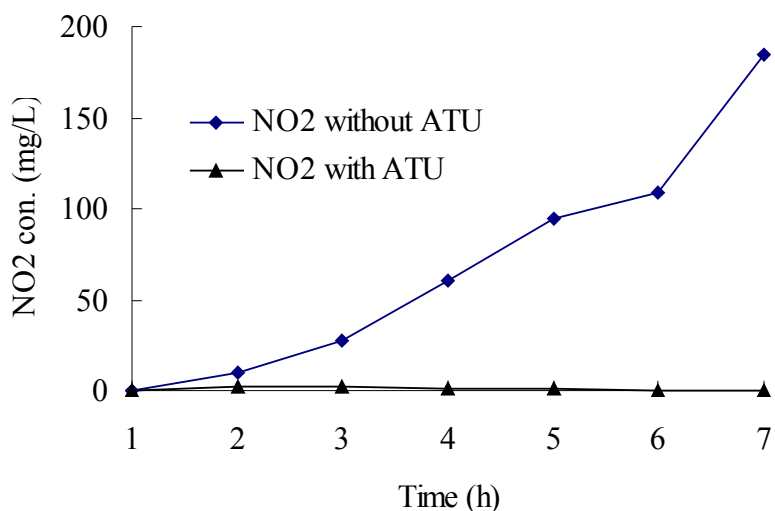
Figure 5-7. Changes in nitrite accumulation during batch experiment with allylthiourea and without allylthiourea 94

allylthiourea. Allylthiourea at 6mg/L was effective to control ammonia oxidation. Results from batch experiments provide evidence for AMO involvement in the antibiotics (Figure 5-4.) biodegradation. The degradation rates are consistent with nitrite accumulation. There were no significance changes in antibiotics concentration in the presence of ATU .The very little change in the antibiotics concentration in the presence of allylthiourea is corresponds to sorption and the activity of other microbes is negligible as it is similar to sorption pattern .

5.4 Conclusions

Biodegradation capability of five antibiotics clarithromycin, enrofloxacin, sulfamerazine, sulfamethoxazole and trimethoprim by nitrifying activated sludge and the role of ammonia oxidizer within the sludge were investigated and the main conclusions are:

● Nitrifying activated sludge (NAS) can biodegrade the tested antibiotics with different biodegradation rate between   2.74 to 9.95 L/gSS/d. Sulfamethoxazole and sulfamerazine degraded faster than trimethoprim, clarithromycin and enrofloxacin. Sulfamethoxazole, sulfamerazine and trimethoprim represented a lower sorption potential (sorption coefficient less than 0.05 L/gSS) to NAS and hardly sorbed,   whereas clarithromycin (0.3 L/gSS) and enrofloxacin (0.86 L/gSS)) represent a relatively high sorption potential.

● In the presence of allylthiourea, it was confirmed ammonia oxidizer in NAS were not metabolically active and there were no significant biodegradation. Ammonia oxidizer played the key role in biodegradation by ammonia monooxygenase enzyme as allylthiourea is a selective inhibitor of ammonia monooxygenase enzyme of ammonia oxidizer.

● The antibiotics degradation by autotrophic nitrifying micro-organisms is a cometabolic process. The nitrifiers degraded antibiotics without prior adaptation and are widespread in the environment. So, these bacteria represent a sink0 for the antibiotics   in biological sewage treatment, in soil or even in surface water ecosystem. Promoting nitrification can enhance the antibiotics biodegradation at or bellow the tested concentration.

● The overall removal efficiency was 80% for sulfamethoxazole, 76% for sulfamerazine, 60% for enrofloxacin, 50% for trimethoprim and 44% for clarithromycin.

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CHAPTER VI

OSELTAMIVIR CARBOXYLATE (THE ACTIVE METABOLITE OF TAMIFLU®) AND AMANTADINE IN WASTEWATER TREATMENT PLANTS EFFLUENT AND IN RIVER WATER IN JAPAN.

6.1 Introduction

During the 20th century, influenza pandemics caused millions of deaths, social disruption and profound economic losses worldwide. The outbreak of Spanish flu in 1918 was the most major one, causing estimated 40 million deaths around the world including 390,000 in Japan. Every year people in Japan and around the globe are suffering due to common seasonal influenza. Influenza experts agree that another pandemic is likely to happen but are unable to say when. According to the World Health Organization (WHO), even in one of the more conservative scenarios, it has been calculated that the world will face up to several 100 million outpatient visits, more than 25 million hospital admissions and several million deaths globally, within a very short period during upcoming influenza pandemic. At present, two groups of antiviral drug have become available for the treatment of influenza infections are the neuraminidase inhibitors (*e.g.* Tamiflu®) and the M2 ion channel inhibitors (*e.g.* Amantadine).WHO recommend the use of antiviral drugs such as Tamiflu® during pandemic, as they are easy to use (Ward *et al.,* 2005). On the other hand, development of new vaccine require at least several months. In Japan, the pandemic influenza preparedness action plan was formulated by the initiative of the Ministry of Health, Labour and Welfare, and approved at the Inter Ministerial Committee on November 14, 2005, to minimize health risks of people and prevent possible damage to social and economic functions. The plans aim to maintain essential services, reduce disease transmission and the socio-economic consequences of a pandemic, and minimize the number of infectious cases, hospitalizations and deaths. The pandemic influenza preparedness action plan of Japan determined the amount of antiviral drug to be secured and stockpiling based on CDC model of USA (around 25% of total population). Target amount of stockpile of Tamiflu® for total number of patients requiring treatment is doses for 25 million patients (amount reserved by the government and prefectures: doses for 21 million patients, and amount of domestic circulation: doses for 4 million patients, dose for one patient is 2 capsules

(752 mg) daily for 5 days, total 10 capsules) which corresponds to several tons of drugs, additionally several tons of antibiotics and anti inflammatory drugs will be used to protect post infection . Target amount of reserved Relenza® is 750,000 thousands patients. Currently only Japan uses ninety percent of Tamiflu® prescribed globally for common seasonal influenza. The above target stockpile amounts shall be increased as necessary from the viewpoint of crisis management, considering the possibility of viruses obtaining tolerance to Tamiflu®, and referring to the status of surveillance on drug-resistance strains. So, it is clear that a huge amount of antiviral drugs and antibiotics will be used during an influenza pandemic condition and effluent to sewage treatment plants (STPs). Unfortunately, these compounds behaviors are mostly unknown during conventional STPs. As they are design to be highly bioactive, these may have significant environmental health impact on non target organism. The wind foul are the natural reservoir of influenza viruses (Olsen *et al.,* 2007). The exposure of antiviral drug in the wild fowl gut and its implications for hastening the generation of antiviral-resistance in avian influenza is also an emerging issue due to wide spread use of these drugs as well as antibiotics resistance (Singer *et al.,* 2007) .

Influenza virus infections continues to cause significant morbidity and mortality worldwide and placing a considerable economic burden on individuals, families, businesses and healthcare providers. Seasonal influenza epidemics causing tens of millions of respiratory illnesses and 250,000 to 500,000 deaths worldwide each year (WHO 2003). During the 20th century, influenza pandemics caused millions of deaths, social disruption and profound economic losses. The outbreak of Spanish flu in 1918 was the most major one, causing estimated 40 million deaths worldwide including 390,000 in Japan (MHLW 2005). Influenza pandemics occur when a new strain of the influenza virus is transmitted to humans from another animal species. Species that are thought to be important in the emergence of new human strains are ducks, chickens and pigs. Recent emergences of highly pathogenic avian influenza virus (H5N1) and report on influenza virus resistance against available antiviral drugs are of great concern..The available options for the control of influenza are limited and vaccination serve as the primary defense against influenza but due to the rapid change in viral antigenic determinants, annual vaccination is required and is not always effective.   At present, two groups of antiviral drug have become available for the treatment of influenza infections are the neuraminidase inhibitors (*e.g.* Tamiflu®) and the M2 ion channel inhibitors (*e.g.* Amantadine). Oseltamivir Phosphate (OP) (Figure 1) which is marketed as Tamiflu®, is recommended by World Health

Organization (WHO) for both treatment and prophylaxis of influenza, and considered an important first-line defense in the event of a future influenza pandemic. OP is a prodrug which is rapidly and extensively hydrolyzed *in vivo* to its active metabolite oseltamivir carboxylate (OC) (Figure 1), a potent and selective inhibitor of influenza A and B virus neuraminidase. OC is excreted (over 80% of oral dose) unchanged (Sweetman SC, 2007).

Recently, OP is widely used in Japan.Like many other pharmaceutically active compounds, sewage treatment plants (STP) effluent will be the main source of OC (as OC excreted unchanged from body via urine and face) in surface water environment, if this drug is not remove significantly in the STPs. The most subtype of influenza A viruses are circulate in waterfowls where they remain, multiplies and excreted in large number through droppings (Olsen et al. 2006).Interestingly, waterfowls stay close to the treated wastewater effluent points , where temperature is relatively higher than surrounding and have enough available foods, specially in winter-the time of common seasonal influenza. Therefore, widespread use of the antiviral OP to fight seasonal influenza in humans could actually lead to the development of OC resistant strains of the viruses in wild birds (Fick et al. 2007 and Singer et al. 2007). Beside virus resistance, a mass administration of OP during future pandemic condition is also an important issue for drinking water safety and ecological health risk.

In this Chapter VI, I developed method for oseltamivir carboxylate and amantadine investigated the occurrence of OC in treated sewage discharges and in receiving river water during 2008/2009 seasonal influenza at Kyoto city in Japan.

6.2 Methods

6.2.1 Site description

All the sampling points located in the Katsura river watershed, comprising river water samples and treated sewage effluent water sample (Figure 6-1). Katsura River receive high anthropogenic inputs, over 80% of wastewater generated from Kyoto City (a city of around 1.5million people) The Katsura river is the final outlet of the STP-A (capacity 975000 m^3/day), STP-B (capacity 114000 m^3/day)   and STP-C (capacity 225,000m^3/day). STP-A has two effluent points: one is in Katsura river (points S1 in the Figure 6-1) and other one is in Nishitakase river (points S3 in the Figure 6-1). STP-B effluent to Nishitakase river (sampling point S1) and STP-C effluent to Katsura river (sampling point S4) (Figure 2). Around 90% of water in the Nishitakase river is sewage water discharged from STP-A and STP-B, during dry days. Sampling point R1 was the

most upstream point and R7 was the most downstream point of the Katsura river.

Occurrence of OC in STPs discharges and receiving river water in three sampling camping
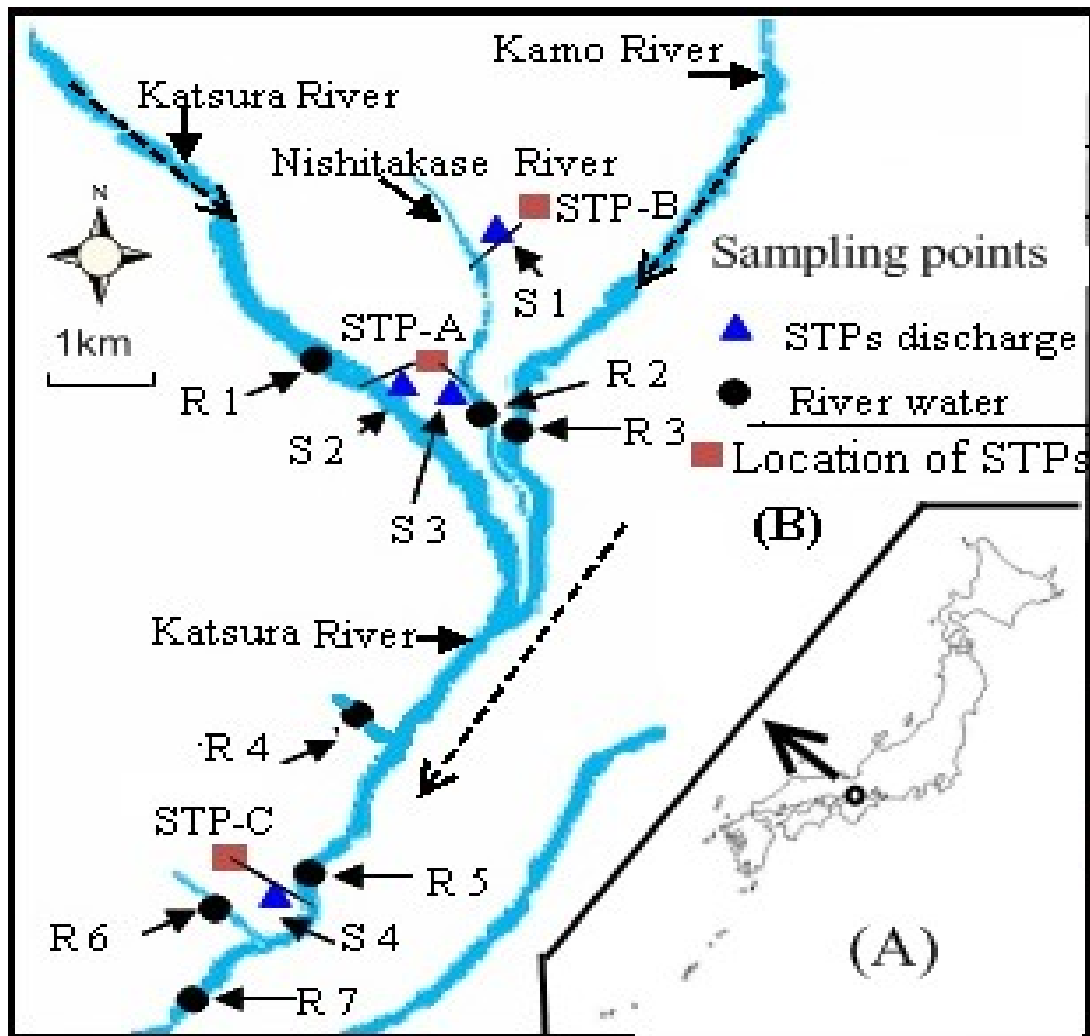
Figure 6-1

Location of sampling points along the Katsura River (B) in the context of Japan (A). S1, S2, S3 and S4 indicate sampling point of sewage treatment plants (STPs) effluent (before mixing with river water). R1, R2, R3, R4, R5, R6 and R7 indicate river water sampling point.

during influenza season (49th week of 2008, 5th and 8th week of 2009) was studied by means of samples collection from 11 locations. Sampling point S1, S2, S3, and S4 indicated STP and R1 ,

R2, R3, R4, R5, R6 and R7 indicate river water sample (Figure 2). Sewage effluent samples were always gathered from outlet of STPs and river sample from bridge in the centre of the stream.

6.2.2 Sample Collection, Preparation and analysis

Grab samples of the STP effluent and the river water were collected into glass bottles. After filtration sodium chloride (1 g/L) was added and sample pH was adjusted to 4 with sulfuric acid if needed, and the surrogate standard D3-OC (50 ng) was added. Solid-phase extraction was performed on 6-mL Oasis HLB sorbent cartridges (200 mg: Waters, Corp., Milford, MA) using concentrator. The cartridges were preconditioned with 3ml of methanol and 3ml of milli-Q water (pH 4). All the samples were passed through the cartridges at a flow rate of 5 mL/min. After concentration, the cartridges were dried completely by air on vacuum manifold for 2 h. The analyte were then eluted from the cartridge with 6 mL of methanol containing 2% ammonia and passed through Sep-Pak® Plus NH2 (350mg: waters Corp., Milford, MA) cartridge (for clean up) into 10-mL graduated glass vessels and dried by a gentle flow of nitrogen at 37^0C temperature. The sample final volume was adjusted to 0.5 mL with water containing 20% methanol (concentration factor of 600).

Chromatographic separation of OC and D3-OC were achieved with a Waters Acquity Ultra Performance™ liquid chromatography (UPLC™) separation module with a binary pump system equipped with UPLC BEH C18 column (100×2.1mm, 1.7 μm particle size). The gradient elution setting was: 0 to 2 min: 10% B; 2 to 4 min: 10 to 75% B; 4 to 4.30 min: 75 to 90% B; 4.30 to 5.30 min: 90% B; 5.30 to 5.80 min: 90% to 10% B; 5.80 to 8.0 min: 10% B (equilibration of the column). The column temperature was kept at 60°C and injected sample volume was 10 μL. The UPLC system was coupled to a Quattro Micro ™ API mass spectrometer with the electrospray ionization (ESI) (Waters company Ltd.). The parameters of the mass spectrometer were as follows: electrospray source block and desolvatation temperature: 120°C and 400°C respectively; capillary voltages: 2.5 kV; cone and desolvatation gas flow 50 and 900 L/h respectively. The instrument control, data acquisition and quantification were performed by Mass Lynx 4.1 software**.**

6.3 Results and discussion

6.3.1 Oseltamivir carboxylate in STP effluent and river water

Three sampling campaign were done at upstream, downstream and effluent point of STP-A, STP-B and STP-C located along the Katsura river during 2008/2009 influenza season in Japan. The result of the sampling in presented in figure 6-2. In this study OC concentration was measured only in liquid phase (GF/B filtered sample). OC has both amine and carboxylate groups in the molecule, a low partition coefficient (log P of 1.1), and high water solubility (588 mg/mL at 25°C) (American Hospital Formulary Service 2006). These physicochemical properties indicating a very low sorption potential to suspended particle as well as sewage sludge during treatment, so load in solid phase in sewage effluent will be less significant

First sampling camping was the beginning of influenza session (Figure 6-2 A). Only twenty seven confirm influenza cases were reported in that week and OC was only detected in all STPs effluent and in river water the concentration of OC was bellow the detection limit (Figure 6-2 B). In this campaign, highest concentration of OC (18.2 ng/L) was detected at sampling point S2, an discharge point of STP-A. A similar level of concentration (16.2ng/L) was detected at points S3, which is also a discharge point of STP-A. STP-A is an activated sludge based biological sewage treatment plants and the biggest sewage treatment plant in Kyoto city. During sampling time average flow rate at discharge point S2 and S3 was 43,1940m^3/d and 105,150 m^3/d, which were correspond to a load of 7.1g/d and 3.2 g/d of OC to the Katsura River, respectively. STP-C is also an activated sludge based biological sewage treatment plants effluent to the Katsura River. At STP-C discharge point S4, OC concentration was 12ng/L with OC load of 1.7g/day. Surprisingly OC concentration was 6.3 ng/L at point S1 (STP-B discharge) which is significantly lower than STP-A and STP-C discharge, which was applicable at all campaign. STP-B operating with activated sludge based secondary treatment with ozonation as tertiary treatment.
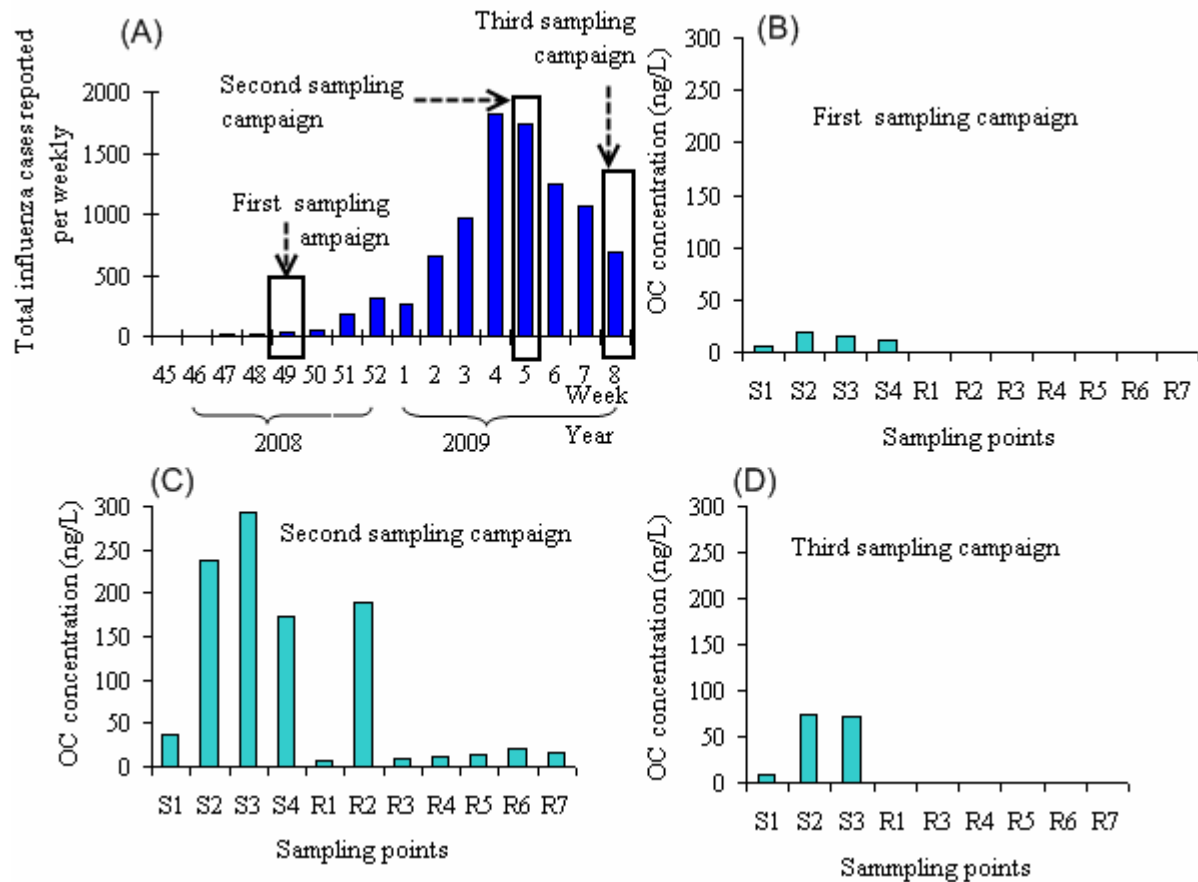
Figure 6-2 Total influenza cases reported per weekly during 2008/2009 influenza outbreak in Kyoto city (until 8th week of 2009) with three sampling campaign time **(A)**, detected concentration of oseltamivir phosphate (OC) in STPs effluents and receiving river water (B) to (D) during three sampling campaign.

Second sampling time was the peak period of 2008/2009 influenza season in the Kyoto city. One thousand seven hundred thirty eight cases were reported in this sampling week. OC was detected in all sampling points, with highest concentration at point S3 (293.3 ng/L) following S2 (238.2 ng/L), R2 (190.2 ng/L), S4 (172.1ng/L) S1 (37.9 ng/L), R6 (19.6ng/L), R4 (11.3 ng/L), R7 (17.1 ng/L) R5 (13.1 ng/L), R3 (8.2 ng/L) and R1 (6.6 ng/L). Sampling point R1 in the Katsura river is located upstream of STP-A (S2) discharge, carried a small amount of wastewater from Kameoka city and hilly area, and have relatively low concentration of OC. Similarly OC concentration was low at the Kamo river sampling point R3 (8.2ng/L), which is also carrying low level of anthropogenic contaminants. On the other hand the Nishitakase river carried treated sewage from STP-B (S1) and STP-A (S3) which is around 90% of its total flow

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and have high concentration of OC at point R2 (190.2ng/L), as expected. The Nishitakase and Kamo rivers join with the Katsura river in the upstream of sampling point R5. At sampling point R5 OC concentration was 13.1 ng/L which is similar to the most down point of Katsura river R7 (11.6ng/L).

Third sample campaign was nearly end of 2008/2009 flue season. In this campaign discharge from STP-C and river sample at R2 was not collected. Similar to first sample campaign, OC was only detected in STPs discharge: S1 (9.0ng/L), S2 (74.6ng/L) and S3 (72.7ng/L).

. Analysis of samples shows an occurrence of OC in all the STPs effluent water during three sampling campaign at concentration reaching up to 293 ng/L during second sampling campaign (Figure 4) which is similar to maximum concentration (311 ng/L) predicted using total number of patient data (Figure 6-3). The prediction was based of some assumption using equation (1) (e.g Singer et al., 2007).

$$PC_{STP}(ng/L) = \frac{(TIC*D)*0.7*0.8*10^6}{P*300} - (1)$$

Where PC_{STP} is predicted concentration of OC in STP effluent in Kyoto city; TIC is total influenza cases per week in Kyoto city during study period , D is OP dose considering 85% adult (two 75 mg OP/day) and 15% (two 45 mg OP/day) child in TIC which is similar to population structure of Kyoto city in 2008; 0.7 (default): seventy percent of cases in a week was considered for a day considering the cases were equally distributed in a week and 5days dose for a patient, 0.8 (default): eighty percent of ingested OP excreted as OC from body, P (1389,000) is total population in Kyoto city, 300 (default) water consumption per person per day. Considering all the cases was treated with OP as first sign of infection and there was zero reduction of OC in STPs.
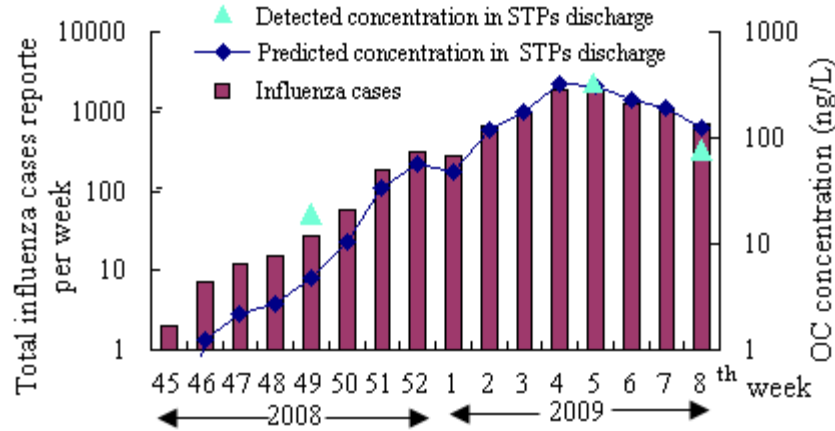
Figure 6-3 Total number of influenza cases reported weekly with predicted and maximum detected concentration of OC in Kyoto city sewage effluent water: assuming *a)* 65 % of weekly confirmed cases represent a single day patient number (a dose continue to 5 days), *b)*Tamiflu® prescribed to all detected cases, *c)* 85% of the detected cases were adult (75X 2 mg dose) and 15% were child (45X2 mg)- same as the population structure of Kyoto city with a total population of 1389,000. *d)* OC dilution factor 300 with sewage water and *e)* no degradation of OC in the sewage treatment plants (Fick et al. 2007).

6.3.2 Amantadine in STP effluent and river water

Amantadine was detected almost all the samples in all three sampling campaigns except at point R1 during first and third campaign (Figure 6-4). Interestingly AMN concentration did not change significantly with increasing the patient number (Figure 6-4). Maximum concentration of AMN was detected during first sampling campaign at point S4 (a STP effluent point). Maximum concentration of AMN in river water was detected at point R2, the river which mostly contains STPs discharge water.
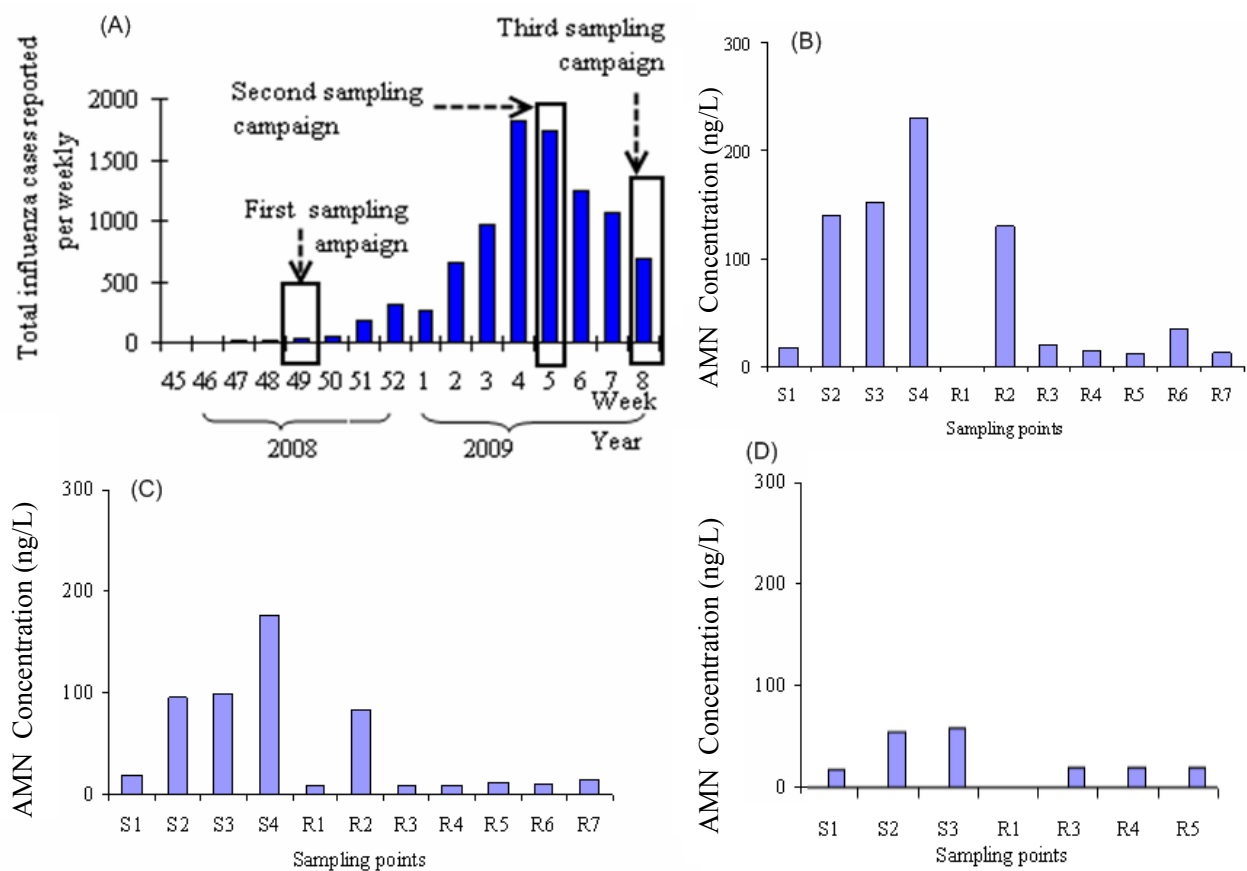
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Figure 6-4. Total influenza cases reported weekly during 2008/2009 influenza outbreak in Kyoto city (until 8th week of 2009) with three sampling campaign time **(A)**, detected concentration of amantadine (AMN) in STPs effluents and receiving river water (B) to (D) during three sampling campaign

6.3.3 Transport and dissipation

During non influenza time OC was not detected in studied sewage discharges and river water samples. The Katsura River is the main river in the study area, receiving most of the wastewater (over 80%) generated from Kyoto city. In the Katsura river profile, OC concentration increased between point R1 (6.6ng/L) and R5 (13.1ng/L) during seasonal influenza. Effluent of STP-A and STP-B were the main source involved in this increase. There was no effect of dilution between R1 and R5 sampling point as the Kamo River (R3 8.2ng/L) also carried higher concentration than R1.OC concentration at most down stream point of the Katsura river (point R7, 17.1ng/L) is relatively higher than R5. The addition of OC from STP-C effluent (point S4,

172 ng/L) and a small canal (point R6, 19.6 ng/L) between R5 and R7 were responsible for the higher concentration at R7 compare to R5. Like pharmaceuticals, OC dissipation in the aquatic environment may primarily occur by three degradation pathways: sorption, biodegradation and photolysis. Amantadine (AMN) is present in the river water and its fate in the environment is not significantly high as concentration not changing with distance. Presence of AMN in the river water during influenza and non influenza time reveled that AMN is not mainly use for influenza treatment , but other disease like Parkinson in Japan.   In n river water, OC removal can be achieved by addition of a low amount (5%) of sediments to water, which promotes microbial degradation processes but need several weeks (more than 8% of ^{14}C-OC evolved as $^{14}CO_2$ from water/sediment samples after 21days of incubation and 60) (Saccà et al. 2009) and OC showed a low sorption affinity to sediments, a negligible reduction is expected due to sorption (Saccà et al. 2009,). In the study area, the time required to water flow from the most upstream and down stream is only several hours (due to steep slop), so contribution of sorption and biodegradation between R1 and R7 will be less significant. On the other hand, photolysis could be less effective as degradation pathway of UV-spectra for OC show no absorbance in the interval 295 to 700 nm (Fick et al. 2007). As a result of low degradation pathways OC will be persist in water environment. A significant load reduction from STPs effluent (similar to STP-B) through implication of advance sewage treatment strategy may reduce the load of OC in the water environment.

6.3.4 Environmental risk

In this study, a maximum OC concentration of 293.3ng/L was detected in STP-A effluent at S3 during second sampling campaign. The highest concentration in river water was 190.2 ng/L at R2 in Nishitakase river during second sampling campaign. The Nishitakase river carried mostly STP-A and STP-B effluent water during dry days. So, it has the higher concentration of OC as expected. A predicted environmental level of OC during a pandemic influenza in Europe and North America was reported between less than 300ng/L to 32 µg/L depending on different characteristics of river basin (Singer et al. 2007).In the Katsura river highest concentration was 17.1ng/l at R7, the most downstream point in this study. In an acute toxicity study in *Daphnia magna*, Tamiflu® was classified as harmful according to the EU Directive 67/548/EEC as amended (EMEA 2005). In sewage treatment process nitrification is generally consider as a sensitive process to toxic compounds. Study shows that concentrations of OC up to 20 µg/mL

did not affect the structure of the microbial community and bacterial nitrification processes (Saccà et al. 2009). However, lower concentration of OC in river water, aquatic flora/fauna and sediments should be considered for waterfowls, a natural reservoir of influenza viruses, which are likely to increase the risk of generating of OC-resistant virus strains. Inhibition concentration of 50% influenza virus (IC_{50}) in some cases was reported between 80ng/L to 230ng/L (Monto et al. 2006 and Gubareva et al. 2001) which is in similar range to the concentration detected in STPs effluent in this study. Resent emergence of highly pathogenic avian influenza virus H5N1 and H1N1 resistance to Tamiflu® in 2008/2009 influenza season are of great concern (WHO 2009). During common influenza season (i.e. winter), waterfowls stay close to STPs effluent due to warm water and higher availability of food. In this circumstances waterfowl would ingest large quantities of OC with virus (virus is believed to be transmitted between waterfowl by the fecal-oral route) in their daily water intake and a high percentage of OC ingested by waterfowls will remain in the intestinal tract (although OC have low sorption potential), the primary site of viral replication and that could promote a selection process towards drug-resistance (Singer et al. 2007 and Fick et al. 2007). As a result, there is a potential risk associated with the widespread use of Tamiflu® during seasonal influenza. The environmental risk of AMN is still hindered by the lack of information.

6.4 Conclusion

This study first time reported the detection of OC in sewage discharge and in river water. The highest concentration of OC detected in sewage effluent was 293ng/L at STP-A. Sewage treatment plant operating with   activated sludge based secondary treatment plus ozonation as tertiary treatment (STP-B) discharged significantly low concentration of OC and AMN than activated sludge based sewage treatment plants (STP-A and STP-C) to the rives. Detection of OC in the river water raises the question as to whether or not a risk of OC resistance influenza virus in waterfowls should be taken under consideration as a result of widespread use of Tamiflu®.

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CHAPTER VII

OCCURRENCE AND FATE OF ANTIVIRAL DRUG OSELTAMIVIR CARBOXYLATE AND AMANTADINE IN SEWAGE TREATMENT PLANTS

7.1 Introduction

Recently, a novel influenza A (H1N1) virus has spread rapidly across the globe and a pandemic alert was announced by WHO. On April 29, 2009, the World Health Organization (WHO) announced that the rapid global spread of a strain of influenza A (H1N1) virus detected in the previous week warranted moving the global pandemic alert level to phase 5 (WHO 2009: http://www.who.int/csr_/disease/swineflu/). At present, two kinds of defense strategy are known against influenza viruses, vaccination for prophylaxis and antiviral drugs for prophylaxis and treatment of the viruses infection. Development of vaccines for a novel influenza virus is a time consuming process (Gerdil, 2003), so antiviral drugs are considered as a first defense line against influenza pandemic. Two groups of antiviral drug have become available for the treatment of influenza infections are the neuraminidase inhibitors (Tamiflu® from Gilead/Roche and Relenza® from GlaxoSmithKline) and the M2 ion channel inhibitors (such as amantadine). Oseltamivir Phosphate (OP) which is marketed as Tamiflu®, is recommended by World Health Organization (WHO) for both treatment and prophylaxis of influenza pandemic. OP is a prodrug, extensively metabolized (over 75%) in the human liver to oseltamivir carboxylate (OC), the active metabolote which is excreted unchanged (Singer , 2007). On the other hand amantadine (AMN) is alsocommonly applied during seasonal influenza and it also applied for hepatitis C, parkinsonism, and multiple sclerosis

Species that are thought to be important in the emergence of new human strains are ducks, chickens and pigs. Recent emergences of novel H1N1 swine fllu, highly pathogenic avian influenza virus (H5N1) infection to human and influenza virus resistance against available antiviral drugs are of great concern. The most subtype of influenza A viruses are circulate in wild ducks where they remain, multiplies and excreted in large number from infected ducks through droppings. Influenza A viruses can occasionally be transmitted from wild birds to other species causing outbreaks in domestic poultry and may give rise to human influenza pandemics (Klenk et al 2008).

According to present strategy, a large amount of antiviral drugs will be used during influenza epidemic or pandemic and release of these compounds to environment could pose a serious ecological health risk. Most importantly ,widespread use of the antiviral drugs to fight seasonal

influenza in humans could actually lead to the development of drug-resistant strains of the viruses in waterfowls, if substantial amount of OC pass through the STPs. More than hundrads pharmaceutical compounds are ubiquitously present in the water environment and they are more or less continuously released from multiple source with a wide range of ellimination in STPs. In this chapter VII, I investigated the occurance and fate of OC and AMN in three STPs which applied a wide range of secondary tretments techonology and advance tertiary treatment in one of them with different operation strategies. There is a need to assess the existing technology to understand their effectiveness and their role during a pandemic For the protection of water resource and aquatic organism for the expected widespread use of antiviral drugs. Unfortunately, the behavior of antiviral drug OC and AMN in STPs are still hindered by the lack of information. At present, OC and AMN are widely used during seasonal influenza which provided a good opportunity to evaluate the compounds behavior in STPs.

7.2 Experimental section

7.2.1 Chemicals and reagents

Oseltamivir carboxylate (OC) and oseltamivir carboxylate label with deuterium (OC-d3) was provided by F. Hoffmann-La Roche Ltd, Pharma Research, Basel, Switzerland. Acetonitrile(LC/MS grade), methanol (LC/MS grade), formic acid (99.9%, for MS), acetic acid, sodium chloride and ammonium hydroxide were purchased from WAKO, Japan.

7.2.2 Description of STPs, sample collection and preparation

Sample were collected from three municipal sewage treatment plants (STPs) in Japan. The STP-X and STP-Z are located in Kyoto prefecture. However, STP-Z located in Shiga prefecture. The plants were selected in this study based on differences in secondary and tertiary treatment technologies. The schematic diagrams of these treatment plants and the various sampling points are shown in Figure 7-1 , while the STPs characteristics and operating conditions are listed in Table 7-1. Primary treatment consists of screen, aerated grit removal tank and a primary clarifier for all treatment plants. In case of secondary treatment, STP-X performed two parallel–operated treatment units which is indicated as section 1 and section 2 (Fig. 1A). Section 1 performed AOAO process with ambient air as oxygen source, whereas section 2 is AOAO process with pure oxygen. The AOAO process with pure oxygen has relatively shorter SRT (solid retention time; 8.2days) and HRT (hydraulic retention time; 4.3hours) for substrate removal compare to AOAO process with ambient air for oxygen (SRT 20days and HRT 11hours). The STP-Y (Fig. 7-1B) utilizes an extended aeration conventional

activated sludge (CAS) system for organic matter degradation. The STP-Z includes three parallel-operated treatment units comprising CAS, AOAO and A2O processes, but for this study we selected only A2O (Anaerobic/Anoxic/Oxic) process.

All the samples were collected in same day from all the STPs in the first week of February 2009, the peak time of influenza during 2008/2009 influenza season in Japan. Composite samples (24-h) were collected from primary influent, primary effluent secondary effluent and tertiary effluent, whereas grab samples were collected from biological reaction tank outlet and return sludge. Ascorbic acid (1g/L) was previously placed in the bottle for acidifying the samples, quenched chlorine/ozone and to enhance analyte sorption on solid phase extraction cartridge. All the samples were filtered (GF/B, pore size 1μm) as soon as possible but no later than 4 h after sampling. The filtered samples were immediately extracted or kept at -25 °C in half-filled amber glass bottles in horizontal position until extraction. After filtration sodium chloride (1 g/L) was added and sample pH was adjusted to 4 with sulfuric acid if needed. The sample volume were 200mL for primary influent and effluent, 300 mL for biological reaction tank effluent, return sludge water, secondary effluent and tertiary effluent. Every sample was divided into two representative sample. One is spiked by OC surrogate standard OC-d3 (50 ng) and other one is spiked by amantadine. Surrogate standard OC-d3 spiked one was considered as unspiked for amantadine. Solid-phase extraction was performed on 6-mL Oasis HLB sorbent cartridges (200 mg: Waters, Corp., Milford, MA) using concentrator. The cartridges were conditioned and equilibrate with 3ml of methanol and 3ml of milli-Q water (pH 4), respectively. All samples were passed through the cartridges at a flow rate of 5mL/min. After concentration, the cartridges were dried completely by air on vacuum manifold for 2 h. The analyte were then eluted from the cartridge with 3 mL of methanol containing 2% ammonia and 3mL of methanol containing 2% acetic acid, and finally passed through Sep-Pak® Plus NH2 (350mg: waters Corp., Milford, MA) cartridge (for clean up) into 10-mL graduated glass vessels and dried by a gentle flow of nitrogen at 37^0C temperature. The sample final volume was adjusted to 0.5 mL with 100μL of methanol and 400μL water containing 0.1% formic acid (i.e. concentration factor of 400 for primary influent and primary effluent ;and 600 for biological reaction tank effluent, return sludge water, secondary effluent and tertiary effluent).

7.2.3 Liquid chromatography–tandem mass spectrometry

Same as previous chapter III (3.2.2.4)The gradient elution setting was: 0 to 2 min: 10% B; 2 to 4 min: 10 to 75% B; 4 to 4.30 min: 75 to 90% B; 4.30 to 5.30 min: 90% B; 5.30 to 5.80 min: 90% to 10% B; 5.80 to 8.0 min: 10% B (equilibration of the column). The parameters of the mass spectrometer were as follows: electrospray source block and

desolvatation temperature: 120°C and 400°C respectively; capillary voltages: 2.5 kV; cone and desolvatation gas flow 50 and 900 L/h respectively. The instrument control, data acquisition and quantification were performed by Mass Lynx 4.1 software.

7.2.4 Batch experiment: Sorption and Biodegradation

Activated sludge from aeration tanks of STP-Y was collected for sorption and biodegradation experiments. the biomass concentration was determined to be 2354 mg/L. in the degradation experiment 2.5L sample was placed in a 3.0L glass reactor covered with aluminum foil and temperature was kept at 20^0C using an external water jacket. Initial pH of the sample was 7.6, dissolved oxygen (DO) was maintained 2.5 to 3.0 mg/L using online DO sensor connecting with a air flow controller. Mixed liqueur mixing inside the reactor was ensured by a stand mount mixer from top at 200 revolutions per minute. In case of sorption experiment, same reactor setup was used without aeration and 15mg/L sodium azide was added to stop biological activity and ensured through DO consumption monitoring. A mixture of the OC and AMN was spiked to get final concentration of 500ng/L for each analyte. A 75ml aliquot was withdrawn from reactor after 0,1, 3, 6, and 12 h and filtered through GF/B. A 50 ml filtered sample was diluted with 200ml water, ascorbic acid and sodium chloride at 1g/L was added. Finally sample pH was adjusted to 4 by sulfuric acid, if needed and extracted same way as mentioned above. The concentrations of OC and AMN in dissolved phase were determined using the SPE and LC-MS/MS methods described above.

Table 7-1 A summary of the STPs characteristics and operating conditions

STP ID	Average flow (m³/day)	Treatment processes			HRT (h)	SRT (days)	BOD$_5$ (mg/L)		TSS (mg/L)		T-N (mg/L)	
		Primary	Secondary	Tertiary			Influent	Effluent	Influent	Effluent	Influent	Effluent
	32,020	Yes	Section 1 AOAO		11.0	20	170	7.3	77	14	17	7.2
STP-X	31,230	Yes	Section 2[a] AOAO	Ozonation	3.3	8.2	150	3.3	82	2	17	5.1
STP-Y	48,130	Yes	CAS		10	11	96	5.9	210	2.9	22.7	12
STP-Z	23,350	Yes	A2O		9.5	13	110	2.7	112	2	20	5.1

HRT:Hydraulic retention time; SRT:Solids retention time; BOD:Biological oxygen demand; TSS:Total suspended solids; T-N:Total nitrogen
AOAO: Anoxic/Oxic/ Anoxic/Oxic; CAS:Conventional activated sludge; A2O: Anaerobic/Anoxic/Oxic; O3:Ozonation

[a] Pure oxygen was applied in Section 2 of STP-K

Table 7-2 LC-MS/MS parameters and method validation result

Analyte	Precursor ion (m/z)	Product ion (m/z)	CV (V)	CE (eV)	RT (min)	Precision RSD (%); n = 5	Accuracy Recovery ± SD(%); n = 5	LOQ (ng/L)
Oseltamivir carboxylate (OC)	285.1	196.7	20	10	3.24	9.5	Primary effluent :135 ± 10	12
							Secondary effluent :120 ± 7	9
OC-d3	288.1	199.7	20	10	3.24	-	-	-
Amantadine	152	134.86			2.91	4.2	Primary effluent :72 ± 8	6
							Secondary effluent :81 ± 6	4

CV: cone voltage; CE: collision energy; RT: Retention time; RSD: Relative standard deviation, SD: standard deviation; LOQ: Limit of quantification

Table 7-3 Concentration of OC and AMN in samples collected from STP-X, STP-Y and STP-Z

		Primary influent	Primary effluent	Aeration tank outlet	Return sludge	Secondary effluent	After ozonation
STP-X	Sampling point	K1	K2	K3	K5	K4	K10[b]
Section 1	OC (ng/L)	375	359	290	269	280	37.5
(AOAO)	AMN (ng/L)	538	438	280	335	234	19.8
Section 2	Sampling point		K6	K7	K8	K9	
(AOAO pu-	OC (ng/L)	375	365.78	293	270	280	
re oxygen)	AMN (ng/L)	538	480	385.36	375.2	315.89	
STP-Y	Sampling point	O1	O2	O3	O4	O5	
(CAS)	OC (ng/L)	359.8	352	310	255	295	
	AMN (ng/L)	310	289	248	240	230	
STP-Z	Sampling point	T1	T2	T3	T4	T5	
(A2O)	OC (ng/L)	403	392.56	255	313	245	
	AMN (ng/L)	229.73	207	175	141.67	140	

[b] combined flow of secondary effluent from section 1 and 2 of STP-X passed through ozonation process and sample K10 was collected after ozonation. (Figure 7-1)

7.3 Result and discussion

7.3.1 Optimizing LC-MS/MS conditions and method validation

In order to obtain the best instrumental conditions for identification of the very low concentration of OC and AMN expected in aqueous samples, MS/MS parameters were optimized by infusing 10μL of 1000 μg/L individual standard (as described in experimental section (3.2.2.4)) and checked for both positive and negative ionization mode. The protonated molecule $[M+H]^+$ proved to be the most abundant ion for both OC and AMN. The $[M+H]^+$ was selected as the precursor ion for collision induced dissociation fragmentation (Table 7-2). With the protonated molecule $[M+H]^+$ as precursor ion, the product ions of OC and OC-d3 were m/z 196.72 and m/z199.71 were observed as the most abundant in the collisioninduced dissociation spectrum which corresponds to loss of the isopentyl side-chain. In case of amantadine, product ion (m/z 134.86) attributable to a loss of NH_3^+ from the precursor ion (m/z 152) of amantadine . Based on the precursor ion and product ions, the MS/MS parameters such as collision energy, cone voltage, capillary voltage, cone and desolvation gas flow, source temperature, desolvation gas temperature were optimized as described in the experimental section and Table3.
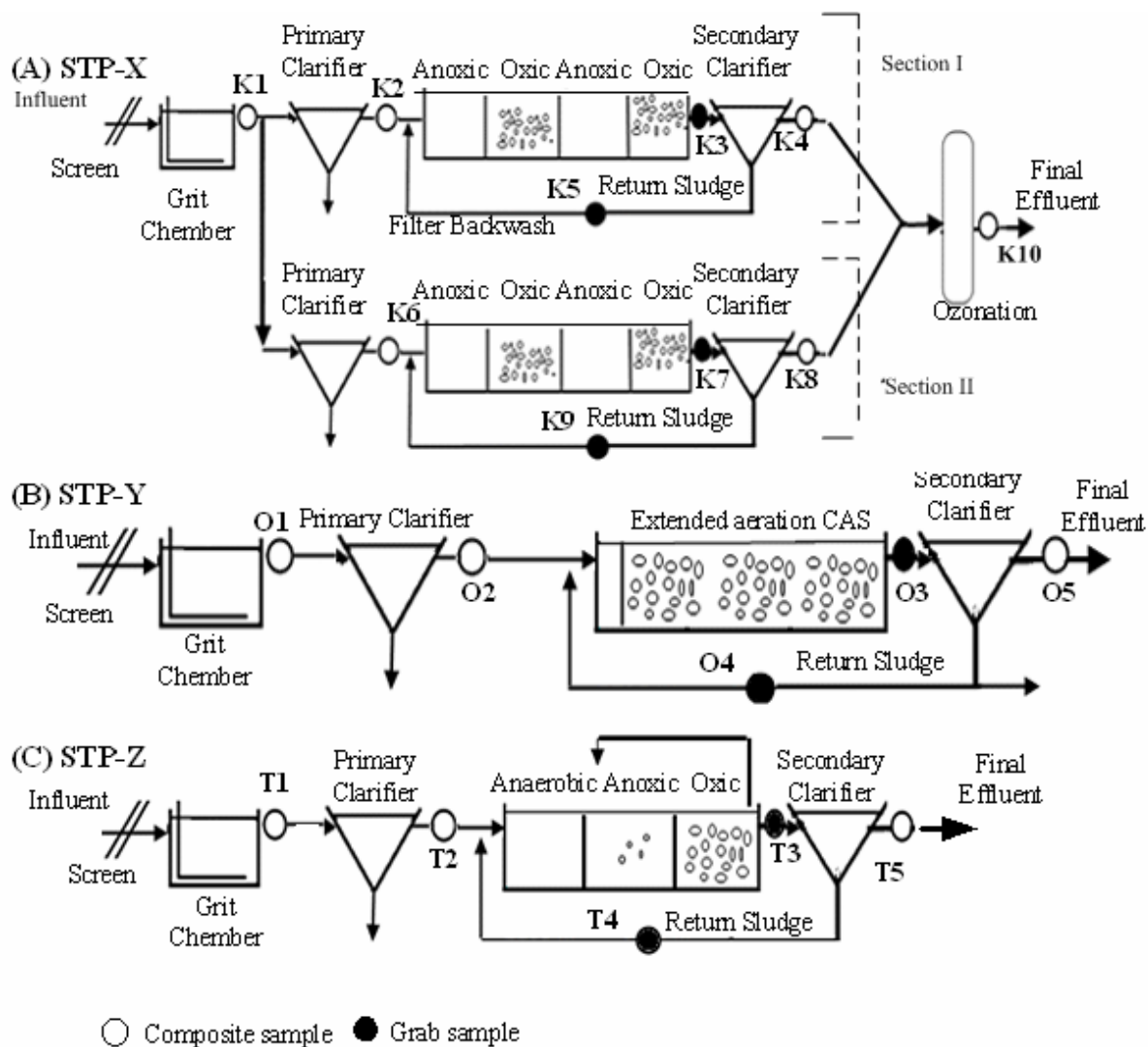
FIGURE 7.1 Flow scheme of the sewage treatment plants : STP-X including section I and sention II (A), STP-Y (B) and STP-Z (C). Sampling points indicated as K1 to K10 for STP-X, O1 to O5 for STP-Y and T1 to T5 for STP-X

The pH of the sample proved to be the most influential variable for OC during sample enrichment on a solid phase extraction cartridge. The effect of pH on the extraction efficiency was studied by adjusting pH value of the sample to 4.0, 7.0 and 11.0. In the light of this result we selected sample pH 4.0. Addition of sodium chloride (1g/L) provided higher recovery of amantadine but independent to OC. According to Ghosh et al. 2009, a cartridge breakthrough studies was performed and sample volume was selected as described in experimental section. The extraction yield of the surrogate standard OC-d3 was determined and it was analogous to OC. Thus, the analyte was quantitatively enriched by one cartridge and exhaustively eluted by the procedure described in methods. The Sep-Pak® Plus NH2 cartridge for clean up during

elution reduced matrix effects and increased the peak sharpness and instrument sensitivity significantly. For the standard curves, linearity was observed with correlation factors typically above 0.99, between 5 pg to 4000 pg on column. The precision, accuracies and instrument LOQ are listed in Table 7-2.

7.3.2 Occurrence and fate of oseltamivir carboxylate and amantadine in STPs (primary and secondary treatment)

This study was performed on February first week in 2009, the peak period of 2008/2009 influenza season in Japan as well as in the study area. Both OC and AMN was detected in all sample investigated and the concentrations are presented in Table 7-3. At STP-X, influent OC and AMN concentrations were 375ng/L and 538ng/L , respectively. It is notable that while AMN significantly decreased during primary treatment (12.6% and 10.8% of influent in section I and section II, respectively), the reduction in OC (4.2 % and 2.5% of influent in section I and section II, respectively) was limited in this treatment process. This could be attributed to sorption mechanisms of molecules to solid particle related to *log P* value of molecule. AMN has relatively higher than OC (Table 7-3). At STP-X, OC and AMN concentration was moderately decreased (21-23% of OC and 30-33% of AMN) in both sections during secondary treatment. STP-X applied AOAO process as secondary treatment, ambient air as oxygen source in section I and pure oxygen in section II. During the sampling period, the HRT and SRT in section I was 11hours and 20days while in section II the value was 3.3hours and 8.2 days respectively. Previously it was observed that activated sludge process with shorter HRT and SRT does not appear effective in the degradation of micropollutants. HRT and SRT are the two key factors which can affect the removal efficiencies of pharmaceuticals in the STPs. Previous studies showed that some compounds were better eliminated in STPs with high HRTor SRT (Clara et al., 2005; Siegrist et al., 2005). Kim et al., (2005) examined the influence of HRT and SRT on the removal of tetracycline in the activated sludge processes. However in this case both section had similar removal performance, so pure oxygen could be the factor for similar removal with a shorter HRT and SRT in section II as of section I. ozonation as tertiary treatment in STP-X provided around 85% removal of   OC and over 90% removal of AMN from secondary effluent.
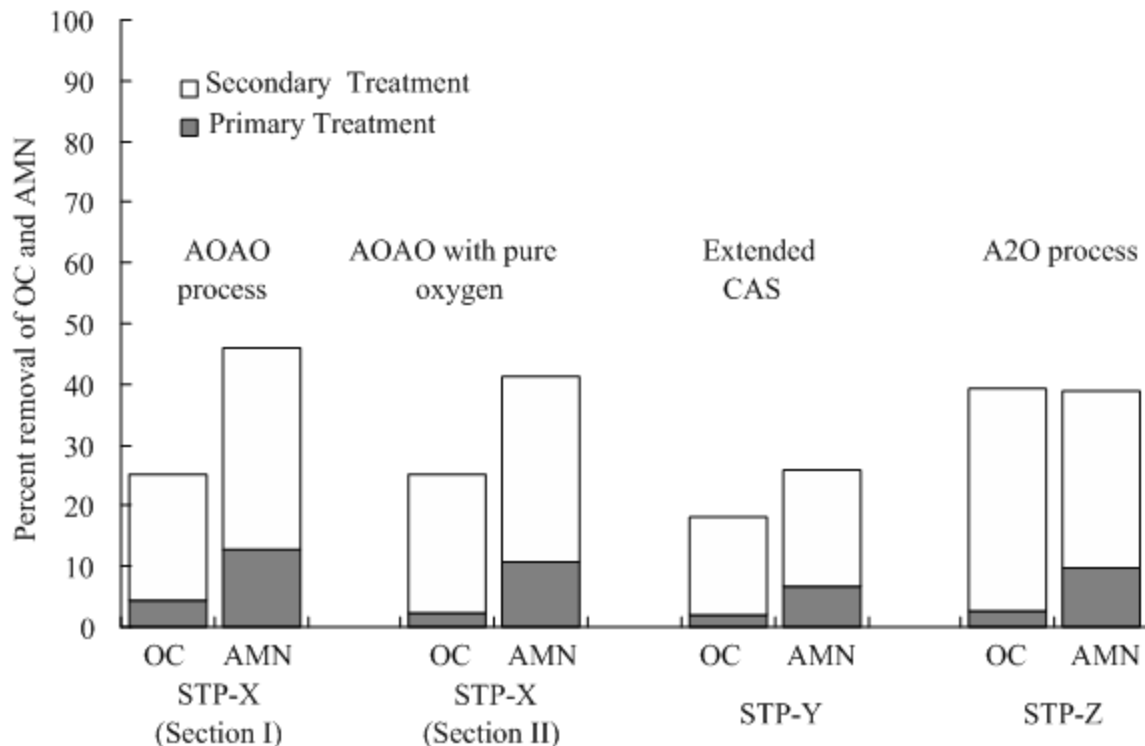
FIGURE 7.2 Removal of OC and AMN during primary and secondary treatment in STP-X, STP-Y and STP-Z

OC concentration (359.8 ng/L) In STP-Y influent was similar to STP-X but AMN concentration was relatively low (229ng/L). Similar removal pattern for both OC and AMN was observed during primary treatment in STP-Y, as of STP-X. During extended aeration based CAS process during secondary treatment in STP-Y OC and AMN were removed 15% and 20% respectively. STP-Y was inferior to AOAO based secondary treatment of STP-X for OC and AMN removal, could be a result of different microbial community due to different operation mode and differences in SRT. Within three STPs, STP-Z shows highest OC removal (36%) during secondary treatment whereas AMN removal was similar to STP-X. since sorption of OC to activated sludge is negligible (Figure 7-2)the removal of OC could be attributed mainly to biodegradation. The difference in OC and AMN removal by three different secondary treatment in three STPs indicates that the nature of the microbial population in STPs have an important role on the biodegradation. Finally, it was observed that only primary and secondary treatment process in STPs can not remove these compound completely and a significant portion still remained in secondary effluent.

7.3.3 Overall elimination of OC and AMN at various STPs

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The difference between removal rates in the STPs probably results from many factors, such as the type of the treatment process (AOAO, CAS A2O), mode of oxygen source, HRT, SRT and even variation of concentration in influent. Therefore, removal rates can vary significantly from one plant to another, and at different time periods in any one plant. In this study, STP-Y and STP-Z are the two activated sludge secondary treatment plants without any tertiary treatment facility, while STP X has secondary treatment facility plus ozonation as tertiary treatment (ozone dose around 4.5mg/L with 30 to 35 min contact time) The present study results showed that the removal rates of OC and AMN in STP-X was higher than those in the other two plants.
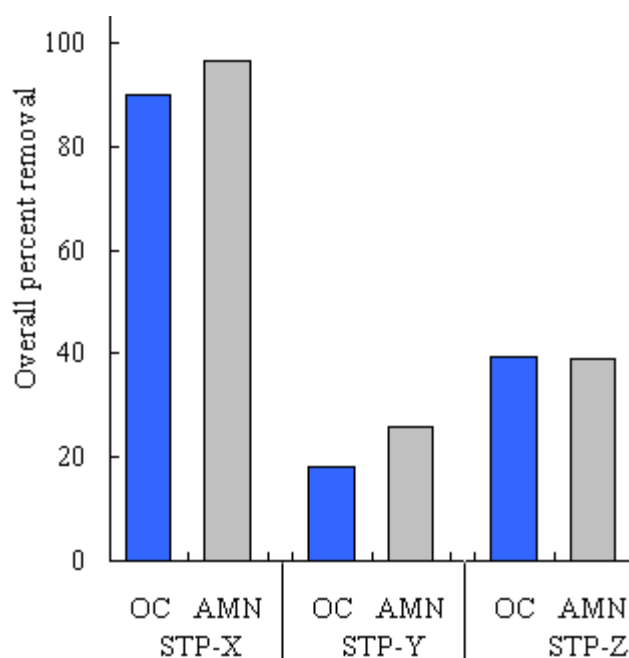

Figure 7.3 Overall removal efficiency of OC and AMN in STP-X, STP-Y and STP-Z

Overall OC and AMN removal in STP-X was 90% and 96% respectively (Figure 7-3). Ozonation as tertiary treatment removed 86% of OC and 93% of AMN from secondary effluent. However, the elimination rates of OC and AMN in STP-Y and STP-Z were significantly lower than STP-X. The STP-Y removed only 15% of OC and 19% of AMN during entire treatment process however STP-Z removed was 36% of and 29% of AMN. From the investigation it is observed, OC and AMN can pass through the STPs which applied only primary plus secondary treatment and ozonation as tertiary treatment can removed these compound over 90%.

7.3.4 Dissolved daily mass fluxes of OC and AMN at the STPs

The dissolved daily mass fluxes for OC and AMN in the STPs are shown in Fig. 7-4. The dissolved daily mass fluxes can vary significantly among different STPs. The dissolved daily mass fluxes of OC and AMM in final effluent of STP Y and STP Z were much higher than STP-X . The dominant factor was application of ozonation as tertiary treatment in STP X. Dissolved mass flux of OC in influent was 24.7, 17.3 and 9.4 g/day in STP X (secton I + section II) , STP Y and STP Z respectively while the value was 34.0, 14.9 and 5.4 for AMN in STP X (secton I + section II) , STP Y and STP Z respectively
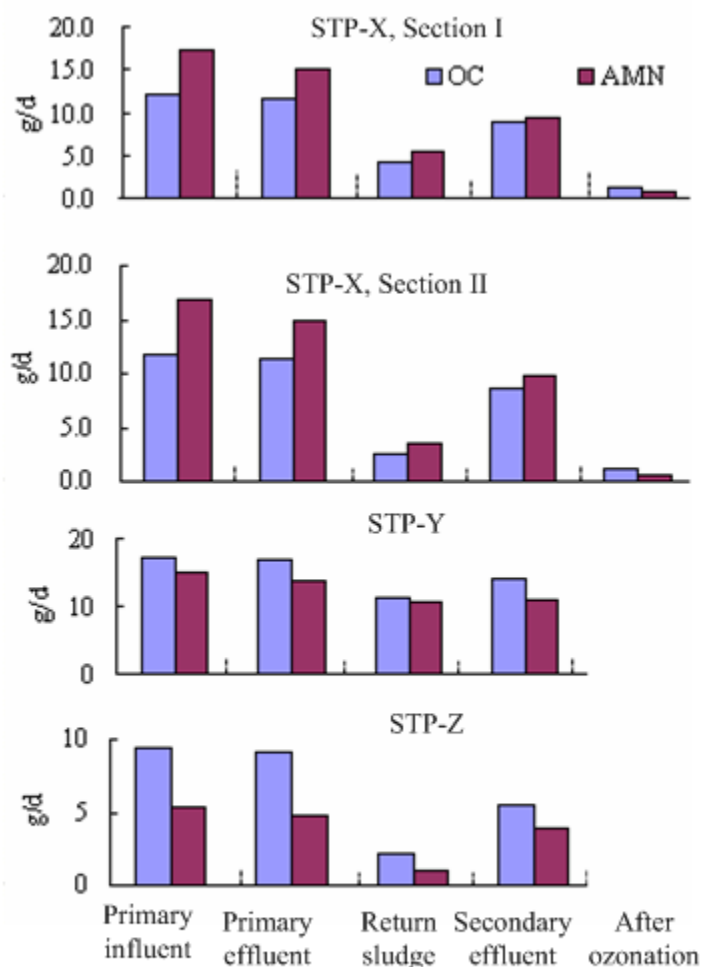

FIGURE 7.4 Dissolved mass fluxes of OC and AMN
at STP-X (Section I and II), STP-Y and STP-Z

Every year large amounts of OC and AMN are transported to the aquatic environment via STPs, mainly during seasonal influenza, however a small amount of AMN used all time for other

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disease like Parkinson .Although the removal of OC and AMN in STPs may be optimized through increased SRT, HRT and/or an additional tertiary treatment step (such as ozonation), the overall elimination of the compounds in STPs is still incomplete. Therefore, residual amounts of these compounds are continuously discharged to receiving surface waters. In addition, little knowledge of the environmental risk assessment for these compounds is available at the moment. Recent emergence of new influenza virus and usages of these drugs during a future pandemic and potential threat to ecosystem are of important concern among researcher. Before it was thought that OC is unchanged degrading wastewater treatment, but this study first time evaluated different full scale STPs and it was observed that OC in removed around 20 to 35% during primary plus biological secondary treatment in STPs. Ozonation as tertiary treatment can reduce both OC and AMN over 90%. STP X and STP Z are two major STPs in Kyoto city. STP-Z is the biggest treatment plant in kyoto city (975000m3/d) but we studies only the A2O line. STP-X and STP-Z combinedly treated 60-70% of wastewater generated from Kyoto city, Japan. The Katsura River receives water from both STPs effluents including other STPs which are around 80% of wastewater produced in Kyoto city. In river water the corresponding concentrations will be ten times lower than STPs effluent. Based on chronic toxicity data it is observeded that predicted no effect concentrations for fish, daphnia and algae are in the milligrams per litter range (Straub in press). However the effect of AMN is still hindered by lack of information and AMN is not considered as a first defense line drug for pandemic. Therefore, the OC in the rives near the sewage discharge points were unlikely to induce lethal toxicity to aquatic organisms or to have a significant impact on the growth of plants and bacteria, during a pandemic , when around 25% of tital population will intake antiviral drug according to present preparedness action plan.. However, there are not enough data to assess the long-term influences caused by continuous discharge of hundreds of antibiotics in combination with antiviral drugs into the aquatic environment, as antibiotics are considered to be use for post infection (Singer et al 2008). Moreover, biomagnifications and avian influenza virus resistance inside waterfowls are of important issues that should be paid more attention. Inhibition concentration of 50% influenza virus (IC_{50}) in some cases was reported between 80ng/L to 230ng/L (Monto et al. 2006 and Gubareva et al. 2001) which is in similar range to the concentration detected in STPs discharge without advance tertiary treatment during seasonal influenza in this study.

7.3.6 Sorption and biodegradation during batch experiment

The biodegradation and sorption behavior of OC and AMN was determined in laboratory

experiment as described in methods. Fig 7-5 demonstrates that sludge sorption is the main removes mechanism for AMN (15%),but not OC (5%) .
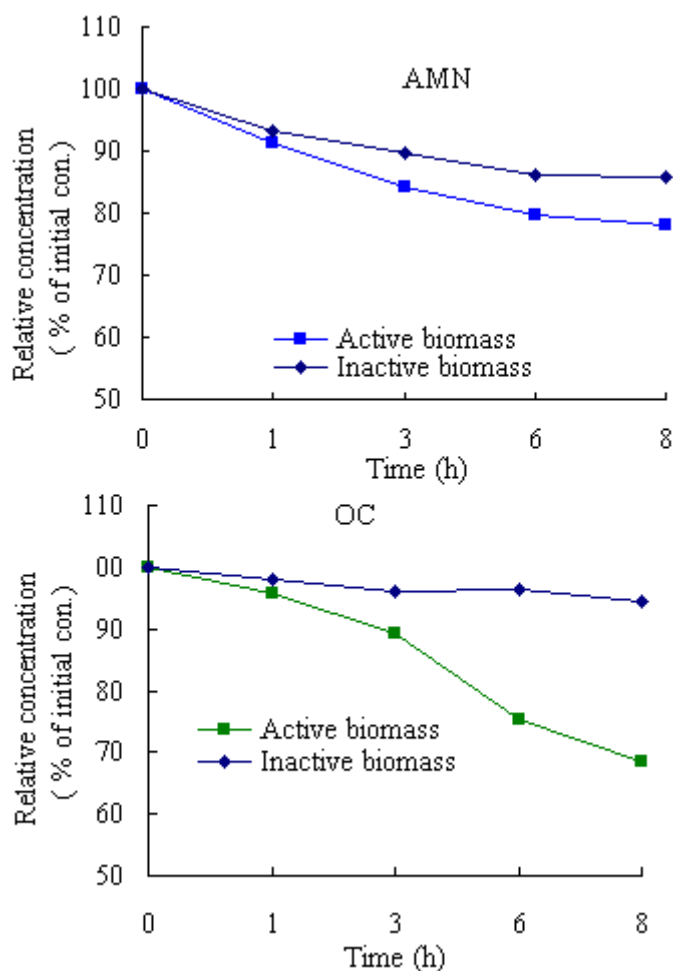

Figure 7-5 Sorption and biodegradation batch experiment of OC and AMN

As dissolved phase was analyzed during the sorption study, it is logical to assume that any decrease in concentration in the solution phase is due to sorption becausethey are not volatile and biodegradation was inhibited by sodium azide. Our observation is in agreement with earlier studies which showed OC sorption to sediment and calculate Freundlich constant (K_f) were between 0.26 to 1.26   (Sacca et al., 2009). On the other hand, other researchers have also demonstrated that OC is highly water soluble and have negligible sorption to sludge biomass (Fick et al., 2007). During biodegradation study OC and AMN removal were 33% and 23% respectively. As OC has low tendency of sorption, this removal can be expected due to biodegradation . Recently it is reported that addition of 5% of sediment to water can promote

biological degradation of OC (Sacca et al., 2009) and from environmental fate study it was also reported that degradation of OC seems to occur as a combination of microbial metabolism and indirect photolysis (Bartles and Tümpling 2009).

7.4 Conclusion

Both OC and AMN were detected in all the sample during the campaign. The highest concentration of OC and AMN in the primary influent was 403ng/L and 538ng/L respectively. Both OC and AMN is not remover significantly in conventional sewage treatment systems (STP-Y and STP-Z). Removal of OC and AMN in primary treatment is relatively low (5 to 10% of the total removal). OC and AMN removal in biological nutrient removal STPs were higher than conventional STP. Ozonation as tertiary treatment can remove OC over 90% and AMN over 95% from secondary effluent and can reduce the risk associated with OC use during a future epidemic and pandemic. Protection of water ecosystem and drinking water supply during a future pandemic is emerging, need to pay more attention, and ozonation will a good choice with prove acceptance.

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CHAPTER VIII

WASTEWATER TREATMENT SYSTEM PREPAREDNESS AND RISK ASSESSMENT FOR ANTIVIRAL DRUG AND ANTIBIOTICS USE UNDER PANDEMIC CONDITIONS

8.1 Background

Influenza pandemics are unpredictable but recurring events that can have severe consequences on societies worldwide. Since the 16th century, influenza pandemics have been described at intervals ranging between 10 and 50 years with varying severity and impact (Table 8-1). A novel influenza A (H1N1) virus has spread rapidly across the globe. On April 29, 2009, the World Health Organization (WHO) announced that the rapid global spread of a strain of influenza A (H1N1) virus detected in the previous week warranted moving the global pandemic alert level to phase 5. Recently WHO announce a pandemic alert and raising up to Phase 6.In 1997, an avian influenza A virus of subtype H5N1 first demonstrated its capacity to infect humans after causing disease outbreaks in poultry in Hong Kong SAR, China. Since its widespread reemergence in 2003-2004, this avian virus has resulted in millions of poultry infections and over four hundred human cases. An unusually high percentage of human H5N1 infection resulted in severe illness and death compared to other influenza viruses and far exceed the proportion of deaths caused by the 1918 pandemic virus. On rare occasions, H5N1 has spread from an infected person to another person - most often a family or other household member acting as a caregiver. However, none of these events has so far resulted in sustained community-level outbreaks. The primary risk factor for a human to acquire a zoonotic H5N1 infection is direct contact or close exposure to infected poultry, although the virus remains difficult to transmit to humans. Five years after the widespread emergence and spread of H5N1, the virus is now entrenched in domestic birds in several countries. Controlling H5N1 among poultry is essential in reducing the risk of human infection and in preventing or reducing the severe economic burden of such outbreaks. Given the persistence of the H5N1 virus, successfully meeting this challenge will require long-term commitment from countries and strong coordination between animal and human health authorities. While the H5N1 virus is currently the most visible influenza virus with pandemic potential, it is not the only candidate. Wild birds form a reservoir for a large number of other influenza viruses and influenza viruses are found in other animal species as well. Any one of these other viruses, which normally do

not infect people, could transform into a pandemic virus. In addition to H5N1, other examples of animal influenza viruses previously known to infect people include avian H7 and H9 subtypes and swine influenza viruses. The H2 subtype, which was responsible for the 1957 pandemic (but has not circulated for decades), could also have the potential to cause a pandemic should it return. The uncertainty of the next pandemic virus means that planning for pandemic influenza should not exclusively focus on H5N1, but should be based on active and robust surveillance and science-based risk assessment.

Influenza experts agree that another pandemic is likely to happen but are unable to say when. According to the World Health Organization (WHO), even in one of the more conservative scenarios, it has been calculated that the world will face up to several 100 million outpatient visits, more than 25 million hospital admissions and several million deaths globally, within a very short period during upcoming influenza pandemic. WHO recommend the use of antiviral drugs such as Tamiflu® during pandemic, as they are easy to use (Ward *et al.,* 2005). On the other hand, development of new vaccine require at least several months. In Japan, the pandemic influenza preparedness action plan was formulated by the initiative of the Ministry of Health, Labour and Welfare, and approved at the Inter Ministerial Committee on November 14, 2005, to minimize health risks of people and prevent possible damage to social and economic functions. The plans aim to maintain essential services, reduce disease transmission and the socio-economic consequences of a pandemic, and minimize the number of infectious cases, hospitalizations and deaths. The pandemic influenza preparedness action plan of Japan determined the amount of antiviral drug to be secured and stockpiling based on CDC model of USA (around 25% of total population). Target amount of stockpile of Tamiflu® for total number of patients requiring treatment is doses for 25 million patients (amount reserved by the government and prefectures: doses for 21 million patients, and amount of domestic circulation: doses for 4 million patients, dose for one patient is 2 capsules (150 mg) daily for 5 days, which corresponds to several tons of drugs, additionally several tons of antibiotics and anti inflammatory drugs will be used to protect post infection . Target amount of reserved Relenza® is 750,000 thousands patients. Currently only Japan uses ninety percent of Tamiflu® prescribed globally during common seasonal influenza. The above target stockpile amounts shall be increased as necessary from the viewpoint of crisis management, considering the possibility of viruses obtaining tolerance to Tamiflu®, and referring to the status of surveillance on drug-resistance strains. Population coverage of antiviral drug Tamiflu® of Japan still far bellows than other developed countries (figure.) So, it is clear that a huge amount of antiviral drugs and antibiotics will be used during an influenza pandemic condition

and discharge to sewage treatment plants (STPs). Unfortunately, these compounds behaviors are mostly unknown during conventional STPs. As they are design to be highly bioactive, these may have significant environmental health impact on non target organism. The wind foul are the natural reservoir of influenza viruses (Olsen *et al.,* 2007). The exposure of antiviral drug in the wild fowl gut and its implications for hastening the generation of antiviral-resistance in avian influenza is also an emerging issue due to wide spread use of these drugs as well as antibiotics resistance (Singer *et al.,* 2007) .
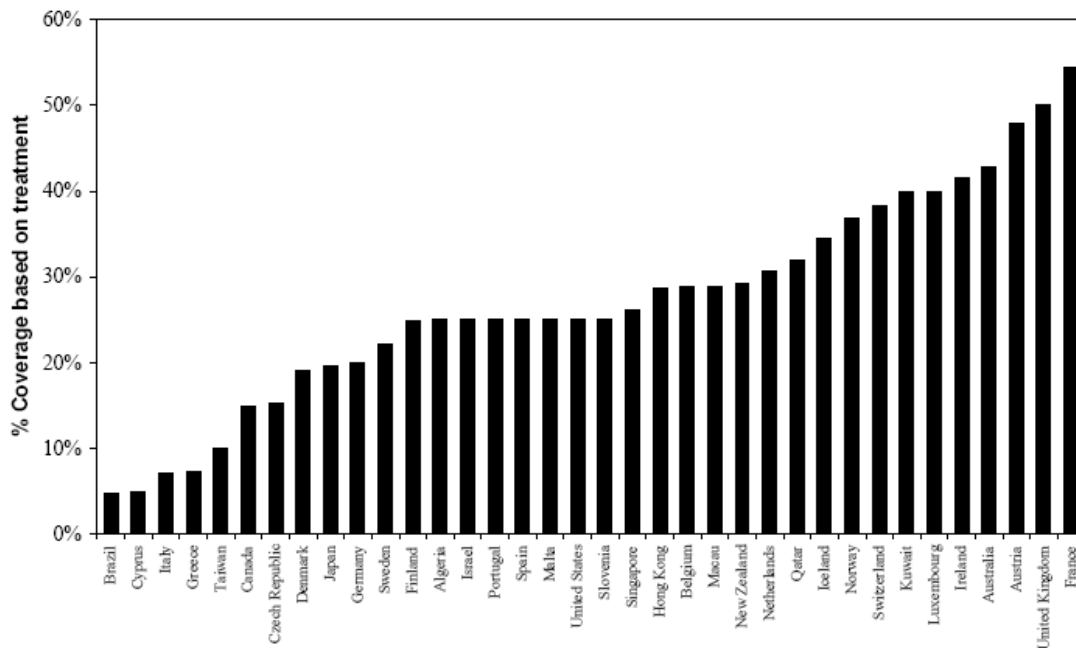

Figure 8-1. Government Tamiflu targets for population coverage (%). (Singer et al., 2008)

Table8-1Characteristics of the three pandemics of the 20th century[#]

Pandemic (Date and Common name)	Area of Emergence	Influenza A virus Subtype	Estimated Reproductive Number	Estimated fatality Rate	Estimated Worldwide	Age group Most Affected	GDP (Percentage Change)[*+]
1918-1919 "Spanish Flu"	Unclear	H1N1	1.5-1.8	2-3%	20-50 million	Young adults	-16.9 to 2.4
1957-1958 "Asian Flu"	Southern China	H2N2	1.5	<0.2%	1-4 million	Children	-3.5 to 0.4
1968-1969 "Hong Kong Flu"	Southern China	H3N2	1.3-1.6	<0.2%	1-4million	All age groups	-0.4 to (-1.5)

[#]Adapted from European Centre for Disease Prevention and Control, Pandemics of the 20th Century
(http://ecdc.europa.eu/Health_topics/Pandemic_Influenza/stats.aspx
[*] McKibbin and Sidorenko., 2006
[+]McKibbin and Sidorenko., 2007

8.2 Pandemic plan of Japanese Government

The 'Pandemic Influenza Preparedness Action Plan of the Japanese Government' has been drafted in compliance with WHO Global Influenza Preparedness Plan (May 2005) to facilitate quick and trustworthy countermeasures and revised in 2007. Estimation model of US Centers for Disease Control (FluAid 2.0) was used for the assessment of new influenza epidemics/pandemics. It was estimated that, if 25% of the Japanese population are infected by the virus, 13 to 25 million (median approx. 17 million) will visit medical facilities (MHLW 2005).

Target amount of stockpile of oseltamivir phosphate (Tamiflu)

Total number of patients requiring treatment: The number of patients requiring treatment is the estimated number of patients who   visit medical institutions if 25% of the Japanese population are infected with new   influenza (calculated based on a CDC model) which is corresponding to doses for 25 million patients

(1) Amount reserved by the government and prefectures: doses for 21 million patients.Government: doses for 10.5 million patients; Prefectures: doses for 10.5 million patients

(2) Amount of domestic circulation: doses for 4 million patients   (dose for one adult patient is 2 capsules (150mg)daily for 5 days, total 10 capsules).

Target amount of reserved zanamivir hydrate （product name: Relenza） This could be the second line defense in case of Tamiflu resistance.

(1) Amount of domestic circulation: doses for 150 000 patients

(2) Amount reserved by the government: doses for 600 000 patients

The above target stockpile amounts shall be increased as necessary from the viewpoint of crisis management, considering the possibility of viruses obtaining tolerance to oseltamivir phosphate, and referring to the status of surveillance on drug-resistance strains.

8.3 Predicted maximum OC concentration in STPs influent, secondary effluent and after advance tertiary treatment (ozonation) during a pandemic with three scenarios.

Table 8-2 and Figure 8-3, shows the predicted OC concentration in influent , effluent in Kyoto city STPs and downstream of the Katsura river ant Miyamaibashi during a pandemic with three different scenario. According to CDC flueAid model it is estimated than from 15% (minimum) to 35% (maximum)., 25% (most likely) population may affected during pandemic

and will take antiviral drugs (Singer et al., 2008, CDC flu aid model . The predicted concentration The prediction was based of some assumption using equation (1).

$$PC_{STP}(ng/L) = \frac{(TIC*D)**0.8*10^3}{P*300} - (1)$$

Where PC_{STP} is predicted concentration of OC in STP influent in Kyoto city; TIC is total influenza in Kyoto city , D is OP dose considering 85% adult (two 75 mg OP/day) and 15% (two 45 mg OP/day) child in TIC which is similar to population structure of Kyoto city in 2008; 0.8 (default): eighty percent of ingested OP excreted as OC from body, P (1389,000) is total population in Kyoto city, 300 (default) water consumption (Litter) per person per day. After biological treatment average 20% reduction was considered (as of Chapter 7), after ozonation 90% load from secondary effluent is considered (as it was observed in the STP ; Chapter 7). In case of river water delusion factor 10 was taken in calculation.

In this study , scenario 1 indicate 15% of the total population will be infected by influenza virus and will administrated antiviral drug Tamiflu®. Scenario 2 and scenario 3 represent 25% and 35 % of the population will be infected and will take Tamiflu, respectively. From the scenario, it is expected that OC concentration will be in the range of 60 to 140 µg/L in influent for scenario 1 to scenario3. This concentration will reduced to 48 µg/L to 112 µg/L after biological treatment. Ozonation after biological can reduce OC load significantly. After ozonation as tertiary treatment concentration of OC will be reduced to 6µg/L for scenario 1, 10µg/L for scenario2 to 14 µg/L for scenario 3 (it is expected that ozonation will reduced 90% of OC from secondary effluent according to the result from STPs survey of Chapter VII). With existing treatment facility OC concentration at Miyamaibashi (down stream of Katsura river) OC concentration may reach up to 10µg/L as it's a fast flowing river and rater retention time is less than a day (10 time dilution of STPs effluent and no degradation in the river due to short hydraulic retention time between discharge points and Miyamaibashi). This concentration could be high if the dilution factor decreased. But this concentration can be reduced to ng/L level with application of ozonation after biological based secondary treatment in upstream STPs discharge. In some case in Europe and USA the maximum concentration was predicted around 80µg/L in river water where OC degradation in STPs was considered zero (Singer 2007).

Table 8-2 predicted concentration of oseltamivir in different compartment of water environment in Kyoto city.

		Scenario 1	Scenario 2	Scenario 3
		15%	25%	35%
OC excreted (mg)				
From adult		21251700	35419500	49587300
From child		3750300	6250500	8750700
Total		25002000	41670000	58338000
OC concentration (µg/L) in different water compartment Under different condition	Series 1	60	100	140
	Series 2	48	80	112
	Series 3	6	10	14
	Series 4	3.84	6.4	8.96
	Series 5	0.48	0.8	1.12
	Series 6	4.32	7.2	10.08

Series 1 = STPs influent concentration

Series 2 = STPs effluent concentration after biological treatment

Series 3 = OC concentration in STPs effluent after Ozonation as tertiary treatment treatment

Series 4 = OC concentration in downstream Katsura river water at Miyamayabashi (with only biological treatment in STPs)

Series 5 = OC concentration in downstream Katsura river water at Miyamayabashi (with ozonation as tertiary treatment in STPs with present operation conditions)

Series 6 = scenario with existing facility

OC concentration in STPs influent and effluent and receiving water during influenza pandemic scenario : minimum (15% of total population), most likely (25% of total population) and maximum (35%), according to U.S.A CDC(center for disease control) model. (All concentrations are in microgram/L and dilution factor 10 in river water)

Series 1: OC concentration in STPs influent (Kyoto City)
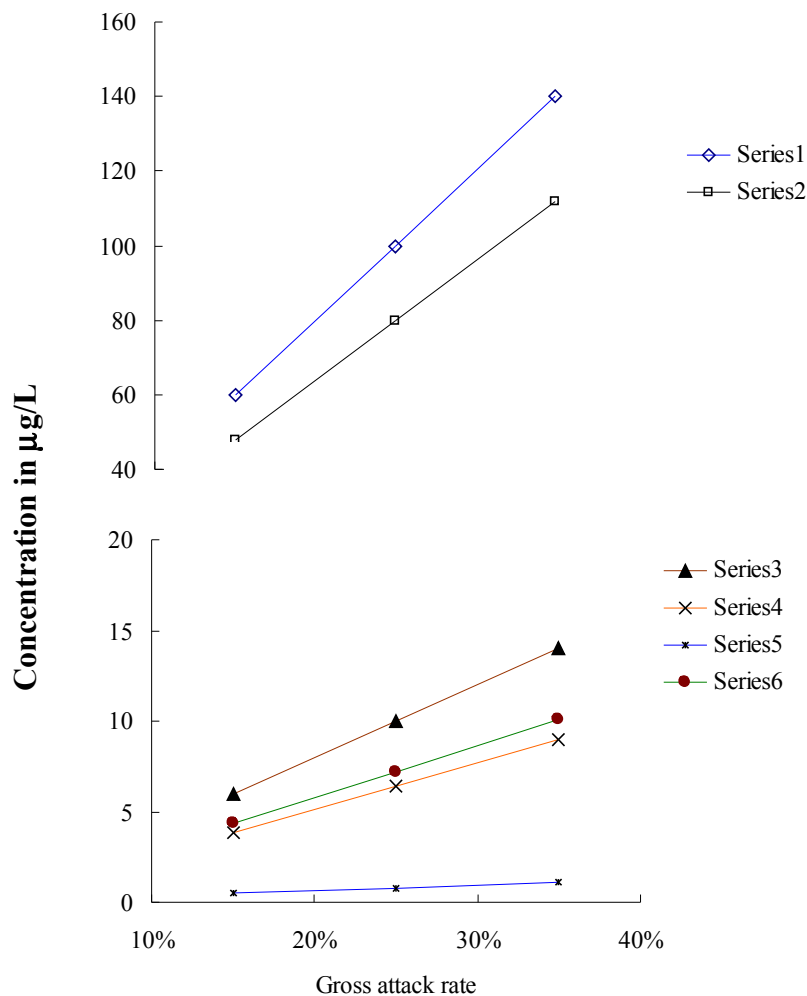Series 2: OC concentration in STPs effluent concentration after biological treatment (Kyoto City)
Series 3: OC concentration in STPs effluent after Ozonation as tertiary treatment treatment (if applied)
Series 4: OC concentration in downstream Katsura riverwater at Miyamayabashi (with only biological treatment in STPs)
Series 5: OC concentration in downstream Katsura riverwater at Miyamayabashi (with ozonation as tertiary treatment in STPs with present operation conditions)

Series 6: OC concentration in downstream Katsura riverwater at Miyamayabashiscenario with existing facility

Figure 8-2 Predicted maximum concentration of oseltamivir in different compartment of water Environment in Kyoto city for three different pandemic influenza scenarios (Zero reduction in pipeline and river water)

8.4 Risk assessment of OC under pandemic conditions

8.4.1 Preliminary Hazard Characterization

Fick et al., (2007) suggested that the release of OC into rivers could generate OC resistance in avian influenza in wildfowl. It was proposed that OC could enter the gut of wildfowl from ingested river water and interact with the avian influenza neuraminidase. The concentration of OC in the gut might be higher than in river water owing to recycling of the urine in waterfowl,

thereby further increasing the selection pressure for OC resistance. From clinical study it was observed that IC$_{50}$ value of OC in some influenza is less than 100ng/L (Gubareva et al., 2001; Monto et al., 2006) which is similar to the value detected in STPs in Japan during seasonal influenza epidemic.

8.4.2 Ecotoxicity

Table 8-3 shows ecotoxicity data and based on the data published in peer-reviewed journal, OC was not likely to be an area of primary concern. There is a need for broader range of ecotoxicologic work. However, the level of concern regarding the ecotoxicity was generally quite low. Considerably greater concern was expressed regarding the potential inhibition of non-target neuraminidases, in organisms other than influenza (e.g., microorganisms). From the prediction it was observed that OC concentration in Katsura river could reached up to around 10μg/L, whereas lowest PNEC value of OC from the published data is more than or equal to 100μg , so the PEC/PNEC is lower than one, which indicated low risk associated with OC.

Table 8-3 Ecotoxicological risk associated with oseltamivir carboxylate.

Tests, organisms	Test Method	Endpo-int	Dur-ation	OP (mg/L)	OP+OC 1:4 (mg/L)	Chronic PNEC	Ref.
Freshwater							
Algal growth inhibition *P. subcapitata*	OECD 201	NOEC	96h	7.6			Roche (2007)
			72h		10	1 (mg/L)	Roche (2007)
Acute daphnid immobilisation, *D. magna*	OECD 202	NOEC	48h	14			Roche (2007)
		EC50	48h	25			Roche (2007)
Acute fish toxicity, *C. carpio*	OECD 203	NOEC	96h	76			Roche (2007)
		LC50	96h	>76			
Chronic daphnid reproduction, *D. magna*	OECD 211	NOEC	21d		1HTC	0.1(mg/L)	Cafarella (2008a)
Fish early life stage test, *D. rerio*	OECD 210	NOEC	32d		1HTC	0.1(mg/L)	Cafarella (2008b)
Marine							
Algal growth inhibition *Isochrysis galbana*	OECD 201	NOEC	72h		100 HTC (OC)		Hutchinson (2009)

8.4.3 Antiviral resistance

There was uncertainty among scientist regarding the potential for generation of OC resistance in avian influenza viruses as a result of the exposure of wildfowl to OC in surface waters.

Virologists agreed that reassortment, or mutation, would be required to generate a virus with human specificity, and such viruses would have to find a path to circulate back to human. Concerns were somewhat alleviated by the knowledge that OC is not readily absorbed from the gut and therefore would not be present in significant amounts in the urine to recycle as proposed in Singer et al. (2007). The consensus opinion was that it is difficult to predict the exposure of OC in the wild fowl gut and its implications for hastening the generation of OC-resistance in avian influenza. The highest concentrations of OC in river water will only be reached during relatively short spells of very high drug usage during an influenza pandemic outbreak. Peak concentrations (between 3 to 6 weeks) will only be reached after thousands of infected patients have been treated with the drug. Resistant virus will inevitably have been selected in some of these patients in advance of any possible selection in waterfowl. Thus, should the waterfowl be infected with the pandemic strain, the resistant virus strains and genetic mutations encoding OC resistance selected will be common to both species but selected in man first. Resistant pandemic virus in waterfowl alongside the same viruses already in man will have little or no effect on the human situation. As in man, resistant viruses in waterfowl are likely to be cleared or revert to wild type once river OC levels fall and selection pressure is removed. Available data regarding the nature of OC resistance indicate that mutations responsible for conferring resistance severely compromise viral growth and infectivity (Aoki et al. 2007; Chutinimitkul et al. 2007; Hayden 2006; Hurt et al.2007; Lipsitch et al. 2007; Yen et al. 2005). If resistance were to be selected or acquired by reassortment in other avian virus strains, again the resistant viruses are likely to revert to wild-type once selection pressure is removed. If for some reasons this does not happen and resistant virus persists as one of the circulating avian virus strains in waterfowl, then its chance of becoming a human pathogen (by chance mutation or recombination) will be no greater than that for any other of the 16 avian influenza A virus serotypes in circulation. Such events are rare; highly pathogenic H5N1 viruses have been circulating and infecting the occasional human who has direct contact with birds since 1997 and the virus has still not achieved the capability of transmitting between humans. Thus, the idea of a "second wave" pandemic arising by this mechanism is considered highly unlikely.

8.4.4 Sewage treatment plant (STP) failure
 STPs will receive higher concentrations of OC than river. This factor will pose a widespread problem to STPs should these compounds be found to inhibit the process organisms at concentrations anticipated following use in a pandemic. One of the concerns highlighted was

that OC might exhibit activity on neuraminidases held by bacteria within STPs. From a preliminary study by Sacca et al (2009), OC concentration up to 20µg/L not affecting the nitrifier-a sensitive microbial group in STPs. Tamiflu is a neuraminidase inhibitor that was rationally designed to inhibit the influenza A and B neuraminidase; however, there is a basis for considering that a neuraminidase present in a bacterium could also be inhibited by Tamiflu. Soong et al. (2006) demonstrated the efficacy of OC and a similar antiviral, peramivir, to inhibit biofilm formation in the microorganism *Pseudomonas aeruginosa*. These authors were interested in the potential to use the NAI for alleviating symptoms of cystic fibrosis, hence environmentally relevant concentrations of OC were not investigated. Nevertheless, 1 µg/L of OC demonstrated a 0.6-fold inhibition of biofilm formation, which might be applicable to concentrations in sewage treatment plants (STP) during an influenza pandemic (Soong et al. 2006).

8.5 Antibiotics and pandemic flu

While antivirals may reduce the number of complications, there are still likely to be significant numbers of complications occurring in a pandemic. Some of the most common include bacterial infections in the respiratory tract and lungs. Antibiotics are needed to treat such complications. Antibiotics will be used to treat people in the community if they develop complications. In hospitals, antibiotics will be used to treat the sickest patients and may reduce the length of hospitalization. If a pandemic started soon no effective vaccine would be available and there would probably be a shortage of antiviral drugs. There is no evidence (yet) of the effectiveness of neuraminidase inhibitors(like Tamiflu®) in case of avian and pandemic influenza viruses, (Jefferson et al., 2006) and mortality among patients infected with H5N1 bird flu remains high, despite the use of neuraminidase inhibitors (Beigel 2005). Resistance to antiviral drugs, which may even develop during treatment, might further limit the efficacy of these drugs (De Jong 2005). Given that secondary bacterial infection is an important and often fatal complication of influenza, antibiotics will also have a critical role in the event of a human pandemic. How should antibiotics be used in the event of a flu pandemic? And how many patients with influenza will develop secondary bacterial pneumonia? In a largely healthy population of adolescents and adults who developed acute influenza (predominantly caused by the virus H3N2) the rate of respiratory events diagnosed by doctors and treated with prescribed antibiotics was around 17%, most commonly acute

bronchitis and acute sinusitis. Pneumonia was diagnosed in only 1-2% of patients (Kaiser et al., 2000 and 2003).

The neuraminidase inhibitors (NIs) zanamivir and oseltamivir are antiviral agents that were developed during the 1990s specifically for the treatment and prophylaxis of influenza. These agents have a therapeutic advantage over older antiviral drugs, e.g., rimantadine and amantadine, in that they are active against influenza B as well as influenza A viruses, they are tolerated much more readily, and the emergence of resistance occurs infrequently. Clinical trials have established that NIs is effective in reducing the severity and duration of influenza in adults and adolescents((Hayden FG et al., 1997; Treanor JJ 2000) as well as in children (Whitley RJ, 2001, Hedrick JA 2000). Several large controlled studies of the use of NIs for disease prevention have demonstrated that zanamivir and oseltamivir are effective in preventing the clinical symptoms of influenza in healthy adults when the drugs are used either as prophylaxis for close contacts, e.g., household members after exposure, or as seasonal prophylaxis in the community. These studies revealed that the incidence of both influenza A and influenza B infections was reduced by 70–90% when NIs were used for prophylaxis either before or after exposure to the virus An additional benefit of treating influenza with NIs is the reduction in the need for antibiotics to treat secondary infections. Oseltamivir (Tamiflu®) reduced the incidence of complications requiring antibiotics in children by 40% as compared with placebo, and reduced the relative risk of antibiotics media by 44% (Whitley RJ,2001).

8.6 Predicted antibiotics concentration in STPs influent, secondary effluent and after advance tertiary treatment (ozonation) during a pandemic with three scenarios.

Antibiotics concentrations in influent, effluent and in river water were predicted as of OC was done in above section. The assumption was: antibiotics doses will be around 500mg/ day, excretion from body is 60% of oral dose (as most of the antibiotics have); removal in STPs will be 60% in average Chapter IV 4.1.3) and others factors are as of OC scenario. The predicted concentration of antibiotics in influent and effluent after different treatment in STPs in Kyoto city is presented in Figure 8-4. It is difficult to predict what type of antibiotics will be prescribed as we have many options for antibiotics. From the prediction is observed that antibiotics concentration may reach to 25μg/L in maximum combiendly or individually and after secondary treatment in STPs it can be reached to 15μg/L. however if ozonation is applied the concentration can reduced to maximum 2.5μg/L .
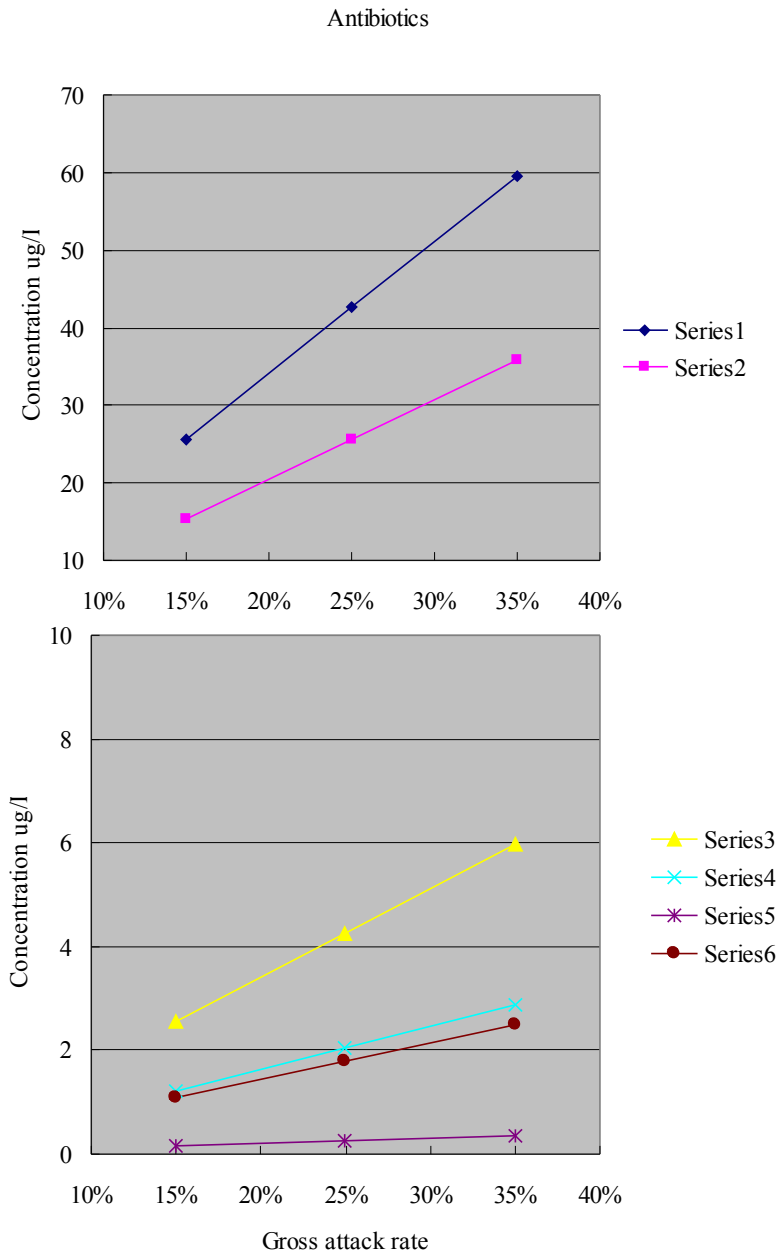
Antibiotics concentration in STPs influent and effluent and receiving water during influenza pandemic
scenario : gross attack rate minimum (15% of total population), most likely (25% of total population) and maximum
(35%), according to U.S.A CDC(center for disease control) model. Maximum level of secondary infections subjected to
antibiotics is expected to be 17% of the influenza patient (according to Kaiser et al., 2000 and 2003) . Oseltamivir
(Tamiflu®) reduced the incidence of complications requiring antibiotics by 40% (Whitley RJ 2001)
(All concentrations are in microgram/L)
Series 1: Antibiotics concentration in STPs influent
Series 2: Antibiotics concentration in STPs effluent concentration after biological treatment
Series 3: Antibiotics concentration in STPs effluent after Ozonation as tertiary treatment treatment
Series 4: Antibiotics concentration in downstream Katsura riverwater at Miyamayabashi (with only biological treatment in
        STPs)
Series 5: Antibiotics concentration in downstream Katsura river water at Miyamayabashi (with ozonation as tertiary
         treatment in STPs with present operation conditions)
Series 6: Antibiotics concentration in downstream Katsura river water at Miyamayabashi with existing facility

Figure 8-3 Predicted maximum concentration of antibiotics in different compartment of water
environment in Kyoto City for three different pandemic influenza scenarios.

8.7 Environmental risk assessment (ERA) of antibiotics in pandemi

8.7.1 Ecotoxicology

The use pattern of antibiotics is different from antiviral drugs because of a wide range to available antibiotics. From our STPs survey (Chapter IV) and antibiotics use pattern in Japan, it is assumed that clarithromycin, azithromycin and levofloxacin are widely used following sulfamethoxazole, trimethoprim and ciprofloxacin. These antibiotics combined share over 90% of human antibiotics use (Chapter IV; Table 4-2). Considering the similar pattern of use during a future pandemic the ecological risk assessment of antibiotics were determined according to the figure 8-5 and the ecotoxicological data of *Pseudokirchneriella subcapitata* (Fukunaga 2008)
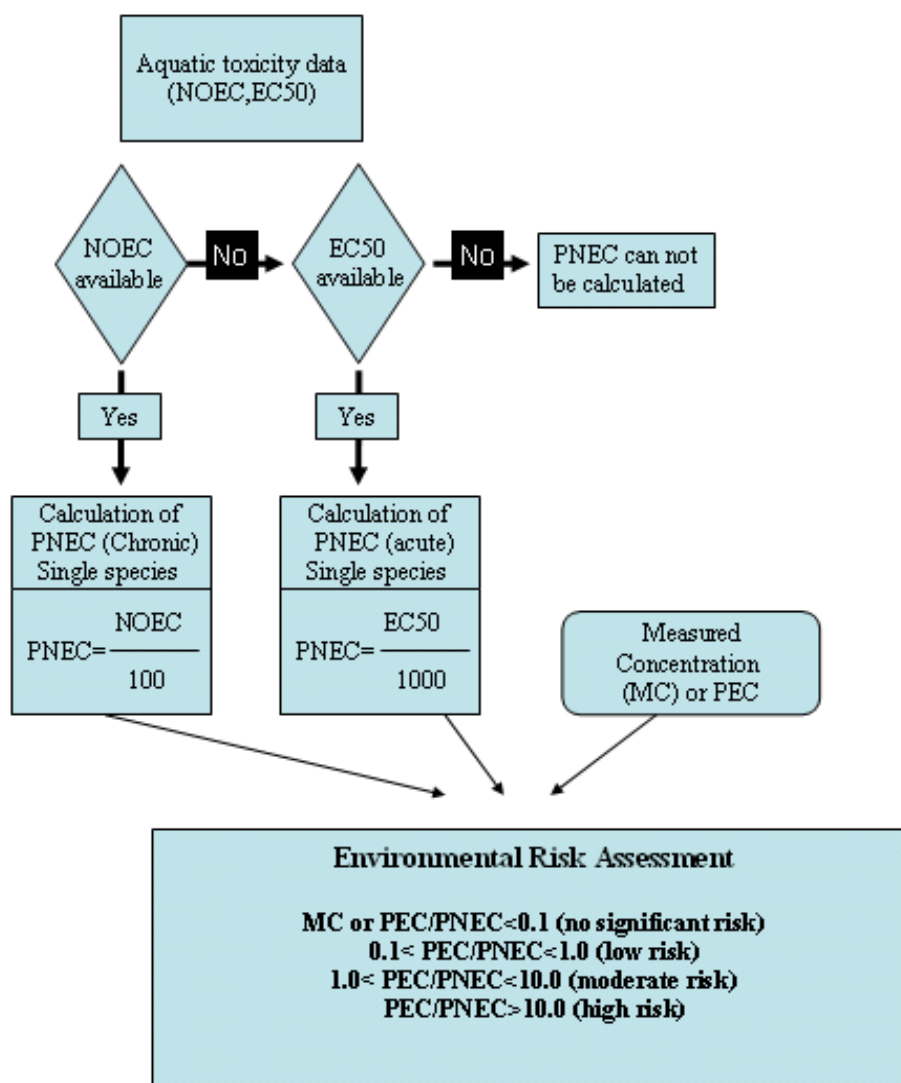
Figure 8-4 Flow diagram of ERA of antibiotics.

From the prediction it is observed that use of antibiotics during pandemic may have high risk to aquatic organism. According to the present use pattern clarithromycin use can have a high risk during pandemic with only conventional treatment (Figure8-6) and existing STPs (Figure,8-7) in the upstream of Katsura river. But application of ozonation in all STPs (Figure 8-8) can reduce the risk from moderate to low in the down stream of Katsura river. As it is only from the algae study there could be a different scenario for other sensitive organism, specially bacteria, invertebrate and other aquatic organism.
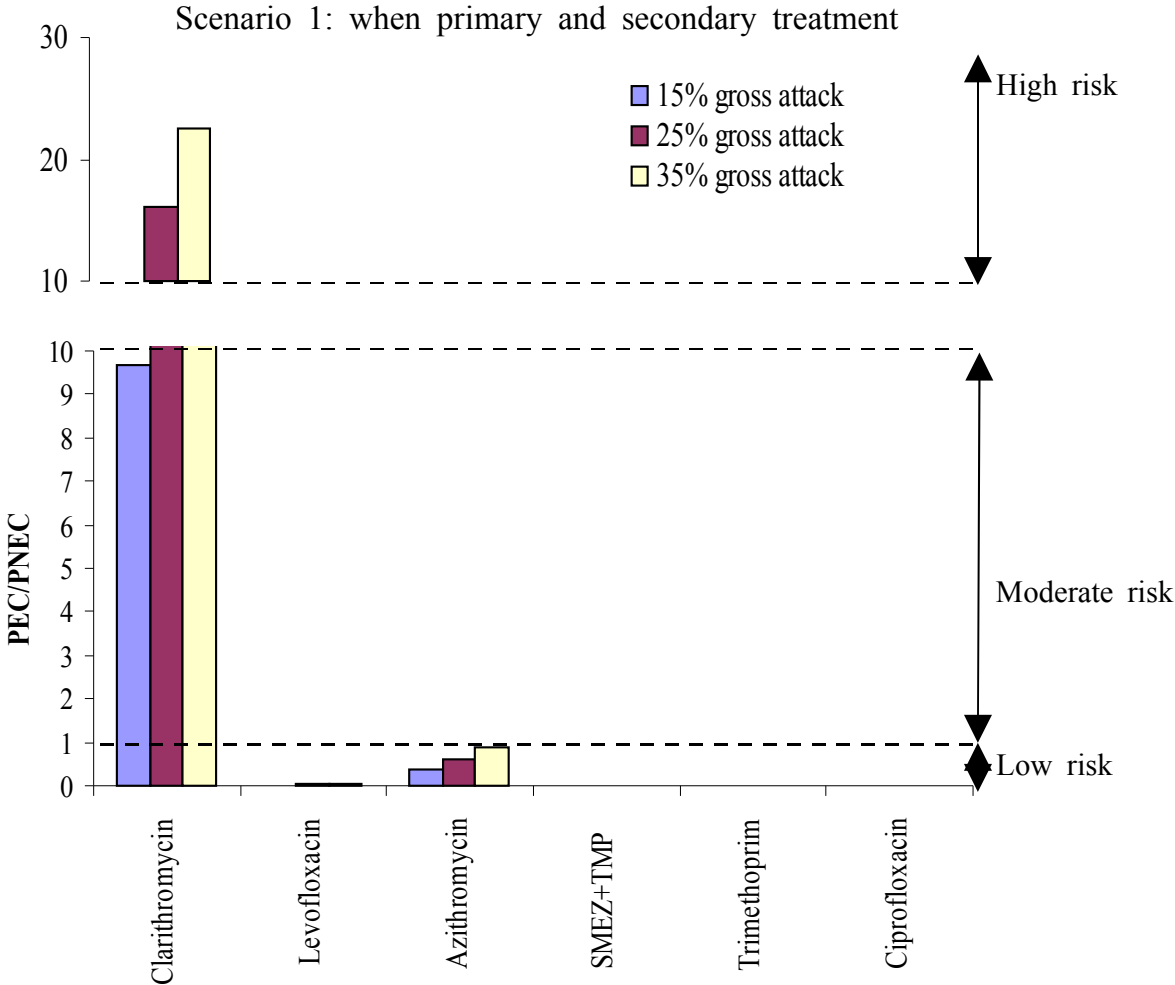

Figure 8-5 ERA of antibiotics at down stream of Katsura river with considering only conventional STPs in upstream of Katsura River.
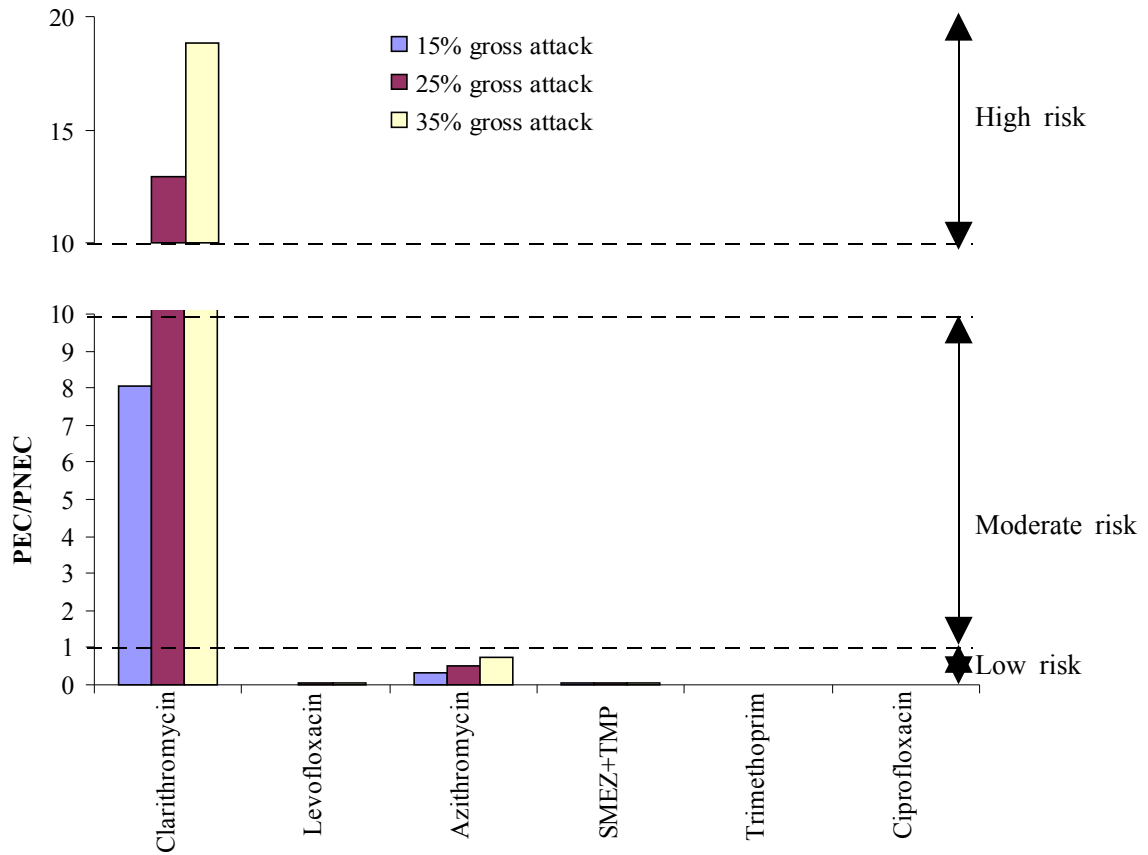
Figure 8-6 ERA of antibiotics at down stream of Katsura river with existing STPs
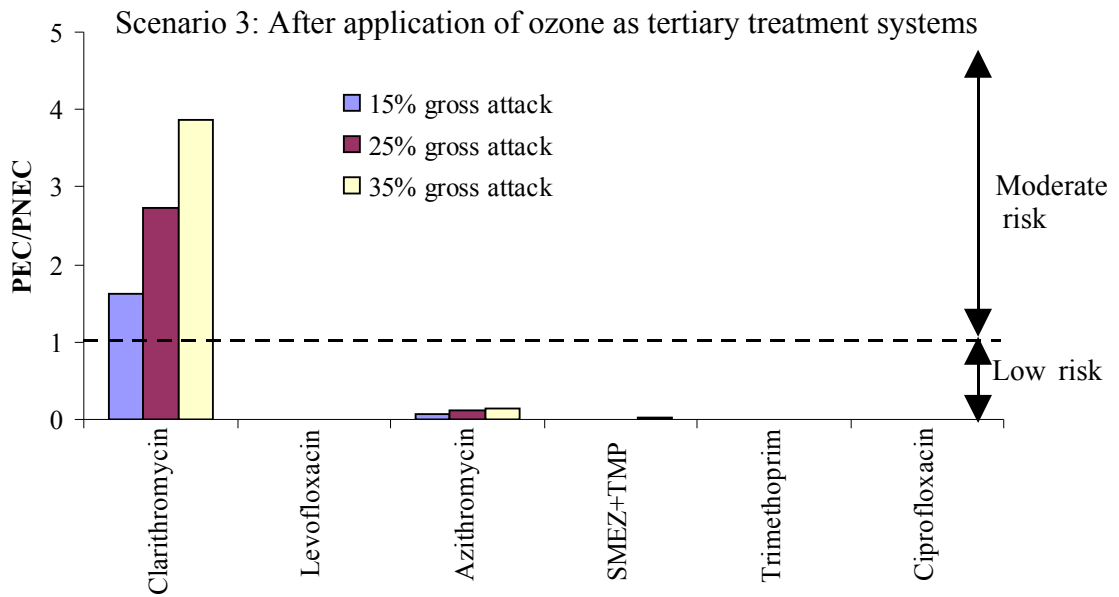

Figure 8-7 ERA of antibiotics at down Stream of Katsura River after application of ozonation as tertiary treatment in all STPs

8.7.2 Antibiotics effects on Sewage treatment plants during pandemic

Additional concerns regarding STP failure were raised after acknowledging the potential scale of antibiotic use to treat 16 secondary infections stemming from influenza-infected patients, although the use of antivirals might decrease the need for antibiotic use (Kaiser et al. 2003; Nicholson et al. 2000; Treanor et al. 2000; Whitley et al. 2001).Changes in activated sludge floc integrity, or the biofilms in trickling filter works could lead to a loss of effective sewage treatment. This would have catastrophic environmental consequences, as untreated   sewage entering rivers would kill a large number of the aquatic organisms and be an additional threat to human health. Combined effect of antibiotics in microgram level can inhibit nitrification (oxidation of ammonia by nitrifier) in STPs (Gopal et al, 2009). In a study by Sorensen B.H. (2001) it was observed that nitrifier *N. europaea* EC_{50} value is as low as $2.0\mu g/L$ and EC 50 (growth rate) of activater sludge which is as low as $70\mu g/L$ for oxolinic acid antibiotics. From the toxicity data of bacteria and predicted concentration of antibiotics during a pandemic, there might be a serious effect on biological treatment system and there is a possibility that the STPs will fail to meet the regulatory standard. As a result surface water will be polluted and ecosystem will be affected. Most importantly after shut down of STPs , the startup of the system again is a time consuming and tough work.

8.8 Wastewater treatment system preparedness:

Application of Ozone to Municipal Sewage Treatment plants for antibiotics and antiviral drugs load reduction during pandemic influenza

8.8.1 Background

A wide range of pharmaceutically active compounds – including antibiotics, anticonvulsants, analgesics, cardiovascular drugs, etc. – have been found in the drinking water supplies and/or wastewaters of many countries (Environmental News Network, 2008). Since some of these compounds are not easily degradable and usually get away intact from conventional treatment plants, it is inferred that the presence of residual pharmaceuticals in the environment and in aquatic systems in particular, represent a serious environmental problem. Antibiotics and antiviral drugs may inflict serious toxic and other effects to humans and other living organisms, as they are design to be highly bioactive. In general antibiotics are other pharmaceutical are present in wastewater in very low concentration all time. Additionally, widespread use of antibiotics and antiviral drugs during a future pandemic are of important concern and their huge amount of discharge to aquatic environment are also a long pending research.   Ozone is a water treatment technology with an increasing acceptation and applicability in drinking water facilities and wastewater treatment plants dealing with pharmaceuticals (Ternes et al., 2003; Vogna et al., 2004; Snyder et al., 2006; Gagnon et al.,

2008; Klavarioti et al., 2009). Ozone is an oxidizing agent showing a high reactivity with a large number of organic compounds. Ozone either decomposes in water to form free hydroxyl radicals (more powerful oxidants agents than ozone itself) or reacts selectively with certain functional groups (direct ozone reactions). This work tries to investigate the applicability of ozonation as tertiary treatment for antibiotics and antiviral drugs removal during a future pandemic.

8.8.2 Overview of ozone application to sewage treatment in Japan

In 1988, the first ozonation plant for polishing sewage effluent was constructed in Oita Treatment Plant to supply the water for the castle's moat water in Japan. Then the number of ozonation plants constructed for sewage treatment has been increased year by year and reached to more than 60 in 2004 as shown in Figure 8-9. The application points of ozonation are mainly after coagulation and sedimentation, sand filtration and/or biofiltration of secondary effluent. And the most popular and important parameter for design and operation is ozone dosage, amount of ozone dosed per unit volume of treated water. The ozone dosage ratio is 5-20 mg /L of water, typically 10-15 mg ozone /L of water.
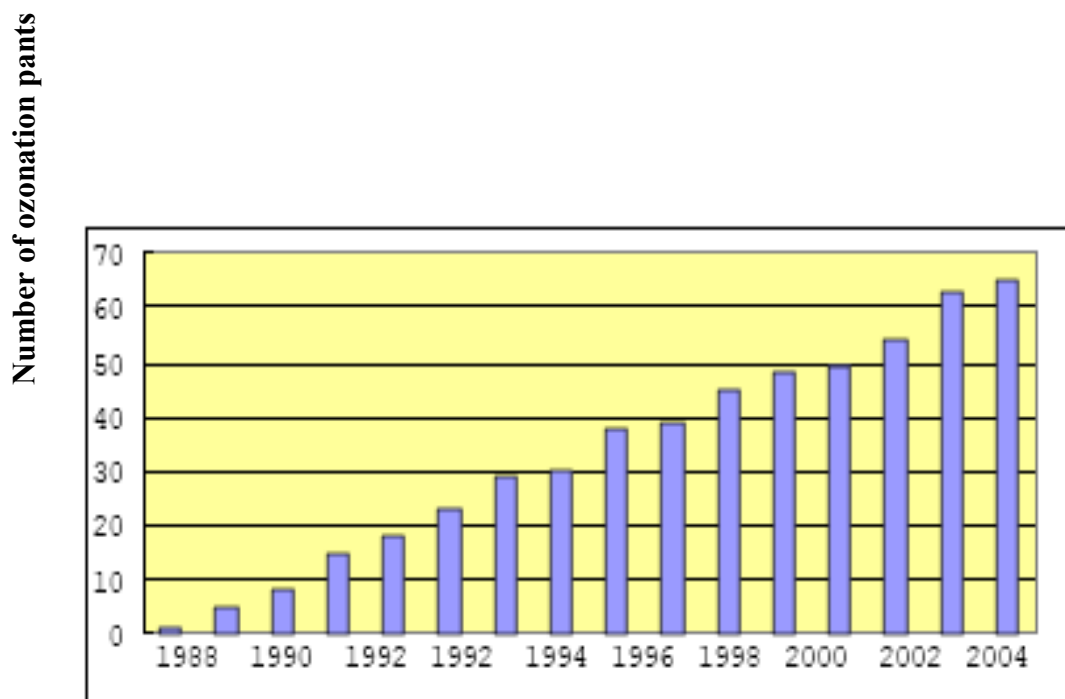

Figure 2   Trend of numbers of installed ozonation plants [2)]

Figure 8-8 Installed number of ozonation pants in sewage treatment facility (Source: JAO)

8.8.3 Effectiveness of ozone treatment for antiviral drugs and antibiotics removal

Kisshoin Treatment Plant (STP-K), Kyoto

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STP-K receives a large amount of wastewater from dyestuff industries and suffered from color and COD problems. Then ozonation process was introduced after pure oxygen and air activated sludge process for removal of color and reduction of COD concentration as well as disinfection. The outline of the ozonation process and water quality of the effluent is shown in Table 8-4. Ozone treated water quality clears the guideline of discharged water quality.

Outline of ozonation process (STP-K)

Capacity 120,000 m3/day (Line A 40,000 m3/day ,Line B 80,000m3/day

Amount of ozone generation : 22.5kg/h 3 number

Ozone concentration : :110g/Nm3, diffuser ,Contact time: 11.3 min

Ozone dosage : 4 to Max 20mg/l

Power consumption : 0.1kw/ m3

Table 8-4 Water quality of treated sewage

	PH	BOD	C-BOD	COD	SS	T-N	NH4-N	T-P	Color
Raw Water A	7.3	91		63	84	21		2.8	30
Raw Water B	7.6	230		190	204	21		4.0	77
SedimentaionA	7.2	60		49	43	19	12	1.6	31
SedimentaionB	7.3	64		57	52	21	13	1.8	37
Treated WaterA	7.1	3.1	2.0	8	2	5.1	0.3	0.33	16
Treated WaterB	6.5	5.1	2.3	11	3	13	2.6	0.7	20
Discharged	6.8	5.1	3.6	8.3	2	8.4	1.5	0.46	5.9

Efficiency of ozonation process for antibiotics and antiviral drugs removal at STP-K

As mentioned in chapter VII, ozonation in STP-K can reduce over 90% of OC from secondary effluent and AMN over 95%. Whereas entire biological system can removed only 20-30% in average of influent OC and AMN. Figure 8-10 shows the concentration variation of antibiotics concentration in final effluent of two STPs located in Kyoto city. These antibiotics are frequently detected in high concentration. Except clarithromycin, levofloxacin and azithromycin, all antibiotics were detected less than 10ng/L in STP-K, but their concentration level were much higher in STP-K (have only primary and secondary biological treatment). Both STPs are located in same geographical area have a similar concentration in influent. In Chapter IV it was also observed that ozonation can removed antibiotics over 80% from secondary effluent in a STP investigated in China (ozone dose was 4.5mg/L and contact time around 10min).
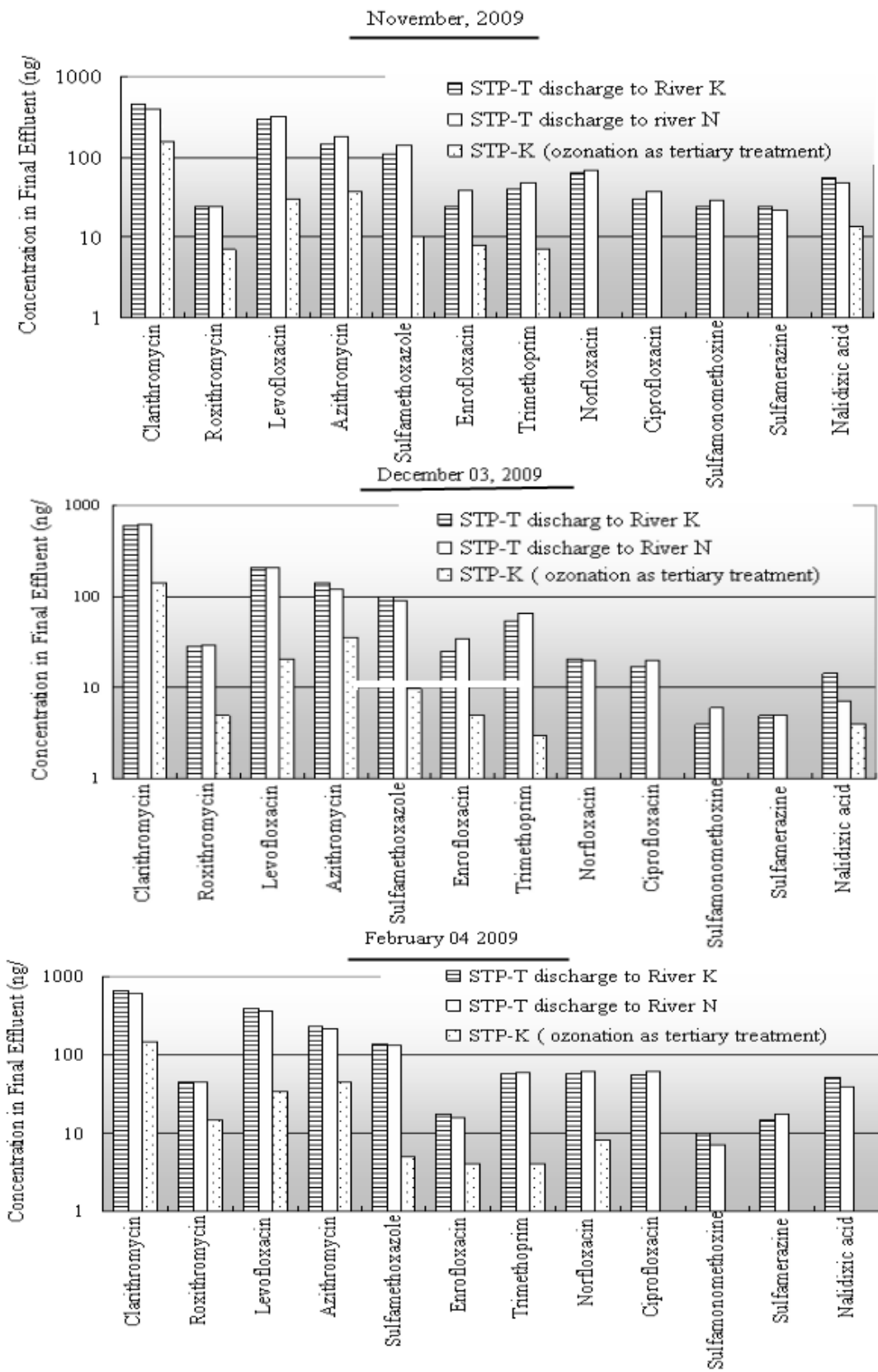
Figure 8-9 Performance of ozonation process for antibiotics removal.

8.9 Ozonation batch experiment
8.9.1 Methods and materials

8.9.1 .1Target compounds and preparation of tested water

The fifteen antibiotics (Tetracycline, Sulfamerazine, Livofloxacine, Norfloxacin, Trimethoprim, Sulfadimizine, Ciprofloxacin, Sulfomethoxazole (SMETH), Enrofloxacin, Ampicillin, Sulfadimethoxin , Azithromycin , Erithromycin, Roxithromycin, Clarithromycin) and two antiviral drugs Amantadine (AMN) and Oseltamivir Carboxylate (OC) were used for this study. The entire target compounds standards were dissolved in methanol except amantadine (ethanol). Milli-Q water generated from Millipore system was used for this experiment. Initial concentrations of the compounds in the tasted were 1.0μg/L in mixed condition. Initially the target compounds standards were put in a flux as a mixer, then nitrogen was purged to dryness to remove the organic solvent. Finally the compounds were dissolved in Milli-Q water.

8.9 .1.2 Experimental setup and

Ozonation experiments of the target antibiotics and antiviral drugs were conducted in a cylindrical stainless reactor with 1.5L (Fig. 4-2). The temperature of tested water was maintained at 20°C by circulating controlled temperature water into a water jacket outside the reactor by a water circulator. The pH of tested waters was controlled to 7.2 with phosphate buffer after addition of target compounds. All the experiments started by sparging O_3 gas continuously into the reactor filled with tested water.

◄ Sampling port

F:
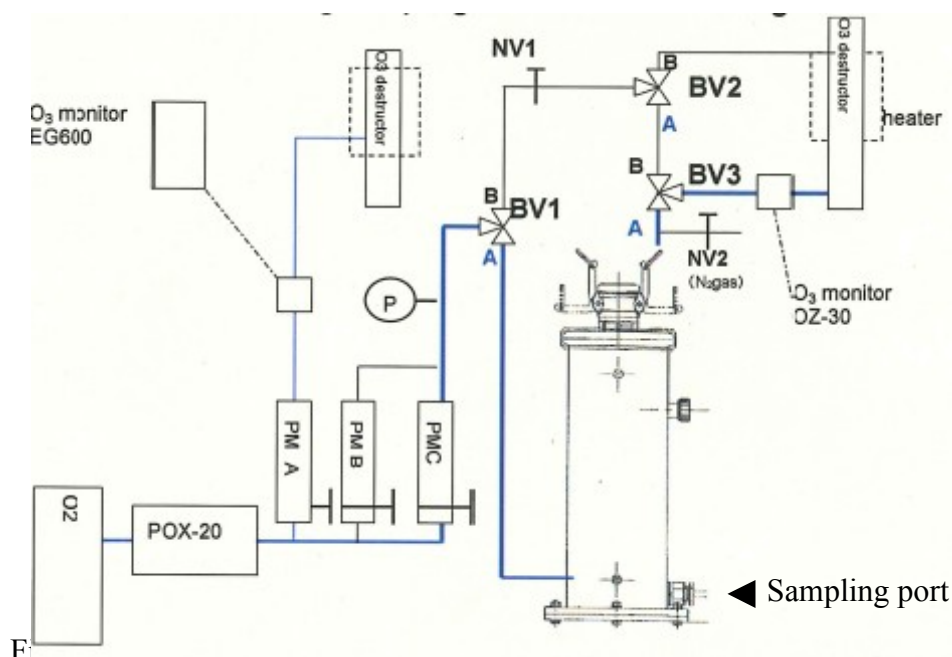
8.9.1.3 Experimental conditions for O₃ treatment

Previously the effect of O_3 feed rate on (PhACs) degradation was investigated using tested water prepared by spiking the 30 PPCPs into Pure Water (Kim, 2008) and O_3 feed rates 0.6 mg/L/min, with 10.0 min contact time was suitable for over 90% reduction of most of the target compounds. Due to the limitation of OC amount (not commercially available), I selected the suitable conditions observed before in our research group. In this study I conducted three experiments (10mim, 5min and 3min contact time were denoted as EXP1 EXP2 and EXP3 respectively). The experimental conditions were as follows: feed ozone concentration 4.0mg.L, ozone gas flow rate 0.23l/min to maintain ozone feed rate of 0.6 mg/L/min.

8.9.1.4 Analytical method

The concentrations of the target compounds were determined by the methods described in Chapter III, antibiotics and antiviral drugs were determined separately. Dissolved O_3 concentration was measured with indigo method (Bader *et al*., 1981) measuring the absorbance at 600 nm wavelength by a spectrophotometer (UV-16000, Shimadzu). DOC (dissolved organic carbon) concentration was measured with a TOC analyzer (TOC-5000A,

Shimadzu) and calculated from the difference of TOC (total organic carbon) and IC (inorganic carbon)

8.9.2 Results and discussion of ozonation batch experiments:

8.9.2.1 Determination of rate constants of Antibiotics and Antiviral drugs

The degradation reaction of an organic compound with O_3 in batch reactor is expressed as equation (1):

$$-\frac{d[C]}{dt} = k[C][O_3] \quad (1)$$

where, $[C]$ is the concentration of the organic compound; $[O_3]$ is the concentration of dissolved O_3; k is the rate constant. If O_3 is not consumed by organic compounds *etc*, the increasing rate of dissolved O_3 in batch reactor is expressed as equation (2):

$$\frac{d[O_3]}{dt} = k_L a([O_3]^* - [O_3]) - k_{O3}[O_3] \quad (2)$$

where, $[O_3]^*$ is the saturated concentration of dissolved O_3, which is determined by the partial pressure and distribution coefficient of O_3 gas; $k_L a$ is the volumetric overall mass transfer coefficient (/h); k_{O3} is the rate constant of O_3 self decomposition (/h). As known in equation (2), when O_3 gas is supplied to the reactor the concentration of dissolved O_3 in the reactor increases with time. Moreover, if O_3 gas is supplied continuously, finally the concentration of dissolved O_3 will become constant $(k_L a([O_3]^* - [O_3]) = k_{O3}[O_3])$. Therefore, equation (1) can be expressed as pseudo first-order reaction such as equation (3):

$$-\frac{d[C]}{dt} = k'_{O3}[C] \quad (3)$$

where, k'_{O3} is affected by the concentration of dissolved O_3 because k'_{O3} equals $k[O_3]$. However, for experiments using the same reactor, k'_{O3} value can be used as an indicator for the reactivity of an organic compound with O_3. By integrating equation (3), next equation is obtained.

$$\ln(C_t / C_0) = k'_{O3}t \quad (4)$$

where, C_t is the concentration of an organic compound at the reaction time of t; C_0 is the initial concentration of the organic compound.
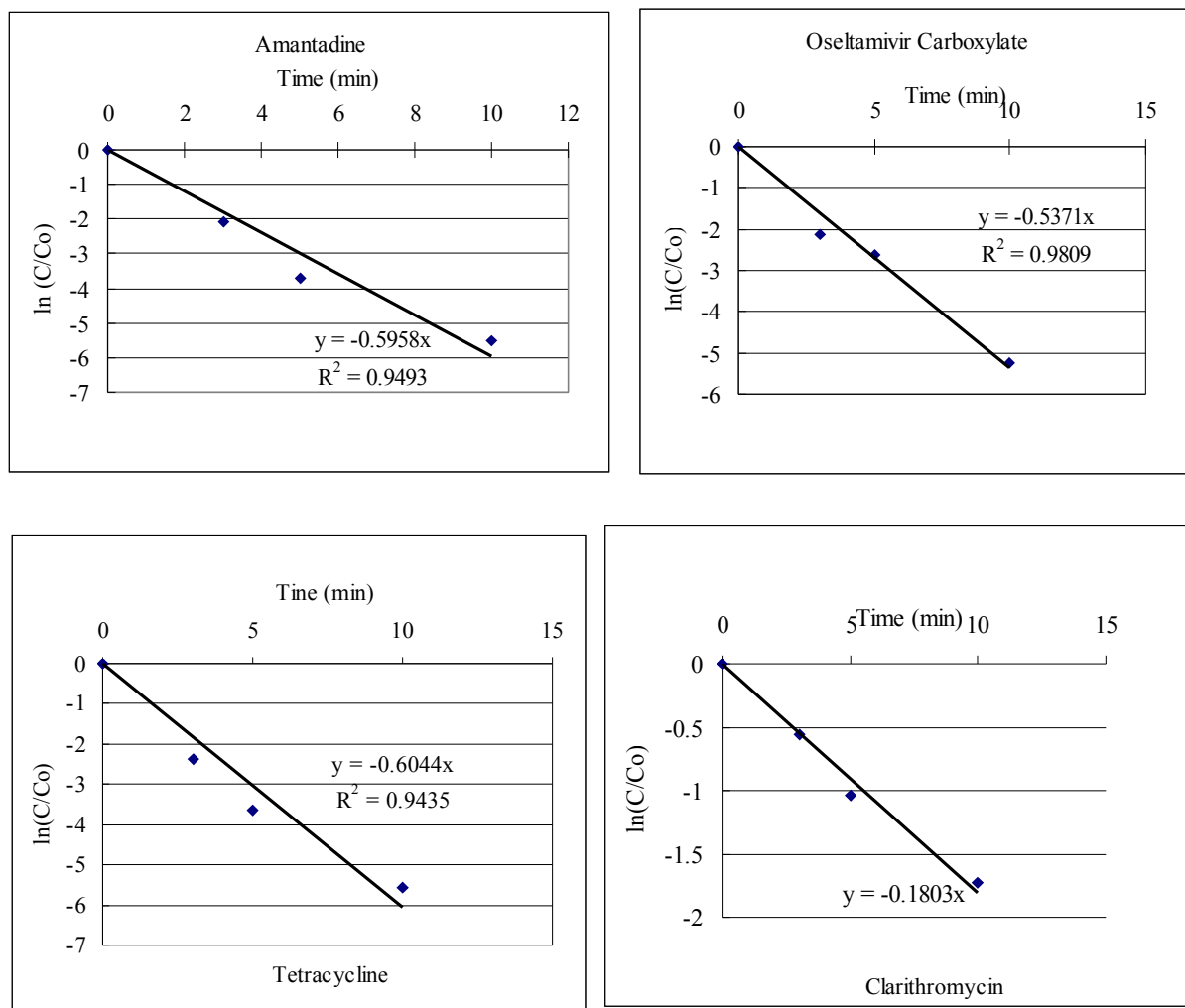
Figure 8-11 concentration changes of Amantadine, Oseltamivir Carboxylate , tetracycline and clarithromycin during ozonation (ozone feed rate 0.61 mg/L/min, Feed ozone gas concentration 4.0mg/L, pH 2 at 20^0C)

Fig. 8-12 shows the concentration decrease of amantadine (AMN) and oseltamivir carboxylate (OC) for the reaction time of 10 min during O_3 treatments, which were conducted using milli-Q water spiked by fifteen antibiotics. O_3 was supplied to the reactor at an O_3 feed rate of 0.61 mg/L/min. Previously it was observed that in a semi-batch experiment with an O_3 feed rate of 0.6 mg/L/min antibiotics were removed more than 90% within 10 min (Kim I., 2008). In this study the logarithmic concentrations of AMN and OC decreased linearly with time in all the experiments and it can be, therefore, said that the degradation reactions follow pseudo first-order reaction. As shown in Fig. 8-3, k'_{O3} (pseudo first-order rate constant for O_3) of AMN was 0.596 /min (0.00993/sec), and OC was 0.524 /min (0.008725/sec).

Fig. 8-13 compares pseudo first-order rate constants of the 15 antibiotics with two antiviral drugs with ozone feed rate of 0.6 mg/L/min. Beta lactam antibiotics ampicillin and tetracycline antibiotics have relatively high degradation rates compared to the other antibiotics. It has been reported that tetracycline reacted very quickly with O_3, even though total organic carbon analyses revealed that it was not mineralized at all (Dalmazio *et al*., 2007). They proposed that during O_3 treatment the C11a-C12 double bond of tetracycline was attacked by O_3 and O_3 reaction at the C2-C3 double bond occurred by subsequent O_3 attack Dodd *et al* (2006) have measured O_3 and OH radical reaction kinetics for 14 antibacterial compounds from nine structural families. In their study, C11a-C12 double bond, C2-C3 double bond and tertiary amine were proposed as expected sites of O_3 attack, and they showed that tetracycline reacted rapidly with O_3 in a wide range of pH. AMN and OC also have a relatively high degradation rate constants, this could be due to presence of NH_2 side chain with aromatic structure of AMN and amide in OC. In this study we have four sulfonamide antibiotics (sulfamerazine, sulfadimizine, sulfamethoxazole and sulfadimethoxine). All the sulfanilamide antibiotics have moderate degradation rates within the selected compounds. Huber *et al* (2003) investigated the oxidation of pharmaceuticals using conventional O_3 treatment and O_3-based advanced oxidation processes (AOPs). The study showed that when O_3 of 1 mg/L was utilized at pH 7~8, half-life of sulfamethoxazole was below 0.5sec, indicating that it is completely transformed during O_3 treatment and O_3-based AOPs. In addition, they expected the aromatic amino group as the main reaction site of O_3 during O_3 degradation of sulfamethoxazole. They also suggested that rate constants of all the sulfonamides will be very similar to the rate constant ($\sim 2.5 \times 10^6 M^{-1} s^{-1}$) of sulfamethoxazeol for O_3 reaction because the reactive group (aromatic amine) is characteristic for all the compounds in sulfonamides group. In this study, 4 sulfonamides showed almost same rate constants (3.5 E-03/sec ~ 4.0 E-03/sec) for O_3 feed rates of 0.6 mg/L/min, although a rather wide range of rate constants were obtained for 0.15 mg/L/min and 0.3 mg/L/min.
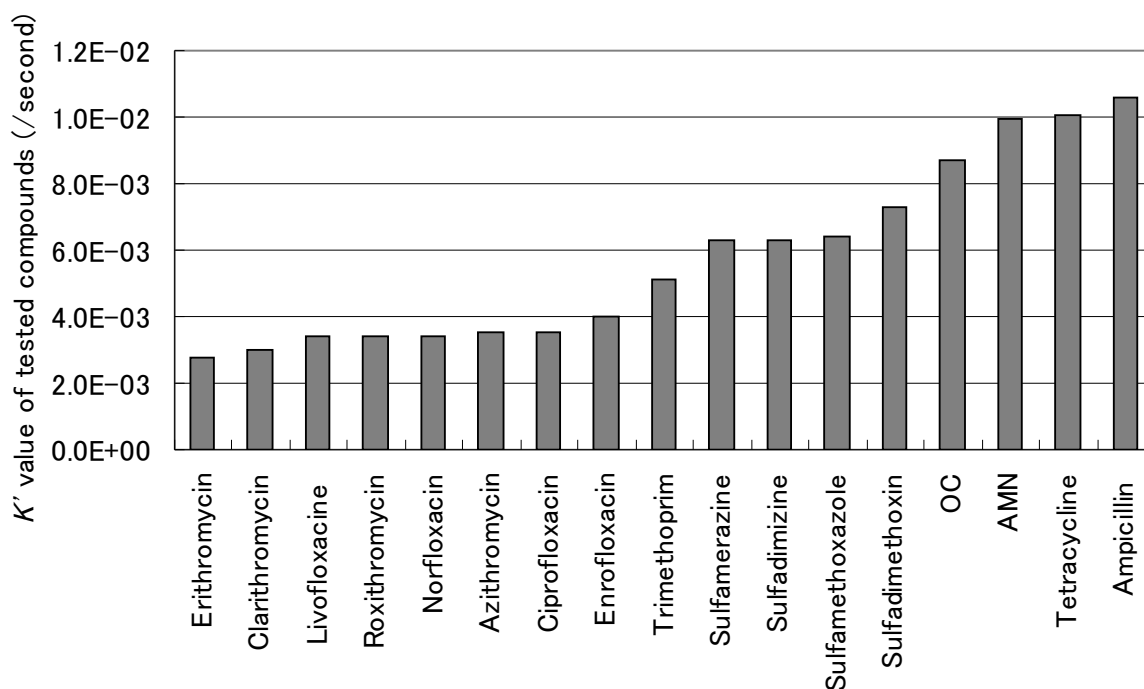
Figure 8-12 rate constants of Antibiotics and Antiviral drugs (OC and AMN)

8.9.2.2 Ozone consumption and removal efficiency of tasted compounds.

Table 8-5 shows O_3 consumptions and removal efficiency of the target compounds in three experiments. The experiments were varied with contacts time, EXP 1, EXP 2 and EXP 3 were for 3min, 5min and 10min. Ozone consumption for the three experiments were 1.25 (EXP1), 1.57(EXP 2) and 2.46 (EXP 3) mg/L. From the EXP1, it was observed that only tetracycline, ampicillin, amantadine and oseltamivir carboxylate were removed signigacently (over 80%). On the other hand during EXP2 seven compounds removed over 90% and others were between 60 to 80%. In EXP3 all the compounds were removed over 80%. From the experiments it was observed that amantadine and oseltamivir carboxylate removal efficiencies were between sulfanilamide antibiotics and betalactams (ampicillin) or tetracycline. Macrolide and fluroquinolol antibiotics are less degradable during ozonation in compare with other antibiotics.

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Table 8-5 summery of batch ozonation experimental conditions and removal efficiency of antibiotics and antiviral drugs

Experiment		EXP1	EXP2	EXP3
Feed O3 gas conc. (mg/L)		4.0	4.0	4.0
Gas flow (L/min)		0.23	0.23	0.23
O3 feed rate (mg/L/min)		0.61	0.61	0.61
O3 consumption (mg/L)		1.254	1.571	2.458
Reaction time (min)		3	5	10
Removal %				
Tetracycline	Antibiotics	96.0	97.0	100.0
Sulfamerazine	Antibiotics	55.2	93.3	96.9
Livofloxacine	Antibiotics	51.4	66.5	86.1
Norfloxacin	Antibiotics	45.0	62.9	87.6
Trimethoprim	Antibiotics	52.9	77.3	95.6
Sulfadimizine	Antibiotics	57.5	74.8	98.4
Ciprofloxacin	Antibiotics	48.8	71.4	86.9
Sulfamethoxazole	Antibiotics	75.3	91.1	97.0
Enrofloxacin	Antibiotics	63.4	72.6	89.6
Ampicillin	Antibiotics	92.0	98.0	100.0
Sulfadimethoxin	Antibiotics	78.9	93.9	98.2
Azithromycin	Antibiotics	54.6	66.5	84.3
Erythromycin	Antibiotics	54.5	58.4	79.2
Roxithromycin	Antibiotics	68.8	76.6	83.1
Clarithromycin	Antibiotics	43.0	64.5	82.3
Amantadine	Antiviral	87.0	97.0	100.0
Oseltamivir Carboxylate	Antiviral	86.0	92.0	100.0

8.10 Conclusion

According to CDC flueAid model it is estimated than from 15% (minimum) to 35% (maximum), 25% (most likely), population may infected by influenza virus during pandemic and will take antiviral drugs, according to Government influenza preparedness action plan. From the prediction, it is expected that OC concentration will be in the range of 60 to 140 μg/L in influent in Kyoto city. This concentration will reduced to 48 μg/L to 112 μg/L after

biological treatment. Ozonation after biological can reduce OC load significantly. After ozonation as tertiary treatment concentration of OC will reduced to 6μg/L (minimum), 10μg/L (most likely) to 14 μg/L (maximum). With existing treatment facility, OC concentration at Miyamaibashi (downstream of Katsura river) may reach up to 10μg/L (if dilution factor is 10 and may vary with dilution capacity) as it is a fast flowing river, retention time is less than a day. This concentration could be high if the dilution factor decreased. However, this concentration can be reduced to ng/L level with application of ozonation after secondary treatment in upstream STPs.

From the prediction it was observed that OC concentration in Katsura river could reached up to 10μg/L, whereas lowest PNEC value of OC from the published data is more than or equal to 100μg/L, so the PEC/PNEC is lower than one, which indicated low risk associated with OC. Whereas clinical study it was observed that IC_{50} value of OC in some influenza is less than 100ng/L which is similar to the value detected in STPs in Japan during seasonal influenza epidemic and may have a selection pressure of growing antiviral resistance virus in waterfowl.

Antibiotics use during pandemic will increase due to secondary infection related to respiratory track infection. It is difficult to predict what type of antibiotics will be prescribed as we have many options for antibiotics. From the prediction is observed that antibiotics concentration may reach to 25μg/L in maximum combiendly or individually and after secondary treatment in STPs it can be reduced to 15μg/L (maximum). However if ozonation is applied the concentration can reduced to maximum 2.5μg/L in effluent. In the receiving river water it will reduced 10 times than STPs due to dilution.   .

From our STPs survey and antibiotics use pattern in Japan, clarithromycin, azithromycin and levofloxacin are widely used, following sulfamethoxazole, trimethoprim and ciprofloxacin. these antibiotics combined share over 90% of human antibiotics use. Considering the similar pattern of use during a future pandemic the ecological risk assessment of antibiotics were determined according to the NOEC data of

Pseudokirchneriella subcapitata. From the prediction it is observed that use of antibiotics during pandemic may pose a risk to aquatic organism. According to the present use pattern clarithromycin use can have a high risk during pandemic with conventional treatment and existing STPs (PEC/PNEC 8 to 25) in Katsura river water downstream of STPs. But application of ozonation can reduce the risk (PEC/PNEC< 4).

From the toxicity data of bacteria and predicted concentration of antibiotics during a

pandemic, there might be a serious effect on biological treatment system and there is a possibility that the STPs will fail to meet the regulatory standard as a result surface water will be polluted and ecosystem will be affected. Most importantly after shut down of STPs, the startup of the system again is a time consuming and tough work.

Ozonation as tertiary treatment can provide a good option to reduce the ecotoxicological effect of antibiotics and antiviral drugs use during a pandemic. A full scale STP antiviral drugs (OC and AMN) reduction was over 90% from secondary effluent after ozonation was observed during seasonal influenza outbreak. In ozonation batch experiment (feed ozone gas concentration 4.0mg/L, ozone gas flow rate 0.23L/min to maintain ozone feed rate of 0.6 mg/L/min), it was observed that logarithmic concentration of AMN and OC decreased linearly with time in all the experiments and it can be, therefore, said that the degradation reactions follow pseudo first-order reaction. k'_{O3} (pseudo first-order rate constant for O_3) of AMN was 0.596 /min (0.00993/sec), and OC was 0.524 /min (0.008725/sec) and over 99% removal within 10min. Beta lactam antibiotics ampicillin and tetracycline antibiotics have relatively high degradation rates compared to the other antibiotics. AMN and OC also have relatively high degradation rate constants; this could be due to presence of NH_2 side chain with aromatic structure of AMN and amide in OC and the degradation rate constant fall between tetracycline and sulfanilamide antibiotics. From the experiment, it was observed that only tetracycline, ampicillin, AMN and OC were removed significantly (over 80%) with in 3min contact time. On the other hand during seven compounds were removed over 90% and the others were between 60 to 80% during 5 min contact time. for 10 min contact time all the compounds were removed over 80%. From the experiments it was observed that amantadine and oseltamivir carboxylate removal efficiencies are between sulfanilamide antibiotics and betalactams (ampicillin) or tetracycline. Macrolides and fluroquinolol antibiotics are less degradable during ozonation in compare with other antibiotics.

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CHAPTER IX

CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

Sewage treatment plants (STPs) are the main route of human pharmaceuticals in the environment. The principal aim of this research work was to increase the knowledge on occurrence and fate of antibiotics and antiviral drugs in the STPs. Due to emergence of novel influenza virus (H1N1: swine flu) and highly pathogenic avian influenza virus (H5N1: bird flu), there is a possibility to widespread use of antiviral drugs in a future pandemic. Under the guidance of the World Health Organization (WHO), most of the developed and developing nations have developed pandemic preparedness action plans describing the role different organizations will play when confronted with an influenza pandemic. According to present plan both developed and developing countries stockpile a huge amount of antiviral drugs and antibiotics that will be use during a pandemic. This study for the first time investigated the occurrence and fate of a antiviral drugs in STPs, and predicted the concentration of antibiotics and antiviral drug in different compartment in the rout of entry to the environment and provides a technological solution to reduce the effects of the compounds under a pandemic conditions. The main findings of this study are described chapter by chapter as follows.

Chapter III mainly focused on development of analytical methods for the selected antibiotics and antiviral drugs in wastewater and river water. One method for antibiotics and other for antiviral drugs. The main findings are as follows:

- Satisfactory precision (relative standard deviation < 15%) and accuracies (percent recoveries 70 to 120%) were obtained for the antibiotics in pure water and sewage effluents. Methods detection limits were in the range of 2 to 18ng/L. Spiking of standard to evaluate recoveries was successfully applied.

- A sensitive and reliable method also developed for oseltamivir carboxylate (OC) and amantadine (AMN) was also developed successfully. OC and AMN precision were 9.5 and 4.2% respectively. Average recoveries were in the range of 100 to 130 % for OC (tri-deuterium label OC was used for recovery calculation) and 65 to 95% for AMN (standard spiking method), in primary influent/final effluent. Method detection limit were as low as 6ng/L for OC and 4ng/L for AMN.

In Chapter IV, occurrence and fate of antibiotics in different sewage treatment plants

were investigated in Japan and China. Levofloxacin (6800 ng/L), norfloxacin (2775 ng/L), trimethoprim (1578 ng/L) sulfamethoxazole (1280 ng/L) and clarithromycin (1134 ng/L) were detected in higher concentration. The occurrences of antibiotics varied with geographical location and among STPs. Most of the antibiotics were detected in higher concentration in influent and secondary effluents from STPs in Beijing, China compare to Japan. Similar to influent sample, levofloxacin (8628 ng/L), norfloxacin (1116 ng/L) trimethoprim (960 ng/L) sulfamethoxazole (683 ng/L) and clarithromycin (561 ng/L) were detected in higher concentration in effluent. Removal efficiency of the antibiotics varied with individual treatment technologies applied, in some extend A2O and AOAO based biological process was superior to CAS and OD, which indication a possible relation between biological nutrient removal and the targeted compounds. Ozonation as tertiary treatment for wastewater reclamation provided significant elimination of antibiotics. Fifty present of the selected antibiotics were removed over eighty percent during ozonation and there was no elimination of antibiotics in dissolve phase during ultra filtration.

In Chapter V, biodegradation capability of five antibiotics - clarithromycin, enrofloxacin, sulfamerazine, sulfamethoxazole and trimethoprim by nitrifying activated sludge was observed to elucidate the relationship between nitrification and antibiotics biodegradation, and the role of ammonia oxidizing bacteria within the sludge were investigated and the main conclusions are:

- Nitrifying activated sludge (NAS) can biodegrade the tested antibiotics with different biodegradation rate between 2.74 to 9.95 L/gSS/d. Sulfamethoxazole and sulfamerazine degraded faster than trimethoprim, clarithromycin and enrofloxacin. Sulfamethoxazole, sulfamerazine and trimethoprim represented a lower sorption potential (sorption coefficient less than 0.05 L/gSS) to NAS and hardly sorbed, whereas clarithromycin (0.3 L/gSS) and enrofloxacin (0.86 L/gSS)) represent a relatively high sorption potential.

- In the presence of allylthiourea, it was confirmed ammonia oxidizer in NAS were not metabolically active and there were no significant biodegradation. Ammonia oxidizer played the key role in biodegradation by ammonia monooxygenase enzyme as allylthiourea is a selective inhibitor of ammonia monooxygenase enzyme of ammonia oxidizer.

- The antibiotics degradation by autotrophic nitrifying micro-organisms is a cometabolic process. The nitrifiers degraded antibiotics without prior adaptation and are widespread in the environment. So, these bacteria represent a sink for the antibiotics in biological sewage treatment, in soil or even in surface water ecosystem. Promoting nitrification can

enhance the antibiotics biodegradation at or bellow the tested concentration.

- The overall removal efficiency was 80% for sulfamethoxazole, 76% for sulfamerazine, 60% for enrofloxacin, 50% for trimethoprim and 44% for clarithromycin.

Chapter VI, for the first time reported the detection of OC and amantadine in sewage effluent and river water. The detected highest concentration of OC in sewage effluent was 293ng/L at a STP operated with mechanical primary treatment plus activated sludge based secondary treatment, however OC concentration was 39.0 ng/L in a STP that has ozonation as tertiary treatment system, both STPs discharges were collected in same day during peak period of seasonal influenza in Kyoto city in 2009. The detection pattern of AMN was similar to AMN. Detection of OC in the river water raises the question as to whether or not a risk of OC resistance influenza virus in waterfowls should be taken as a result of widespread use of Tamiflu. OC in river waster was only detected in the peak period of influenza. Outside the influenza season OC was not detected in both STPs discharge and river water. However AMN was also detected in both influenza and non influenza season in STPs which indicated that there is a potential use of AMN for other diseases outside influenza like Parkinson.

Chapter VII describe the Occurrence and fate of oseltamivir carboxylate and amantadine in STPs . Both OC and AMN was detected in all sample investigated during peak period of seasonal influenza in the study area, in ng/L level. Both OC and AMN were not removed significantly during primary and secondary treatment. During primary treatment OC was removed around 5%, however AMN was removed around 10% , which could be explained due to their $logP$ value differences. In AOAO based STP, 21-23% of OC and 30-33% of AMN was removed during secondary treatment. However in CAS based process, OC and AMN was removed 15% and 20% respectively. During secondary treatment, A2O based process was superior to other investigated process for both OC and AMN removal. Finally, it was observed that only primary and secondary treatment process in STPs can not remove these antiviral drugs completely and a significant portion still remained in secondary effluent. Overall OC and AMN removal in a STP with ozonation as tertiary treatment was 90% and 96%, respectively. Ozonation can provide a significant role to reduce the OC and AMN load during seasonal influenza epidemic or in a future pandemic

Chapter VIII predicted maximum concentration of OC and antibiotics in STPs influent, secondary effluent, after advance tertiary treatment (ozonation) and in receiving water during a pandemic with three scenario, Kyoto city STPs and the Katsura river as a reference. According to CDC flueAid (2.0) model it is estimated that from 15% (minimum) to 35% (maximum) and 25% (most likely) people from total population may infected during a

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pandemic. All the infected people will take antiviral drugs, according to Government influenza preparedness action plan. From the prediction, it is expected than OC concentration will be in the range of 60 to 140 µg/L in influent of Kyoto city. This concentration will reduced to 48 µg/L to 112 µg/L after biological treatment. Ozonation of biologically treated effluent can reduce OC load significantly. After ozonation, the concentration of OC will reduce to 6µg/L (as minimum), 10µg/L (as most likely) to 14 µg/L (as maximum). With existing treatment facility, OC concentration at Miyamaibashi (down stream of Katsura river) may reach upto 10µg/L (dilution factor 10) as it is a fast flowing river; retention time is less than a day, as a result zero reduction was expected in the river. This concentration could be high if the dilution factor decrease. However, this concentration can be reduced to ng/L level by application of ozonation after biological secondary treatment.

From the prediction it was observed that OC concentration in Katsura river could reached up to 10µg/L as maximum, whereas lowest PNEC value of OC from the published data is more than or equal to 100µg , so the PEC/PNEC is lower than one, which indicated low risk associated with OC use during a pandemic. However, from clinical study, it was observed that IC_{50} value of OC for some influenza virus are less than 100ng/L, which is similar to the value detected in STPs in Japan during seasonal influenza, and it may have a selection pressure of growing antiviral resistance in waterfowl.

Antibiotics use during pandemic will increase due to secondary infection related to respiratory track infection. It is difficult to predict what type of antibiotics will be prescribed as we have many options for antibiotics. From the prediction, it is observed that antibiotics concentration may reach to 25µg/L (maximum) for combined or individual antibiotics, and it can be reduced to 15µg/L after secondary treatment in STPs . However, if ozonation is apply, the concentration can reduce to 2.5µg/L in effluent. In the receiving river water it will reduced 10x than STPs as a result of dilution.   .

From the prediction it is observed that use of antibiotics during pandemic may pose a risk to aquatic organism. According to the present use pattern clarithromycin use can have a high risk during pandemic with conventional treatment and existing STPs (PEC/PNEC: 8 to 25) in Katsura river water downstream of STPs. But application of ozonation can reduce the risk (PEC/PNEC< 4).

Ozonation as tertiary treatment can provide a good option to reduce the ecotoxicological effect of antibiotics and antiviral drugs use during a pandemic. In a full scale STP, antiviral drugs (OC and AMN) reduction was over 90% from secondary effluent after

ozonation was observed during seasonal influenza in Kyoto City. In ozonation batch experiment (feed ozone gas concentration 4.0mg.L, ozone gas flow rate 0.23L/min to maintain ozone feed rate of 0.6 mg/L/min), AMN and OC decreased linearly with time in all the experiments and it can be, therefore, said that the degradation reactions follow pseudo first-order reaction. k'_{O_3} (pseudo first-order rate constant for O_3) of AMN was 0.596 /min (0.00993/sec), and OC was 0.524 /min (0.008725/sec) and over 99% removal within 10min.

Finally, this research provided a unique opportunity to make a preliminary holistic assessment of whether safety for the environment and human health can be assured if Tamiflu and antibiotics are used under pandemic conditions. While questions of safety assurance depend on professional judgment, the consensus was that OC release to the environment might still pose risks associated with the generation of antiviral resistance or destabilization of microbial biofilms or flock or sensitive microbial community that are key to the performance and function of sewage treatment plants. This is a critical issue given the unprecedented quantities of antibiotics and antiviral drugs are likely to be used in a pandemic. The case of seasonal use of Tamiflu in Japan provided a valuable surrogate for assessing the behavior of the compounds during sewage treatment processes and implications of release under pandemic conditions.

9.2 Recommendations

Detail study on ozonation and advance oxidation process applicability for antiviral drugs destruction/removal from secondary effluent.

Biodegradation test of antibiotics and antiviral drugs in surface water environment.

The toxicity of by product produced during oxidation treatments, and identification of intermediate.

The risk of antiviral drugs resistance of influenza viruses in wildfowl is an important concern need to pay more attention along with the effects of mixtures of pharmaceuticals in biological sewage treatment processes during a future pandemic.