**commentletters - FW: Comment Letter – proposed Water Recycling Policy, addendum to prior submissions**

12/4/07 Bd. Mtg.
**Water Recycling Policy**
Deadline: 10/26/07 Noon

**From:** Edo McGowan <edo_mcgowan@hotmail.com>
**To:** <commentletters@waterboards.ca.gov>, DrevHunt <drev@lawyersforcleanwater.com>, Elizabeth Erickson <eerickson@waterboards.ca.gov>, <jstone1@dhs.ca.gov>, "Jeffrey.Stone@cdph.ca.gov" <jeffrey.stone@cdph.ca.gov>, <layne@lawyersforcleanwater.com>, "Katherine@venturahydraulics.com" <katherine@venturahydraulics.com>, "SupervisorCarbajal@co.santa-barbara.ca.us" <supervisorcarbajal@co.santa-barbara.ca.us>, <woody.maxwell@venturausd.org>
**Date:** 10/25/2007 5:24 PM
**Subject:** FW: Comment Letter – proposed Water Recycling Policy, addendum to prior submissions

To: Ms Townsend, SWRQB
Fm: Dr E McGowan
Re: Addendum to prior Comment Letter â€“ proposed Water Recycling Policy

This is a version of the WERF paper on reclaimed water in which one of the plants studied was a Title 22 plant. Again, it is doubtful that the state through its lab testing has considered the inability of MPN using typical coliform indicators to properly evaluate the total risk, especially when newly emerging infectious diseases are considered as well as viable but non-culturable. Thus the standards, as extant are deficient. Accordingly, the state can not protect the public health as required in statute. This defect is something that an EIR would address and thus again demonstrates the need for an EIR.

It will be noted that these authors indicate----------

The failure of measurements of single indicator organism to correlate with pathogens suggests that public health is not adequately protected by simple monitoring schemes based on detection of a single indicator, particularly at the detection limits routinely employed.

The current monitoring approach to assess the microbial safety of reclaimed water is the measurement of total or fecal coliform concentrations in a single daily grab sample. Utilities and regulatory agencies have assumed a predictive relationship between indicator organism and pathogen levels to protect the public from exposure to pathogens; however, the imperfect relationship between coliform bacteria and pathogens, such as viruses (12, 13, 25) and protozoa (5), through wastewater treatment has been known for some time (see reference 16 for a review).

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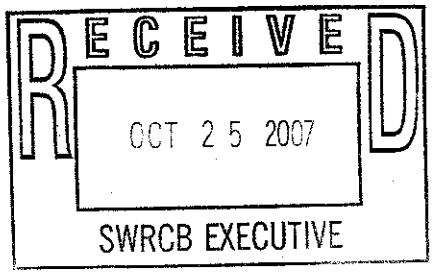
Validity of the Indicator Organism Paradigm for Pathogen Reduction in Reclaimed Water and Public Health



Protectionâ€

Valerie J. Harwood,[1*] Audrey D. Levine,[2] Troy M. Scott,[3] Vasanta Chivukula,[1] Jerzy Lukasik,[3] Samuel R. Farrah,[4] and Joan B. Rose[5]

Department of Biology, SCA 110, University of South Florida, 4202 E. Fowler Ave., Tampa, Florida 33620,[1] Department of Civil and Environmental Engineering, ENB 118, University of South Florida, 4202 E. Fowler Ave., Tampa, Florida 33620,[2] Biological Consulting Services of N. Florida, Inc., 4641 N.W. 6th Street, Suite A, Gainesville, Florida 32609,[3] Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611,[4] Department of Fisheries and Wildlife and Crop and Soil Sciences, 13 Natural Resources Building, Michigan State University, East Lansing, Michigan 48824[5]

*Corresponding author. Mailing address: Department of Biology, SCA 110, University of South Florida, 4202 E. Fowler Ave., Tampa, FL 33620. Phone: (813) 974-1524. Fax: (813) 974-3263. E-mail: vharwood@cas.usf.edu .

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ABSTRACT

The validity of using indicator organisms (total and fecal coliforms, enterococci, *Clostridium perfringens*, and F-specific coliphages) to predict the presence or absence of pathogens (infectious enteric viruses, *Cryptosporidium*, and *Giardia*) was tested at six wastewater reclamation facilities. Multiple samplings conducted at each facility over a 1-year period. Larger sample volumes for indicators (0.2 to 0.4 liters) and pathogens (30 to 100 liters) resulted in more sensitive detection limits than are typical of routine monitoring. Microorganisms were detected in disinfected effluent samples at the following frequencies: total coliforms, 63%; fecal coliforms, 27%; enterococci, 27%; *C. perfringens*, 61%; F-specific coliphages, ~40%; and enteric viruses, 31%. *Cryptosporidium* oocysts and *Giardia* cysts were detected in 70% and 80%, respectively, of reclaimed water samples. Viable *Cryptosporidium*, based on cell culture infectivity assays, was detected in 20% of the reclaimed water samples. No strong correlation was found for any indicator-pathogen combination. When data for all indicators were tested using discriminant analysis, the presence/absence patterns for *Giardia* cysts, *Cryptosporidium* oocysts, infectious *Cryptosporidium*, and infectious enteric viruses were predicted for over 71% of disinfected effluents. The failure of measurements of single indicator organism to correlate with pathogens suggests that public health is not adequately protected by simple monitoring schemes based on detection of a single indicator, particularly at the detection limits routinely employed. Monitoring a suite of indicator organisms in reclaimed effluent is more likely to be predictive of the presence of certain pathogens, and a need for additional pathogen monitoring in reclaimed water in order to protect public health is suggested by this study.

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Reclaimed water is derived from treated municipal wastewater. The treatment processes used for production of reclaimed water provide multiple barriers (biological treatment, physical removal, and chemical disinfection) for control of pathogens. Reclaimed water is used for nonpotable applications such as irrigation, cooling water, industrial process water, and environmental enhancement (17). Indirect potable reuse occurs through groundwater recharge or surface water replenishment, and is assuming greater importance with increased production of reclaimed water. As water use in the United States (7) and worldwide increases, the importance of reclaimed water to sustainable water resources will continue to increase (17).

A major goal of wastewater reclamation facilities is to reduce pathogen loads in order to decrease public health risks associated with exposure. The effectiveness of pathogen control is indirectly assessed through routine monitoring of the reclaimed water (final effluent) by using grab samples to detect standard indicator bacteria such as total or fecal coliforms. Treatment practices for production of reclaimed water vary depending on the ultimate intended use(s) of the water and local regulatory requirements. Currently, there are no universal standards

governing the production and quality of reclaimed water, although the World Health Organization has developed guidelines for the use of reclaimed water (35) that recommend monitoring fecal coliforms and intestinal nematodes. In the United States, there are no federal standards controlling the quality of reclaimed water, and individual states have developed guidelines or implemented specific treatment and monitoring requirements that are intended to protect the public from exposure to pathogens. Due to the inherent constraints associated with pathogen monitoring, indicator organisms are employed as surrogates for pathogens. In some states, total coliform bacteria are used as the indicator organism (6); however, in the majority of states that have specific regulations, the microbiological safety of reclaimed water is evaluated by daily monitoring of fecal coliform bacteria in the disinfected effluent based on a single, 100-ml grab sample (3). In addition, periodic monitoring of viruses and/or protozoan pathogens has been required by a few states, including Arizona, California, and Florida (3).

It has been widely demonstrated that coliform bacteria do not adequately reflect the occurrence of pathogens in disinfected wastewater effluent due to their relatively high susceptibility to chemical disinfection (18) and failure to correlate with protozoan parasites such as *Cryptosporidium* (5) and enteric viruses (13). Alternative microbiological indicators have been suggested for evaluation of wastewater, drinking water, and environmental waters, including *Enterococcus* spp. (18), *Clostridium perfringens* (9, 20), and coliphages (8, 10, 20).

To date, there have been only a few studies of reclaimed water in which the levels of indicator organisms have been directly compared to those of viral, bacterial, or protozoan pathogens at each stage of treatment (23, 24). In this work, the validity of using coliform bacteria and alternative microbial indicators to predict the presence or absence of pathogens, and thus to assess the public health risk, was evaluated using disinfected effluent from six wastewater reclamation facilities in the United States. The facilities varied in location (Arizona, California, and Florida), size, and treatment practices and were each sampled at least five times over a 1-year period. Each sample was analyzed for a suite of indicator bacteria, coliphages, enteric viruses, and protozoan pathogens, and predictive relationships among the microbial groups were evaluated by several statistical methods, including binary logistic regression and discriminant analysis (DA).

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## MATERIALS AND METHODS

Facilities. Six wastewater reclamation facilities in the United States were each samp least five times over a 1-year period. A comparison of the treatment characteristics given in Table 1. The facilities represent a cross-section of typical treatment approa that are used for production of reclaimed water.

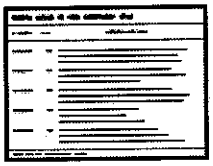

**TABLE 1.**
Comparison of wastewater reclamation facilities sampled for indicator organisms and pathogens in this study

Sampling. All samples were aseptically collected in sterile containers (or sterile filters). Samples were immediately placed on ice in coolers and were kept on ice until processed. At each facility, samples were collected from the influent (untreated wastewater), secondary clarifier (biological treatment), filtered effluent, and disinfected effluent (reclaimed water). Samples were collected under peak flow conditions to provide a â€œworst-caseâ€□ scenario for each facility. Each facility was sampled approximately bimonthly over a 1-year period, resulting in at least five samplings per facility.

Sample volumes collected for bacterial enumeration were 50 ml of influent, 500 ml from the secondary clarifier, 2 liters of effluent from the filtration stage, and 2 liters of disinfected effluent. Assays were performed in triplicate. Large volumes (up to 53 liters) were filtered for protozoan parasite and virus assays. Detection limits for bacterial indicators in disinfected effluent were 0.2 to 0.6 CFU Â· 100 $ml^{â^{'}1}$, those for coliphages were 10 PFU Â· 100 $ml^{â^{'}1}$, those for enteric viruses were 0.3 to 1.4 most probable number (MPN) Â· 100 $liters^{â^{'}1}$, those for *Cryptosporidium* oocysts were 2.0 to 6.9 oocysts Â· 100 $liters^{â^{'}1}$, those for infectious *Cryptosporidium* were 0.29 to 4.1 MPN Â· 100 $liters^{â^{'}1}$, and those for *Giardia* were 1.8 to 5.2 cysts Â· 100 $liters^{â^{'}1}$.

Bacterial enumeration. Indicator bacteria were quantified by membrane filtration using 47-mm cellulose acetate filters with a nominal pore size of 0.45 Î¼m. Total coliform bacteria were cultured on mEndo LES agar (Difco, Sparks, MD) for 24 h at 37Â°C. Colonies that produced a green sheen were enumerated as total coliforms (2). Fecal coliform bacteria were cultured on mFC agar (Difco, Sparks, MD) for 24 h at 44.5Â°C in a water bath. Blue colonies were enumerated as fecal coliforms (2). *Escherichia coli* (ATCC 9637) was used as the positive control for all coliform measurements. Enterococci were cultured on mEI agar (31, 32). Plates were incubated at 41Â°C for 24 h, and colonies with a blue halo were enumerated as enterococci. *Enterococcus faecalis* (ATCC 19433) was used as a positive control. *Clostridium perfringens* was isolated on mCP agar (Acumedia Manufacturers, Inc.) (4). Plates were transferred to gas pack bags (BBL GasPak; Becton Dickinson) and sealed. After 24 h of incubation at 45Â°C, colonies were exposed to ammonium hydroxide fumes. All of the yellow/straw-colored colonies that turned pink/magenta were counted. *C. perfringens* (ATCC 13124) was used as a positive control.

Bacteriophage analysis. Coliphages were analyzed by the agar overlay method of Adams (1). Two *E. coli* host strains were used in separate assays: *E. coli* HS (pFamp) R (ATCC 700891), which infects male-specific ($F^+$) coliphages very efficiently and somatic coliphages poorly (8), and *E. coli* C3000 (ATCC 15597), which should host both somatic and $F^+$ coliphages (14). Serial dilutions of samples were made in phosphate-buffered saline according to expected phage concentrations at each treatment step. Five replicate volumes of 0.1 ml to 2 ml were plated for each dilution, except in the case of the disinfected effluent samples, for which 10 replicates of 2 ml each were plated. PFU Â· 100 $ml^{â^{'}1}$ were calculated after 24 h of incubation (2).

Enteric viruses. The U.S. Environmental Protection Agency (EPA) methodology (30) was used for the detection of enteric viruses. Influent sample volumes were

based on the amount of water that could be processed without clogging the filter. Typically less than 100 liters was filtered for each influent sample, depending on water quality (i.e., content of suspended solids). Larger sample volumes were used for the other sample locations, i.e., ~190-liter samples from the secondary clarifiers and ~380-liter samples from the filtration and disinfection processes. Water samples were pumped through Virusorb 1MDS filters (Cuno, Inc.), which were eluted with 1 liter of 1.5% beef extract (BBL V) in 0.05 M glycine (pH 9.5, ~25Â°C) (U.S. EPA/ICR). The eluted sample was concentrated by organic flocculation and assayed for enterovirus by the observation of cytopathic effects (CPE) on recently passed (<4 days) cell lines. Three cell lines, i.e., buffalo green monkey, rhabdosarcoma (ATCC CCL-136), and MA-104 (ATCC CRL-2378.1) cells, were used for this purpose. Positive controls were processed in a separate room, using poliovirus I. CPE on each cell line were observed, and the most dilute sample showing CPE was recorded. MPN determinations were performed using EPA-released software (Most Probable Number Calculator version 4.04; http://www.epa.gov/microbes/other.htm).

Protozoa. For the detection of *Giardia* and *Cryptosporidium*, samples were concentrated by filtration using Gelman Envirochek HV cartridge filters and processed according to the manufacturer's instructions. Following filtration, samples were processed by immunomagnetic separation (Dynal, Inc.) and immunofluorescent antibody detection (Easy Stain; Biotech Frontier, Australia) according to the procedure outlined in U.S. EPA method 1623 (33). Sample volumes varied depending upon the treatment stage and the amount of water that could be filtered, i.e., 0.5 to 1.0 liters influent, ~19 liters secondary effluent, ~38 liters effluent from filters, and up to 53 liters disinfected effluent. Detection limits varied with the total volume of sample filtered and processed. Each concentrated sample was divided into two aliquots: one for cell culture viability testing and the other for microscopic enumeration. Equivalent volumes were calculated, and the results were reported as cysts or oocysts Â· 100 liters$^{â^'1}$.

*Cryptosporidium* infectivity. Concentrates from the immunomagnetic separation procedure were inoculated onto HCT-8 cell monolayers in eight-well chamber glass cell culture slides. The cultures were incubated in a 5% $CO_2$ atmosphere at 37Â°C for 48 h. Infective *Cryptosporidium* was enumerated by the focus detection method-MPN assay (27). Results were reported as infectious oocysts Â· 100 liters$^{â^'1}$.

Statistical analysis. Statistical analyses were conducted using SAS software version 8.2 (SAS Institute, Cary, NC) or SPSS version 12.0. Data distributions were evaluated with the Shapiro-Wilk test, which was conducted on the raw data, $\log_{10}$-transformed data, and square-root-transformed data. Nonparametric statistical tests were utilized for nonnormally distributed data. Parametric tests were used for analysis of variance, and the Tukey post-hoc test was used to compare treatment means. The Spearman rank correlation was used to test the relationship between indicator organism and pathogen concentrations in the final effluent. A binary logistic regression model (SPSS 12.0) was utilized to determine whether indicator organism concentrations predicted the probability of the occurrence of pathogens in disinfected effluent samples. The dependent variable (pathogen) was treated as a binary variable; that is, a score of 0 was assigned when the organism was not detected, and a score of 1 was assigned when the

organism was detected. The independent variables were continuous, and values for samples in which organisms were not detected were reported as 0. True-positive, true-negative, false-positive, and false-negative values were calculated as the number of samples falling into each category divided by the total sample number.

Discriminant analysis was performed on data from effluent samples by using the DISCRIM procedure of SAS (prior probabilities, equal; covariance matrix, pooled). The results of six assays for indicator organisms (total coliform, fecal coliform, *C. perfringens*, enterococci, and F-specific coliphage assays on two hosts) were converted into a string of binary variables representing the presence or absence of each indicator. The ability of the indicator data string to predict the presence or absence of each pathogen (*Giardia*, *Cryptosporidium*, and enteric viruses) was assessed separately. Results are expressed as the percentage of samples correctly classified into the â€œpathogen presentâ€□ and â€œpathogen absentâ€□ categories.

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## RESULTS

The results presented here represent multiple samplings from six facilities producin reclaimed water and focus on microbial concentrations in the influent and in the reclaimed water (disinfected effluent), which is distributed to end users.

Microbial concentrations through treatment. Concentrations of indicator organisms pathogens before (untreated wastewater) and after (disinfected effluent) treatmen shown in Fig. 1 in a box plot format. The limit of detection (see Materials and Meth was substituted for measured values for samples in which the organism was not detected, which was rare in influent samples but common in effluent samples. Tota coliform concentrations were the highest of the microbial measurements in influent samples (>$10^7$ CFU Â· 100 $ml^{â^{'1}}$), followed by fecal coliforms and enterococci (~1 Â· 100 $ml^{â^{'1}}$) (Fig. 1). *Clostridium perfringens* values ranged from $10^4$ to >$10^6$ CFI 100 $ml^{â^{'1}}$. Coliphage levels were highly variable, ranging from $10^3$ to $10^8$ PFU Â· 1 $ml^{â^{'1}}$. Pathogen concentrations in the influent (Fig. 1) were 4 to 5 orders of magni lower than indicator organism concentrations (note that the unit for pathogen concentrations is 100 $liters^{â^{'1}}$). It should be noted that while the enteric virus concentrations represent infectious viruses, *Cryptosporidium* and *Giardia* concentra reflect the total number of cysts or oocysts (infectious and noninfectious) viewed u immunofluorescence microscopy. In the influent samples, about 40% of the detect *Cryptosporidium* organisms were infective as defined by the focus detection metho cell culture assay. Microbial concentrations in disinfected effluents were much lowe expected (Fig. 1), and in most cases were near or below the detection limits for ea assay.

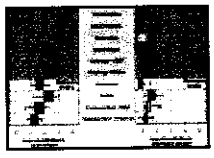

**FIG. 1.**
Mean indicator organism and pathogen concentrations in untreated wastewater and disinfected effluent from six wastewater reclamation facilities ($n$ = 30). $Log_{10}$ concentrations of bacterial indicators (CFU Â· 100 $ml^{â^{'1}}$), coliphages (more ...)

The percentages of samples from each treatment step that contained detectable le each indicator organism and pathogen are summarized in Table 2. Total and fecal coliforms, enterococci, and coliphages were detected in 100% of influent (untreate wastewater samples, in which detection limits were generally 33.3 CFU or PFU Â· 1 $ml^{â^{'1}}$. Three of the 30 untreated wastewater samples were below the detection lin *C. perfringens* (33.3 CFU Â· 100 $ml^{â^{'1}}$). Enteric viruses (detection limit, 100 MPN Â liters$^{â^{'1}}$) and *Giardia* (detection limit, 500 cysts Â· 100 liters$^{â^{'1}}$) were also found i 100% of untreated wastewater samples. *Cryptosporidium* oocysts were detected in of the untreated wastewater samples; however, infective oocysts were identified in 32% of these samples. The detection limit for *Cryptosporidium* in the influent samp depended upon the volume that could be filtered and ranged from 300 to 2,100 oo Â· 100 liters$^{â^{'1}}$. Following biological treatment, the concentrations of indicators an pathogens were reduced by about 1 to 2 $\log_{10}$, thus decreasing the frequency of detection of most organisms; i.e., enteric viruses were detected in only 73% of the secondary effluent samples, compared to 100% of the influent samples. The freque detection of *Cryptosporidium* increased from 75% in the influent samples to 84% i secondary effluent samples, due to the more sensitive detection limits in secondary effluent (21 to 94 oocysts Â· 100 liters$^{â^{'1}}$); however, the frequency of detection of infectious oocysts decreased from 32% to 19%. Filtration further decreased the frequency of detection of microorganisms, particularly for enterococci, the coliphag and *Giardia* (Table 2).

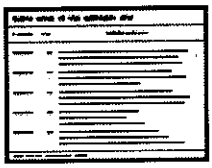

**TABLE 2.**
Percentage of samples with detectable indicator organisms and pathogens

In disinfected samples, total coliforms and *C. perfringens* were detected most frequ and fecal coliforms and enterococci were least frequently detected (Table 2). While frequencies of detection of fecal coliforms and enterococci in disinfected effluents v similar (27%), they were simultaneously detected in only one sample, whereas eith fecal coliforms or enterococci were detected in 50% of the samples. An assessmen correlation between total residual chlorine and fecal coliform concentrations in trea effluent samples from all the facilities showed no significant relationship between th parameters (data not shown).

Pathogens, measured on the scale of 100 liters$^{â^{'1}}$, were detected in 80% (*Giardia*) 31% (enteric virus) of samples. Both *Giardia* and *Cryptosporidium* were detected b microscopy in 60% of disinfected effluent samples. Unlike the trend noted for the c organisms, the percentage of samples in which *Cryptosporidium* oocysts were dete remained fairly consistent through the treatment stages (71 to 84%); however, det limits became progressively more sensitive through the treatment stages, reaching 6.9 oocysts Â· 100 liters$^{â^{'1}}$ in the reclaimed water (disinfected effluents). The perc of samples containing detectable levels of infectious oocysts decreased from 32% i untreated wastewater samples to 20% in the reclaimed water samples.

The frequency of detection of the various microorganisms in disinfected effluent sa was compared using Fisher's exact test. Total coliforms and *C. perfringens* were de

in significantly more samples (63% and 61%, respectively) than enterococci or fecal coliforms (both 27%). Other proportional comparisons between indicator organism detections were not significantly different. The protozoan parasites were detected in significantly more disinfected effluent samples than enteric viruses, but there was no significant difference in the proportion of samples in which *Giardia* cysts versus *Cryptosporidium* oocysts were detected. Infective *Cryptosporidium* was detected in significantly fewer disinfected effluent samples than total *Giardia* or *Cryptosporidium*.

Of all the indicator organisms, including the coliphages, the fecal coliforms were found at the lowest concentrations in final effluent samples (Fig. 1) and were among the least frequently detected (Table 2). At hypothetical detection limits of 2 CFU Â· 100 ml$^{\hat{a}\hat{}'1}$, total coliforms would be detected in 43% of the disinfected effluent samples, whereas fecal coliforms would be detected in only 10% of the samples ($n$ = 30). Reducing the detection limit to 0.2 CFU Â· 100 ml$^{\hat{a}\hat{}'1}$ (the actual detection limit) increased the frequency of detection of fecal coliforms and total coliforms to 27% and 63%, respectively. The relationship between hypothetical detection limit and detection frequency was log linear ($r^2$ = 0.96 for total coliforms and 0.94 for fecal coliforms).

Predictive relationships between microorganisms. Data from disinfected effluent samples were analyzed separately (by facility) and as a pooled data set (all facilities) to determine if the concentrations of any of the indicators (total coliforms, fecal coliforms, enterococci, *C. perfringens*, or coliphages) were correlated with each other or with concentrations of pathogens (enteric viruses, *Giardia*, or *Cryptosporidium*). Analysis of results by facility did not yield significant correlations (probably due to small sample sizes); however, significant correlations between indicator organism concentrations were observed in the pooled data sets: i.e., for total coliform and fecal coliform (Spearman's $r_s$ = 0.5986; $P$ = 0.0005), *C. perfringens* versus coliphages on host *E. coli* 15597 ($r_s$ = 0.5303; $P$ = 0.0031), *C. perfringens* versus coliphages on host *E. coli* 700891 ($r_s$ = 0.4981; $P$ = 0.0060), and coliphages on the two *E. coli* hosts ($r_s$ = 0.7915; $P$ < 0.0001). No significant correlation between concentrations of any combination of indicator organism and pathogen was observed.

Enteric viruses were above detection limits in 31% of the disinfected effluent samples ($n$ = 30); however, coliphages and enteric viruses co-occurred in only 13% of the disinfected effluent samples. Concentrations of coliphages on both *E. coli* hosts were plotted against enterovirus concentrations using only samples in which coliphages and enteric viruses were detected, but the slopes of the relationships were not significantly different from 0 (data not shown).

Binary logistic regression was used to test the hypothesis that indicator organism concentrations were predictive of the presence or absence of pathogens in disinfected effluent. Observations of enteric viruses, *Cryptosporidium* oocysts, and *Giardia* cysts were converted to binary data, and the relationship between the concentration of each indicator organism and the presence or absence of each pathogen was assessed, as well as the relationships between the pathogens. Nagelkerke's $R$-square, which can range from 0.0 to 1.0, denotes the strength of the association; stronger associations have values closer to 1.0. Three indicator-

pathogen combinations displayed very weak correlations: coliphage concentration ( *E. coli* 15597) and enteric virus presence/absence (*R*-square = 0.226), fecal colifor concentrations and *Giardia* presence/absence (*R*-square = 0.222), and total colifor infectious *Cryptosporidium* presence/absence (*R*-square = 0.241). In each case, th variability in *x* accounted for only a fraction of the variability in *y* (odds that a patho would be present). A much tighter association was evidenced, for example, betwee two coliphage assays on different hosts (*R*-square = 0.762), as would be expected two similar assays. No correlations between indicators and pathogens were found u the Spearman correlation; however, this is not unusual as binary logistic regression on maximum likelihood, does not require linear relationships between variables (19 utilizes a binary (0, 1) dependent variable.

The analytical consequences of the failure of indicators to correlate with pathogens shown in Fig. 2. True negatives are samples in which neither indicators nor pathog were detected, true positives are samples in which both indicators and pathogens v detected, false negatives are samples in which pathogens were detected when indi were not detected, and false positives are samples in which indicators were detecte when pathogens were not detected. These values add up to 100% for each indicat pathogen combination. Total coliforms frequently survived the disinfection process; therefore, they tended to be present when pathogens were present, resulting in a relatively high true-positive rate compared to the other indicators (Fig. 2A to D). However, total coliforms also tended to have a low true-negative rate (which woulc ideally be high) and a relatively high false-positive rate, particularly in the cases of viruses and viable *Cryptosporidium*. In contrast, fecal coliforms, which were relative infrequently detected in disinfected effluent, tended to have a high true-negative ra also a low true-positive rate. The percentage of results in the correct categories (tr positive and true negative) was not much greater than 50% for any of the indicato pathogen combinations, although ideally these categories would comprise 100% of observations. Each type of correct and incorrect categorization has distinct implicat for public health protection (see Discussion).

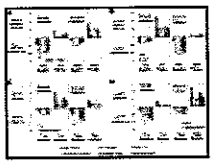

**FIG. 2.**

Relationship between detection of individual indicators and accuracy of pathogen detection in disinfected effluent. All percentages were calculated from the total sample number. Detection limits were 0.2 CFU Â· 100 ml$^{\hat{a}\hat{}'1}$ for total and fecal (more ...)

DA is a multivariate statistical technique that can be used to classify observations ir categories based on a series of independent variables. DA was used to test the hypothesis that the presence or absence of indicator organisms in disinfected efflue samples could predict the presence versus absence of each pathogen (Fig. 3). Indi organism data for each sample were represented as a string of six binary variables (presence or absence of total coliforms, fecal coliforms, enterococci, *C. perfringens* coliphages on *E. coli* 15597, and coliphages on *E. coli* 700891). The presence or ab of each of the pathogens was relatively accurately predicted by the suite of indicat organism data for the 29 effluent samples analyzed (Fig. 3). The data are presente the percentage of samples with pathogens actually present in which pathogen pres was predicted by DA and (ii) the percentage of samples in which pathogens were a

not detected and in which pathogen absence was predicted by DA. When pathoger positive and pathogen-negative samples were considered together, 72% of enteric samples, 79% of *Giardia* samples, 75% of *Cryptosporidium* oocyst samples, and 71 infectious *Cryptosporidium* samples were placed in the correct category (presence absence of the pathogen) by discriminant analysis. The absence of all pathogens e: *Giardia* was more accurately predicted than pathogen presence. In most cases, ren of one variable (indicator organism) from the data string caused the correct classifi rate to decrease by a few percentage points, as one or two additional observations be misclassified. No single indicator was most highly predictive of membership in th â€œpresenceâ€□ or â€œabsenceâ€□ category for pathogens. Interestingly, wher coliphage assayed on *E. coli* 700891 was excluded as a variable, it improved the re the enteric virus analysis by correctly categorizing one additional â€œpresenceâ€□ sample.

**FIG. 3.**
Discriminant analysis, with results showing the percentage of samples correctly categorized with respect to presence or absence of each pathogen. All of the indicators were used as binary dependent variables. Present, percentage of samples with pathogens (more ...)

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## DISCUSSION

The current monitoring approach to assess the microbial safety of reclaimed water is the measurement of total or fecal coliform concentrations in a single daily grab sample. Utilities and regulatory agencies have assumed a predictive relationship between indicator organism and pathogen levels to protect the public from exposure to pathogens; however, the imperfect relationship between coliform bacteria and pathogens, such as viruses (12, 13, 25) and protozoa (5), through wastewater treatment has been known for some time (see reference 16 for a review). A major goal of this work was to examine monitoring strategies and to determine whether any predictive relationship between conventional and alternative indicator organisms and pathogens in reclaimed water could be discerned among a group of treatment facilities producing reclaimed water.

Detection of microorganisms. $Log_{10}$ reduction of microorganisms through wastewater treatment trains is frequently reported (23, 24) but should not be relied upon as the sole measurement of treatment efficacy. Organisms with very high initial concentrations may experience large log reductions while maintaining

detectable levels in disinfected effluents, as illustrated by the total coliforms in this study. Total coliforms experienced an average $\log_{10}$ reduction of >7 from influent to final effluent but were still detected in 67% of disinfected effluent samples.

The linear relationship between hypothetical detection limits and the percentage of samples in which total or fecal coliforms would be detected demonstrates the usefulness of larger sample volumes for detecting indicators, but this ability did not generally translate to a significant predictive relationship between indicators and pathogens. However, if normal volumes (100 ml) had been assayed for fecal coliforms and if we assume that no detection would have occurred in samples in which <1 CFU/100 ml was present, the weak correlations between fecal coliforms versus *Giardia* presence or absence and total coliforms versus infectious *Cryptosporidium* presence or absence would not have been detected (data not shown).

Bacteriophages have been suggested as an alternative indicator for enteric viruses, as their morphology and survival characteristics resemble those of some of the enteric viruses (13, 29). This study found a weak but significant relationship between the presence or absence of enteroviruses and coliphages on *E. coli* 15597 by binary logistic regression. A significant relationship was not found between enteroviruses and coliphages on *E. coli* 700891. This observation, coupled with the improvement in prediction of enterovirus presence or absence by discriminant analysis when coliphage on *E. coli* 700891 was removed as a variable, suggests that the use of other *E. coli* hosts for coliphage assays should be further explored.

The use of U.S. EPA method 1623 for detection of *Cryptosporidium* oocysts does not permit determination of oocyst viability or infectivity, which is crucial information for assessment of the human health risk associated with this parasite. The focus detection method of detecting infectious *Cryptosporidium* (27) has been utilized in a number of studies (11, 15, 21, 22, 26-28, 34), and results coincide well with those of mouse infectivity assays (15). Approximately one-quarter of the disinfected effluent samples with detected *Cryptosporidium* oocysts had detectable levels of infectious *Cryptosporidium*, a disturbing observation in that reclaimed water represents a potential human exposure pathway, depending on how the reclaimed water is used. None of the indicators correlated with *Cryptosporidium* oocysts or infectious *Cryptosporidium*.

Because indicators were not predictive of pathogen presence, the results yielded a high percentage of false-negative or false-positive results for all indicator-pathogen combinations. The relationship of indicators with pathogens that were detected more frequently, such as *Giardia*, tended to show a greater frequency of false negatives (indicators absent but pathogens present). The relationship of indicators with pathogens that were less frequently detected, such as enteric viruses and infectious *Cryptosporidium*, generally showed a higher frequency of false-positives (indicators present but pathogens absent). False-positive results are undesirable because they represent â€œfalse alarms.â€□ An indicator that is frequently present in the absence of pathogens, such as total coliforms in this study, is not very informative as to the true risk to human health but is relatively conservative in terms of human health protection. False negatives, on the other hand, suggest that probable human health risks are not being detected, which certainly compromises efforts to protect public health. This study suggests that

choosing one indicator to predict the survival and/or occurrence of a wide variety of microbial pathogens forces a choice between the two types of error. Although individual indicator organisms and pathogens were weakly correlated or uncorrelated, the use of discriminant analysis on the composite data set resulted in the relatively accurate prediction of the presence or absence of enteric viruses, *Giardia*, *Cryptosporidium* oocysts, and infectious *Cryptosporidium*. With the exception of *Giardia*, errors tended to be false negatives, as the absence of enteric viruses and *Cryptosporidium* was more accurately predicted than their presence. Further analysis of larger data sets and other indicators, perhaps coupled with measurement of key pathogens, may allow us to refine the predictive capabilities demonstrated by this multivariate analysis. Such a monitoring strategy should protect public health better than the one-indicator system currently used.

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commentletters@waterboards.ca.gov

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From: edo_mcgowan@hotmail.com
To: commentletters@waterboards.ca.gov; layne@lawyersforcleanwater.com; drev@lawyersforcleanwater.com; eerickson@waterboards.ca.gov; katherine@venturahydraulics.com; jeffrey.stone@cdph.ca.gov; jstone1@dhs.ca.gov; woody.maxwell@venturausd.org
Subject: Comment Letter â€" proposed Water Recycling Policy, addendum to prior submissions
Date: Thu, 25 Oct 2007 23:29:11 +0000

**To: Ms Townsend, SWRQB**
**Fm: Dr E McGowan**
**Re: Addendum to prior Comment Letter â€" proposed Water Recycling Policy**

**As an example of materials that may slip through the process of making Title 22, the following is submitted. Again, it is doubtful that the state has considered the eventuality of such a situation. Consequently, this is something that an EIR would address and thus again demonstrates the need for an EIR**

# Antiviral Oseltamivir Is not Removed or Degraded in Normal Sewage Water Treatment: Implications for Development of Resistance by Influenza A Virus

Jerker Fick[1*], Richard H. Lindberg[1], Mats Tysklind[1], Paul D. Haemig[2], Jonas WaldenstrÃ¶m[2,3], Anders Wallensten[4,5], BjÃ¶rn Olsen[2,6]
**1** Department of Chemistry, UmeÃ¥ University, UmeÃ¥, Sweden, **2** Section for Zoonotic Ecology and Epidemiology, Kalmar University, Kalmar, Sweden, **3** Department of Animal Ecology, Lund University, Lund, Sweden, **4** Smedby Health Center, Kalmar County Council, Kalmar, Sweden, **5** Division of Virology, Department of Molecular and Clinical Medicine (IMK) Faculty of Health Sciences, LinkÃ¶ping University, LinkÃ¶ping, Sweden, **6** Section of Infectious Diseases, Department of Clinical Sciences, Uppsala University Hospital, Uppsala, Sweden

# Abstract

Oseltamivir is the main antiviral for treatment and prevention of pandemic influenza. The increase in oseltamivir resistance reported recently has therefore sparked a debate on how to use oseltamivir in non pandemic influenza and the risks associated with wide spread use during a pandemic. Several questions have been asked about the fate of oseltamivir in the sewage treatment plants and in the environment. We have assessed the fate of oseltamivir and discuss the implications of environmental residues of oseltamivir regarding the occurrence of resistance. A series of batch experiments that simulated normal sewage treatment with oseltamivir present was conducted and the UV-spectra of oseltamivir were recorded. Findings: Our experiments show that the active moiety of oseltamivir is not removed in normal sewage water treatments and is not degraded substantially by UV light radiation, and that the active substance is released in waste water leaving the plant. Our conclusion is that a ubiquitous use of oseltamivir may result in selection pressures in the environment that favor development of drug-resistance.
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**Competing interests:** The authors have declared that no competing interests exist.
* To whom correspondence should be addressed. E-mail: jerker.fick@chem.umu.se

## Introduction

Influenza A virus is a zoonotic pathogen with a large environmental reservoir in anatids, especially dabbling ducks [1], which also infects a number of mammals, including pigs, horses, seals and canines [2]. Over the past centuries, the virus has been transmitted to humans on several occasions, causing flu pandemics and seasonal epidemic influenzas [3]. At present, there are only few antiviral compounds available to treat human influenza. The most important, oseltamivir, or oseltamivir phosphate (OP), is a prodrug that is extensively metabolized (>75%) in the human liver to oseltamivir carboxylate (OC), the active moiety (Figure 1) [4]. OC is not metabolized further and is excreted unchanged [4]. Oseltamivir is widely used for treatment of seasonal flu and is considered an important first-line defense in the event of a future influenza pandemic [5], [6]. This compound is a neuraminidase inhibitor, which mimics the natural sialic acid substrate and binds to the active site, preventing the viral neuraminidase protein from cleaving host-cell receptors, thereby interfering with the release of new virus particles from infected cells [5]. To investigate whether or not oseltamivir is removed in normal sewage water treatment, we set up and ran batch experiments that simulated normal sewage treatment with oseltamivir present. In these experiments, we used OC, since this is the active moiety and also the molecule excreted by patients.

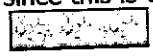

**Figure 1. Oseltamivir phosphate (OP), oseltamivir carboxylate (OC), the internal standard (IS) used, and deuterated oseltamivir carboxylate ($OC_{D3}$).**

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A conventional sewage treatment plant functions in three steps: (1) mechanical treatment, (2) chemical treatment and (3) biological (activated sludge) treatment. In the mechanical treatment phase, raw sewage water passes through a grid that first removes large objects, then lipids and sand. Chemical treatment subsequently reduces nutrients, such as phosphorus, in the aqueous phase by addition of $FeCl_3$ or $FeSO_4$, while biological treatment reduces organic content. The sludge produced is removed in the clarifiers following each step and treated further with different techniques. The treated water is then released and diluted into receiving water courses.

## Methods

## Experimental design

In our experiments we used three different water solutions, each representing one of the three phases in the conventional sewage treatment process outlined in the previous paragraph: (1) raw sewage water, (2) water from combined mechanical/chemical treatment, and (3) water from activated sludge treatment. All three water solutions were collected in one liter bottles as grab samples during two days in June 2006 at UmeÃ¥ Sewage Treatment Plant, (for a detailed description of the plant, see references [7], [8]. During the two days of collecting water, normal conditions were reported for water treatment, with some minor rainfall during the second day. To avoid misinterpretation of the results, quantification of OC in all the raw sewage water samples was made and additional batch experiments (3 Â½ h) conducted using tap water to assess possible OC degradation or adsorption to glass walls.

All batch experiments started within 1 hour of water collection and were conducted as follows: 200 ng of OC, was added to 200 mL of each type of water and gently stirred in an open 500 mL flask at 20Â°C. The duration of each experiment was determined by the hydraulic retention time in the plant: (1) raw sewage water, 2 Â½ h (the assumed time the water spends in the sewage line upon reaching the sewage treatment plant); (2) water from mechanical/chemical treatment, 1 Â½ h; and (3) water from activated sludge treatment, 3 Â½ h. This approach has been used previously with relevant and reproducible results [9]. When the hydraulic retention time was reached, the sample was immediately subjected to solid phase extraction (SPE) and liquid chromatography electrospray tandem mass spectrometry (LC-ESI-MS/MS). Each experiment was conducted in triplicate for each water solution type to assess day-to-day variation of the sewage water treatment process and experiments: (day 1) n = 1; and (day 2) n = 2. The amount of suspended solids (SS) and pH of the various water samples included in the batch experiments can be seen in Table 1.

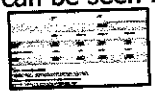

**Table 1. pH and suspended solids (SS) of the water included in the batch experiments**

doi:10.1371/journal.pone.0000986.t001

## Chemicals

Oseltamivir carboxylate (OC), (RO0640802-002; lot: 01007B243804) and Oseltamivir carboxylate labelled with deuterium ($OC_{D3}$), (RO0604802-004; lot: 511-001-2197/4) were obtained from Roche (F. Hoffmann-La Roche Ltd, Basel, Switzerland). Formic acid, ammonium hydroxide 25% and methanol (HPLC-grade) were purchased from JT Baker (Deventer, the Netherlands), acetonitrile (HPLC-grade) from Fischer Chemicals (Zurich, Switzerland) and sulphuric acid from Merck (Darmstadt, Germany). The purified water (resistivity, 18.2 MÎ© cm) was prepared by an ELGA MAXIMA HPLC ultra pure water system (ELGA, High Wycombe Bucks, England), equipped with a UV radiation source. Buffer solutions pH 5 (citric acid/sodium hydroxid) and pH 9 (boric acid/potassium chloride/sodium hydroxide) were bought from Merck (Darmstadt, Germany) and were of ceripure grade. Buffer solution pH 7 (phosphate) was purchased from JT Baker (Deventer, the Netherlands) and was of â€œBaker Analyzedâ€□ grade. Standard stock solutions of OC and $OC_{D3}$, 100 ng $mL^{â^'1}$, were prepared in water (10 mL) and kept at 4 Â°C.

## Solid phase extraction and quantification of OC in sewage water

Sewage water samples were filtered through 0.45 Âµm MFâ„¢-membrane filters (Millipore, Sundbyberg, Sweden) before acidification to pH 3 using sulphuric acid. This low pH ensured a high recovery of the amphoteric OC. 700 ng of the deuterated internal standard $OC_{D3}$ was added to each sample and 200 mL aliquot of each sample was withdrawn and subjected to extraction. The Strata-X-C (200 mg, 6 mL) mixed mode cation exchange sorbent (Phenomenex, email: internationalphenomenex.com) used for the solid phase extraction (SPE) was conditioned and equilibrated by 2.0 ml of methanol and 2.0 ml of deionized water. The samples were applied to the SPE columns at a flow rate of 5 mL $min^{â^'1}$. Impurities were removed by 2.0 ml 0.1% sulphuric acid and the sorbents dried with air during 1 min at 10â€□ Hg. Supposed neutral and acidic components were removed by 2 mL of methanol and wasted, followed by elution of the analytes by 2 mL of 5% $NH_4OH$ in methanol collected with 10 ml glass vials. The eluates were evaporated to approximately 20 Âµl using air and then reconstituted in acetonitrile in water (1:1), containing 0.1% formic acid, to a final extract volume of 1.0 ml. Quantification of OC was performed by internal *standard calibration by comparison of* area ratios $OC/OC_{D3}$ in sample extracts and calibration solution.

## Liquid chromatography electrospray tandem mass spectrometry

A 10 Âµl aliquot of sample extracts and calibration solutions was injected into a YMC Hydrosphere C18 analytical column, 150Ã—4.6 mm i.d., 5 Âµm particle size, (YMC Inc., Wilmington, NC, US) following a 10Ã—4 mm i.d., 5 Âµm particle size, YMC Hydrosphere C18 guard column using an AS 3000 autoinjector (Thermo Finnigan, San Jose, CA, US). OC and $OC_{D3}$ were chromatographically separated during 5 min by 50% $H_20$ balanced with acetonitrile, both containing 0.1% (v/v) formic acid, at a flow rate of 0.8 ml $min^{â^'1}$ generated by a P4000 HPLC pump (Spectra system, Thermo Finnigan) at 25Â°C.
An LCQ Duo ion trap mass spectrometer (Thermo Finnigan) was used together with an electrospray ion source in positive ion mode. The source voltage was maintained at a constant 6.0 kV and the heated capillary temperature set to 250Â°C. The MS/MS parameters were optimised semi-automatically for the analytes using LCQ Duo internal software whilst the collision energy, to produce daughter ions, was manually optimised.
For OC and $OC_{D3}$ parent and daughter ion *m/z*, collision energy, retention time, extraction yields in tap water and raw sewage water and LOQ, see Table 2.

**Table 2. LC-ESI-MS/MS parameters and results of the method validation.**

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The *m/z* obtained for the OC and $OC_{D3}$ parent and daughter ions are consistent with those reported by Wiltshere et al. [10] who also identified that the resultant daughter ion ($M^+$ -88 amu) had lost the pentyloxy sidechain. The retention time of the deuterated internal standard, $OC_{D3}$, was analogous to OC, and the linearity of the calibration curve was above 0.99. Memory effects during LC-ESI-MS/MS were not observed. The extraction yields OC with the mixed mode cation exchange sorbent were in most cases close to 100%, regardless of matrix subjected to extraction, and the precision was acceptable with RSD below 21%. Breakthrough effects were not observed at any level of sample load volume used, and sequential elutions (3Ã—2 mL) with MeOH or $NH_4OH$ did not contain OC above LOQ. Extraction yields of the internal standard $OC_{D3}$ were not determined but assumed to be analogous to OC due to their similarity in physico/chemical properties.

## Method validation

Extraction yields of OC were assessed by fortification experiments. 1000 ng of OC was added to the following matrices prior to extraction with the method presented above (in mL): tap water-10, 100 and 500; and raw sewage water (filtered through 0.45 Âµm)-10, 100, 200 (n = 3) and 500. $OC_{D3}$, 700 ng, were added to the reconstituted extracts (1 mL during these experiments) prior to injection on the LC-ESI-MS/MS. The extraction yields were evaluated by comparison of LC-ESI-MS/MS peak area ratios of $OC/OC_{D3}$ in sample extracts against a calibrate solution of 1000 ng $mL^{â^'1}$ of OC in 1 mL acetonitrile in water (1:1), 0.1% formic acid. Evaluation of matrix effects during LC-ESI-MS/MS was assessed by comparison of the $OC_{D3}$ peak area in chromatograms of the calibrate solution and the sample extracts of the following degree of enrichment during SPE: 0, 10, 100, 200, and 500 times.
An internal standard calibration curve of eight levels, 1â€“1000 ng $mL^{â^'1}$, of OC was injected into the LC-ESI-MS/MS. The limit of quantification (LOQ) was evaluated by using ten times the signal to noise ratio of OC, 1000 ng $mL^{â^'1}$ in sample extract (enriched 200 times).
Blank samples of tap water and raw sewage water subjected to SPE and acetonitrile in water (1:1), containing 0.1% formic acid, were regularly injected into the LC-ESI-MS/MS to control and reduce potential memory effects.

## Ultraviolet absorption spectra

Standard solutions of OC ($10^{â^'3}$ M, 284,35 mg $l^{â^'1}$) were prepared in buffer solutions with pH 5, 7 and 9. UV-spectra were recorded on a UV-VIS-NIR scanning spectrophotometer (UV-3101PC, Shimadzu), which was set to scan over 250â€“800 nm. To correct for differences in cell performance, a baseline correction was made with corresponding buffer solution in both sample and reference cells.

## Results

No OC was detected in the raw sewage water and no losses observed in the batch experiment using tap water, which minimizes the possibilities of positive or negative sampling artefacts. OC and $OC_{D3}$ were readily affected by matrix components in the raw sewage water (Figure S1). However, the combination of a high recovery of OC during solid phase extraction of sewage water, and the use of a deuterated internal standard with almost identical physico-chemical properties, makes the developed analytical methodology very suitable for environmental monitoring in various aqueous matrices.

Removal of OC due to degradation and/or sorption to sludge was not observed in the batch experiments. The day-to-day variation in terms of batch experiment and treatment process seemed to be minor, and all results are close to a 100% recovery of the added OC in the aqueous phase (Figure 2). Recoveries were 107% (S.D. 19) in the raw sewage water, 126% (S.D. 8) in the water from combined mechanical/chemical treatment, and 125% (S.D. 27) in the water from activated sludge treatment.

**Figure 2. Results of batch experiments to assess removal of oseltamivir carboxylate (OC) from the aqueous phase during conventional sewage water treatment.**

Shown is the OC remaining in the aqueous phase after the batch experiments. Abbreviations: RSW, raw sewage water; mech/chem., mechanical and chemical treatment of sewage water; and AST, activated sludge treatment of sewage water. White and grey bars represent day 1 and day 2 of the batch experiments, respectively.

doi:10.1371/journal.pone.0000986.g002

These findings suggest that, since OC is not removed during sewage treatment, it will enter local aquatic environments in areas where oseltamivir is prescribed to patients for therapeutic use.

A way to estimate the levels in the aquatic environment is to calculate the highest predicted environmental concentration (PEC) according to,

$$\mathbf{PEC}(\mu g/l) = \frac{\mathbf{A} \times (100 - \mathbf{R})}{365 \times \mathbf{P} \times \mathbf{V} \times \mathbf{D} \times 100}$$

where A is the total actual pharmaceutical sales (Âµg year$^{â^{'1}}$), R the removal rate due to loss by adsorption to sludge particles, volatilization, hydrolysis or biodegradation (%), P the human population (number of individuals), V the volume of wastewater per capita per day (l day$^{â^{'1}}$) and D a factor for dilution of waste water by surface water flow.

The country where oseltamivir is used most is Japan [11], Roche estimates that 6 million of the 16 million Japanese individuals infected by an influenza virus during the influenza season 2004/2005 received oseltamivir [12]. Environmental concentrations should therefore be the highest in Japan, with the calculated PEC value equal to $PEC_{surfacewater\_Japan}$ = 0.028 Âµg l$^{â^{'1}}$ where A = 2650 kg (Estimated volume during flu season 2004/2005)[12] (Estimation is based on 30% pediatric dosage, 70% adult dosage and OP converted to OC), R = 0 (No removal), P = 127 417 244 (2005), V = 200 (Default) and D = 10 (Default). This calculation provides a national annual average and does not consider local factors such as catchment size, population density or flow rates. Another factor not included is seasonal consumption, such as increased usage during flu season. This PEC level can be compared to the $IC_{50}$ (concentration that causes 50% inhibition) of OC, which depends heavily on type of virus and exposure system, but such low levels as 0.28â€"0.81 nM ($IC_{50}$) have been reported [11], [13]. This corresponds to a concentration of 0.08â€"0.23 Âµg L$^{â^{'1}}$, i.e. a concentration of the same magnitude as the calculated PEC value. Singer et al. [14] estimated the environmental levels of OC during treatment and prevention of a pandemic influenza in Europe and North America. Estimations showed that environmental levels would differ significantly between different catchments and maximum concentrations would range from<0,3 Âµg L$^{â^{'1}}$ to 32 Âµg L$^{â^{'1}}$. Predicted levels in Japan during the flu season are thus comparable to predicted levels in some catchment areas during treatment and prevention of a pandemic.

Incidentally, Japan also has high rate of emerging resistance to oseltamivir [15]. Kiso et al. [16] reported that 18% (9 patients) of the influenza A virus in children had mutations that made them 300â€"$10^5$ times more resistant to oseltamivir.

Once a pharmaceutical enters the aquatic environment, photochemical degradation represents another possible degradation pathway [17]. However, the UV-spectra of OC show no absorbance in the interval 295â€"700 nm, which excludes direct aqueous photolysis as a major degradation pathway. Only chemicals that are able to adsorb solar radiation can be degraded and solar radiation in wavelengths shorter than 290â€"300 nm does not reach the Earth's surface, see Figure 3, and radiation with wavelengths>700 nm do not contain enough energy to break bonds within the molecules.

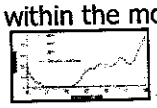

**Figure 3. UV-spectra of oseltamivir carboxylate, at pH 5, 7, 9 and atmospheric transmission at sea level (expressed on a scale of 0 to 1).**

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Pharmaceuticals in the aquatic environment are primarily removed by three degradation pathways: sorption, biodegradation and photolysis. Our experimental results show that OC is not readily degraded by any of these three, which implies that OC is released into aquatic environments at varying concentrations and is not readily removed. Thus, ducks, poultry and humans, or their excretions carrying influenza viruses, may encounter water contaminated with oseltamivir. Under these circumstances, where influenza viruses come in contact with low concentrations of the drug, the stage is set for the evolution of oseltamivir-resistant influenza strains [1].

## Discussion

The problem of environmental contamination becomes even more serious when one considers the ecology of influenza A virus. The life cycle of influenza A virus is intrinsically linked to water where they can remain active for extended periods; and under cold conditions for months [18]â€"[20]. Most subtypes circulate in wild ducks [1] that become infected by contaminated water establish infection in the gastrointestinal tract, where the virus multiplies and is excreted in large numbers via the faeces [2], [21]. Thus, both the infection and the pharmacological effect of OC occur in the gastrointestinal tract. Due to the poor bioavailability of OC [4] it is therefore theoretically possible that exposed ducks have OC at levels close to the $IC_{50}$ in the gastrointestinal tract and that this could promote a selection process towards drug-resistance. Singer and coworkers recently stated this was a potential risk during treatment and prevention of a pandemic influenza [14], and our calculations imply that this can also be a potential risk in Japan today.

In some localities, wild ducks, domesticated ducks, poultry and humans all live in close proximity, transmit influenza viruses to each other, and conceivably ingest low concentrations of OC in treated or untreated sewage water. The water outside a sewage plant may comprise a particularly high risk microhabitat where ducks carrying a multitude of influenza virus strains encounter low levels of oseltamivir. This is particularly so because large numbers of ducks often gather in the warm nutrient-rich waters leaving sewage treatment plants, especially in cold climates where this warmer water remains ice-free year-round.

In some parts of the world, chicken manure is used as fertilizer in fish farming, a practice that can increase the spread of avian influenza [22]. During an outbreak of avian influenza in poultry, this activity could expose ducks, and other animals frequenting fish ponds downstream from sewage outlets, to highly pathogenic influenza virus strains from the chickens and low concentrations of OC from sewage water

Studies have shown that most oseltamivir resistant strains detected so far, have been detected in patients not treated with oseltamivir [11], [23]. It remains to be seen if such resistant strains are transmitted from treated individuals, the result of natural variation in the absence of oseltamivir altogether, or due to the selective pressure of low doses of oseltamivir in the environment. Previous research has, however, shown that it is quite easy for influenza A virus to develop resistance to oseltamivir [24], [25]. For example, a single amino acid substitution, from histidine to tyrosine at position 274 (N2 numbering system; N2 numbering is used throughout this article) of the neuraminidase gene â€œconvertedâ€□ an oseltamivir sensitive H5N1 influenza A virus into a resistant strain, with about a 400â€"600-fold higher resistance to OC [25]. Most resistant influenza A virus have mutations in the neuraminidase gene leading to amino acid substitutions predominantly at positions 119, 152, 274 and 292 of the enzyme's active site [5]. All the resistant variants thus far have contained specific mutations in the neuraminidase molecule; but since neuraminidase serves an essential purpose, mutations that allow the virus to â€œsurviveâ€□ must not inactivate the enzyme [15]. Carr et al. [26] showed, for example, that mutations at position 292 compromised viral fitness to such extent that it was considered to have no clinical consequences. However, an experimental study in ferrets [27] has shown that mutations at position 119 do not compromise viral fitness. The authors state that â€œif such viruses are transmitted, it is uncertain whether, over time, they could predominate over susceptible strainsâ€□ [27].

In conclusion, our experimental results, theoretical calculations and hypothesis imply the possibility that ubiquitous use of oseltamivir may result in selection pressures in the environment that favor development of drug-resistance. This raises the all-important question as to whether or not such a risk should be taken, or if a more restricted use of these agents should be advocated? This is an opinion shared by other researchers [28], and we would like to add that the effects of pharmaceuticals continuously released into the environment should not be underestimated and certainly investigated carefully before widespread use of a drug is encouraged.

## Supporting Information

**Figure S1.**

Peak area of OCD3 in calibrate solution and in raw sewage water (RSW) extracts, as a function of enrichment 10, 100, 200, and, 500 times during SPE.
(0.54 MB TIF)

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**Author Contributions**

Conceived and designed the experiments: BO RL MT. Performed the experiments: JF RL. Analyzed the data: JF RL. Wrote the paper: BO AW JW JF RL MT PH.

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