Influenza virus infections continue to cause significant morbidity and mortality worldwide, and they place a considerable economic burden on individuals, families, businesses, and health care providers. Seasonal flu epidemics cause tens of millions of respiratory illnesses and 250,000–500,000 deaths worldwide each year [World Health Organization (WHO) 2003]. During the 20th century, flu pandemics caused millions of deaths, social disruption, and profound economic losses. The outbreak of the “Spanish flu” in 1918 was the worst, causing an estimated 40 million deaths worldwide, including 390,000 in Japan (Ministry of Health, Labour and Welfare of Japan 2005). Influenza pandemics occur when a new strain of the influenza virus is transmitted to humans from animals. Species 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 reports of flu virus resistance to antiviral drugs are of great concern. Two groups of antiviral drugs are used for the treatment of flu: the neuraminidase inhibitors (e.g., Tamiflu) and the M2 ion channel inhibitors (e.g., amantadine). Oseltamivir phosphate (OP; Figure 1), marketed as Tamiflu, is recommended by the WHO for both treatment of influenza and prophylaxis and is considered an important first-line defense in the event of a flu pandemic. OP is a prodrug that 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 (> 80% of oral dose) unchanged (Sweetman 2007).

OP is widely used in Japan. As with many other pharmacologically active compounds, sewage treatment plant (STP) effluent is the main source of OC in the environment because OC is not removed significantly in STPs (Fick et al. 2007). The most widespread subtypes of influenza A virus are transmitted through waterfowl, where they remain, multiply, and are excreted in large quantities in droppings (Olsen et al. 2006). Notably, waterfowl stay close to STP discharge points, where the water temperature is higher and where they find adequate food, especially in winter, the flu season. Therefore, widespread use of OP to fight seasonal influenza in humans could lead to the development of OC-resistant strains of the viruses in wild birds (Fick et al. 2007; Singer et al. 2007). In addition, a mass administration of OP during a future pandemic could pose a risk to drinking water safety and ecologic health.

In the present study we developed a method for the detection of OC in STP discharge and in river water using solid-phase extraction (SPE) followed by liquid chromatography–tandem mass spectrometry (LC-MS/MS), and investigated the occurrence of OC in STP effluent and receiving river water during the 2008–2009 flu season in Kyoto City, Japan.
we enriched sample matrices on two stacked cartridges.

**Sample collection and preparation.** We assayed for OC in STP effluent and receiving river water during three 1-day-long sampling campaigns (dry weather conditions): the weeks beginning 4 December 2008, 4 February 2009, and 24 February 2009. Samples were collected from 11 locations: sampling points S1–S4 refer to STP discharge sites, and R1–R7 are to river sites (Figure 2). Sewage effluent samples were always gathered from the outlet of STPs, and river samples from a bridge over the center of the stream. No samples were collected at R2 during the first and third campaigns or from S4 during the third campaign.

Grab samples (300 mL) were collected in glass bottles containing ascorbic acid (1 g/L) to acidify the samples and for chlorine and ozone quenching. All the samples were filtered (Whatman GF/B glass microfiber filter, 1 µm pore size) as soon as possible, but no more than 5 hr after sampling. The filtered samples were immediately extracted or kept at –25°C in half-filled amber glass bottles laid horizontally until extraction. Before extraction, sodium chloride (1 g/L) was added and the pH was adjusted to 4.0 with sulfuric acid to enhance OC extraction yield by SPE; the surrogate standard OC-D₃ (50 ng) was added to calculate the recovery rate. SPE was performed with 6-cc Oasis HLB sorbent cartridges (200 mg; Waters Co., Milford, MA, USA) using a Chratec Sep-Pak Concentrator (SPC 10-C; Chratec, Kyoto, Japan). The cartridges were preconditioned with 3 mL methanol followed by 3 mL Milli-Q water (pH 4.0). All samples were passed through the cartridges at a flow rate of 5 mL/min. After concentration, the cartridges were dried completely with air on a vacuum manifold for 2 hr. The analytes were then eluted from the cartridge with 6 mL methanol containing 2% ammonia, passed through a Sep-Pak Plus NH₂ (360 mg; Waters) cartridge for cleanup into 10-mL graduated glass vessels, and dried by a gentle flow of nitrogen at 37°C. The final sample volume was adjusted to 0.5 mL with 0.1% formic acid in Milli-Q water containing 20% acetonitrile (a concentration factor of 600).

**LC-MS/MS.** OC and OC-D₃ were separated in a Waters Acquity Ultra Performance LC (UPLC) separation module with a binary pump system equipped with a UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm particle size). Optimum separation was achieved with a binary gradient of 0.1% formic acid (vol/vol) in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.35 mL/min under the following program: 0–2 min, 10% B; 2–4 min, 10–75% B; 4–4.3 min, 75–90% B; 4.3–5.3 min, 90% B; 5.3–5.8 min, 90–10% B; 5.8–8 min, 100% B (equilibrium of column). The column temperature was kept at 60°C, and the injected sample volume was 10 µL. The UPLC system was coupled to a Quattro Micro API MS with electrospray ionization (Waters).

During quantification optimization, OC and OC-D₃ were individually infused as standard solutions into the initial mobile phase (50% solvent A + 50% solvent B) directly into the MS at 1 mg/L, at a cone voltage of 20 V, a collision energy of 10 eV, a product ion equation: $m/z$ 285.06 (OC) or 288.07 (OC-D₃), a daughter ion $m/z$ of 196.72 (OC) or 199.71 (OC-D₃), and a retention time of 3.24 min. The MS used an electrospray source block temperature of 120°C and a desolvation gas flow of 50 and 900 L/h, respectively. Instrument control, data acquisition, and quantification were performed by MassLynx 4.1 software (Waters).

**Method validation.** We anlyzed OC and OC-D₃ by multiple reaction monitoring at the highest precursor and product ion transitions. Positive identification of OC was based on LC retention time compared with that on LC retention time with a binary gradient of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.35 mL/min under the following program: 0–2 min, 10% B; 2–4 min, 10–75% B; 4–4.3 min, 75–90% B; 4.3–5.3 min, 90% B; 5.3–5.8 min, 90–10% B; 5.8–8 min, 100% B (equilibrium of column). The column temperature was kept at 60°C, and the injected sample volume was 10 µL. The UPLC system was coupled to a Quattro Micro API MS with electrospray ionization (Waters).

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where $P_{CSTP}$ is the predicted concentration of OC in STP discharge in Kyoto City; $TIC \times D$ is the total number of influenza cases per week in Kyoto City during the study period; $D$ is the OP dose assuming 85% adults ($2 \times 75$ mg OP/day) and 15% children ($2 \times 45$ mg OP/day), reflecting the population structure of Kyoto City in 2008; 0.7 reflects that on any day in the week only five of seven patients will be taking OP because the treatment course is 5 days; 0.8 represents the 80% of ingested OP that is excreted unaltered; $P$ is the total population of Kyoto City (1,389,000); and 300 is the average per capita water use (liters per person per day). Further, we assumed that all confirmed influenza cases were treated with OP and that there was zero degradation of OC in the conventional STPs (B and C) (Fick...
et al. 2007). This assumption is not applicable to STP A, which uses ozonation-based tertiary treatment, which efficiently removes pharmaceuticals in wastewater and drinking water (Huber et al. 2003; Ternes et al. 2003).

Results and Discussion

Method validation and application. The pH of the sample proved to be the most influential variable during sample enrichment on the SPE cartridge. Extraction efficiency decreased as pH increased: it was 22% less at pH 11.0 than at pH 4.0. We therefore selected pH 4.0. We used a sample volume of 300 mL, which was the maximum volume without a cartridge breakthrough.

The extraction yield of the surrogate standard OC-D₃ was analogous to that of OC, as expected, owing to their similarities in physicochemical properties. Thus, the analyte was quantitatively enriched by one cartridge and exhaustively eluted as described in “Materials and Methods.” The Sep-Pak Plus NH₂ cartridge for cleanup during elution reduced matrix effects and markedly increased the peak sharpness and instrument sensitivity.

The standard curves were linear, with correlation factors typically > 0.99, between 5 and 4,000 pg on the column. The precision of concentration in effluent without an evaluation of the effect of ozonation on OC may not be appropriate for the prediction of concentration in effluent without an evaluation of the effect of ozonation on OC.

The second sampling campaign ran during the peak period of the 2008–2009 flu season in Kyoto City. The city recorded 1,738 cases during week. OC was detected at all sampling points, with the highest concentration at point S3 (293.3 ng/L). The predicted concentration of OC in STP discharge was 311 ng/L, very close to the actual maximum. The number of patients increased by a factor of 60 between the first and second campaigns, but the concentration of OC in STP discharge increased by a factor of only 20. This difference could be due to a decrease in the prophylactic use of Tamiflu since the beginning of the flu outbreak and to the use of Zanamivir (another neuraminidase inhibitor; GlaxoSmithKline, Tokyo, Japan) instead of Tamiflu in some cases, because Tamiflu-resistant H1N1 virus was recorded during

Figure 3. Representative total ion chromatograms of (A) a blank sewage discharge sample (OC-free) and spiked OC at the LOQ and (B) a blank sewage discharge sample and spiked OC-D₃ at the LOQ of OC.

Figure 4. (A) Total number of influenza cases reported per week during the 2008–2009 influenza outbreak in Kyoto City during three sampling campaigns. (B) OC concentrations at STPs during three sampling campaigns (no sample was collected at S4 during the third campaign). (C) OC concentrations in river water during the second campaign. OC was detected in river water only during the second sampling campaign; no sample was collected at R2 during the first and third campaigns.
the 2008–2009 flu season in Japan (WHO 2009). At sampling point R1, in the Katsura River upstream of STP B (S2), the river incorporates wastewater from a small city and hilly areas, and has a relatively low concentration of OC (6.6 ng/L) (Figure 4C). Similarly, the concentration was low at Kamo River sampling point R3 (8.2 ng/L), which also has low levels of anthropogenic contaminants. On the other hand, the Nishitakase River carries treated sewage from STP A (S1) and STP B (S3), accounting for around 90% of its total flow, and had a high concentration of OC at R2 (190.2 ng/L), as expected. The Nishitakase and Kamo rivers join the Katsura River upstream of R5, where the OC concentration was 13.1 ng/L, comparable to that at the most downstream point, R7 (11.6 ng/L). The OC concentrations at R4 and R6 were 11.3 and 19.6 ng/L, respectively.

**Transport, dissipation, and degradation.** During the non-flu period (June–October 2008), OC was not detected in sewage discharge (S1, S2, S3) or river water (R5). The Katsura River is the main river in the study area, receiving most of the wastewater generated from Kyoto City. The OC concentration increased from point R1 (6.6 ng/L) downstream to R5 (13.1 ng/L) during the flu season (Figure 4C). Discharges from STPs A and B were the main sources involved in this increase. There was no effect of dilution between R1 and R5 because a higher concentration of OC was detected at the Kamo River sampling site (R3; 8.2 ng/L) than at R1. The OC concentration at the most downstream point on the Katsura River (R7; 17.1 ng/L) was higher than that at R5. The addition of OC from STP C effluent (S4; 172.0 ng/L) and a small canal between R5 and R7 (R6; 19.6 ng/L) was responsible for the higher concentration at R7 than at R5. As is the case for other pharmaceuticals, OC may undergo sorption, biodegradation, and photolysis. In river water, OC can be degraded by the addition of a small amount (5%) of sediment, which promotes microbial degradation but requires several weeks (> 8%) of 14C-OC evolved as 14CO2 from water/sediment samples after 21 days of incubation (Saccà et al. 2009). Because OC has a low sorption affinity for sediment, sorption would explain little degradation (Saccà et al. 2009). Because the river flow in the study area is fast (complete transit in a few hours), the removal of OC by sorption and biodegradation between R1 and R7 will be insignificant. On the other hand, direct photolysis plays little or no role in the decomposition of OC. The half-life of OC in unfiltered Elbe River water was 427 hr (Bartels et al. 2005). However, on the basis of “predicted environmental concentration/predicted no-effect concentration” risk ratios, no significant risk of surface waters or STP effluent during both seasonal and pandemic use of Tamiflu was evident (Straub 2009). Nitration in STPs is generally considered to be sensitive to toxic compounds (Pagga et al. 2006). Yet concentrations of OC up to 20 µg/mL had no observed impact on the structure of the microbial community or on bacterial nitrification processes (Saccà et al. 2009). The risk of OC resistance in wildfowl was considered less important than the synergistic effects of pharmaceuticals in sewage on the biological sewage treatment process (Singer et al. 2008). However, the concentration that inhibited 50% of influenza virus (IC50) ranged between 80 and 230 ng/L (Gubareva et al. 2003; Monto et al. 2006), similar to the range detected in STP discharge here. The emergence of resistance to Tamiflu in the influenza virus H1N1 during the 2008–2009 flu season are of great concern (WHO 2009). During a common flu season, waterfowl can ingest large quantities of OC with virus (virus is believed to be transmitted among waterfowl by the fecal–oral route) in their daily water intake, and a high percentage of OC ingested by waterfowl will remain in the intestinal tract, the primary site of viral replication, possibly promoting drug resistance (Fick et al. 2007; Singer et al. 2007).

**Environmental risk.** The highest OC concentrations were detected during the second sampling campaign: 293.3 ng/L at STP A site S3 and 190.2 ng/L at Nishitakase River site R2. The Nishitakase carries mostly STP A and STP B effluent on dry days, resulting in its high concentration. The predicted environmental levels of OC during a flu pandemic in Europe and North America ranged from < 300 ng/L to 32 µg/L, depending on the characteristics of the river basins (Singer et al. 2007). In the Katsura River, the highest concentration was 17.1 ng/L at R7, the most downstream point in the present study. In an acute toxicity study using Daphnia magna, Tamiflu was classified harmful according to European Union Directive 67/548/EEC as amended (European Medicines Agency 2005). However, on the basis of “predicted environmental concentration/predicted no-effect concentration” risk ratios, no significant risk of surface waters or STP effluent during both seasonal and pandemic use of Tamiflu was evident (Straub 2009). Nitration in STPs is generally considered to be sensitive to toxic compounds (Pagga et al. 2006). Yet concentrations of OC up to 20 µg/mL had no observed impact on the structure of the microbial community or on bacterial nitrification processes (Saccà et al. 2009). The risk of OC resistance in wildfowl was considered less important than the synergistic effects of pharmaceuticals in sewage on the biological sewage treatment process (Singer et al. 2008). However, the concentration that inhibited 50% of influenza virus (IC50) ranged between 80 and 230 ng/L (Gubareva et al. 2003; Monto et al. 2006), similar to the range detected in STP discharge here. The emergence of resistance to Tamiflu in the influenza virus H1N1 during the 2008–2009 flu season are of great concern (WHO 2009). During a common flu season, waterfowl can ingest large quantities of OC with virus (virus is believed to be transmitted among waterfowl by the fecal–oral route) in their daily water intake, and a high percentage of OC ingested by waterfowl will remain in the intestinal tract, the primary site of viral replication, possibly promoting drug resistance (Fick et al. 2007; Singer et al. 2007).

**Table 1.** STP characteristics.

| STP | Capacity (m³/day) | Population served | Primary | Secondary | Tertiary |
|-----|------------------|-------------------|---------|-----------|----------|
| A   | 114,000          | 83,000            | Settling | A2O       | O3       |
| B   | 975,000          | 773,000           | Settling | CAS, A2O  | A2O      |
| C   | 228,000          | 338,167           | Settling | A2O       | A2O      |

Abbreviations: A2O, anaerobic/anoxic/oxic; A0A0, anoxic/oxic/anoxic/oxic; CAS, conventional activated sludge; O3, ozonation (4 mg ozone/L, average contact time, 10 min).

**Figure 5.** Total number of influenza cases reported per week with predicted and maximum detected concentrations of OC in Kyoto City sewage discharges (see Equation 1 for assumptions).
Conclusion
We developed and validated an LC-MS/MS-based analytical method for the determination of OC in water samples. Here, we report for the first time the detection of OC in STP discharges and river water during a flu season. The highest concentration of OC detected in STP discharge was 293.3 ng/L at an STP operating with only an activated-sludge-based treatment system. One STP operating with ozonation as a tertiary treatment was highly efficient at removing OC (> 85% from secondary effluent). Ozonation as tertiary treatment in STP will substantially reduce the OC load in STP effluent during an influenza epidemic or pandemic. Further research should investigate the fate of antiviral drugs during every process at every unit in the STPs.

References
Bartels P, Tümpling WV. 2008. The environmental fate of the antiviral drug oseltamivir carboxylate in different waters. Sci Total Environ 405:215–225.

European Medicines Agency. 2005. Tamiflu: European Public Assessment Report. Scientific Discussion. Available: http://www.emea.europa.eu/humandocs/PDFs/EPAR/tamiflu/136102en6.pdf [accessed 25 November 2009].

Fick J, Lindberg RH, Tysklind M, Haemig PD, Waldenström J, Wallensten A, et al. 2007. Antiviral oseltamivir is not removed or degraded in normal sewage water treatment: implications for development of resistance by influenza A virus. PLoS ONE 2(10):e986; doi:10.1371/journal.pone.0000986 [Online 3 October 2007].

Gubareva LV, Webster RG, Hayden FG. 2001. Comparison of the activities of zanamivir, oseltamivir, and RWJ-270201 against clinical isolates of influenza virus and neuraminidase inhibitor-resistant variants. Antimicrob Agents Chemother 45:3403–3408.

Huber M, Canonica S, Park GY, Gunten UV. 2003. Oxidation of pharmaceuticals during ozonation and advanced oxidation processes. Environ Sci Technol 37:1016–1024.

Ministry of Health, Labour and Welfare of Japan. 2005. Pandemic Influenza Preparedness Action Plan of the Japanese Government. Available: http://www.mhlw.go.jp/ english/topics/influenza/dl/pandemic02.pdf [accessed 20 April 2009].

Monto AS, McKimm-Breschkin J, Macken C, Hampson AW, Hay A, Klimov A, et al. 2006. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrob Agents Chemother 50:2395–2402.

Olsen B, Munster VJ, Wallensten A, Waldenström J, Osterhaus ADME, Fouchier RAM. 2006. Global patterns of influenza A virus in wild birds. Science 312:384–388.

Pagga U, Bachner J, Strotmann U. 2006. Inhibition of nitrification in laboratory tests and model wastewater treatment plants. Chemosphere 65:1–8.

Saccà ML, Accinelli C, Fick J, Lindberg R, Olsen B. 2009. Environmental fate of the antiviral drug Tamiflu in two aquatic ecosystems. Chemosphere 75:29–33.

Singer AC, Howard BM, Johnson AC, Knowles CJ, Jackman S, Accinelli C, et al. 2008. Meeting report: risk assessment of Tamiflu use under pandemic conditions. Environ Health Perspect 116:1563–1567.

Singer AC, Nunn MA, Gould EA, Johnson AC. 2007. Potential risks associated with the widespread use of Tamiflu. Environ Health Perspect 115:102–106.

Straub JD. 2009. Environmental risk assessment for oseltamivir (Tamiflu) for sewage works and surface waters under seasonal-influenza- and pandemic-use conditions. Ecotoxicol Environ Saf 72(6):1625–1634.

Sweetman SC, ed. 2007. Martindale: The Complete Drug Reference. Electronic version (edition 070214). London: Pharmaceutical Press.

Ternes TA, Stubber J, Herrmann N, McDowell D, Ried A, Kampmann M, et al. 2003. Ozonation: a tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? Water Res 37:1076–1082.

WHO (World Health Organization). 2003. Influenza (Seasonal). Fact Sheet No. 211. Available: http://www.who.int/mediacentre/factsheets/fs211/en/ [accessed 20 April 2009].

WHO (World Health Organization). 2009. Influenza A (H1N1) Virus Resistance to Oseltamivir - 2008/2009 Influenza Season, Northern Hemisphere. Available: http://www.who.int/csr/disease/influenza/H1N1webupdate20090316%20ed_ms.pdf [accessed 20 April 2009].