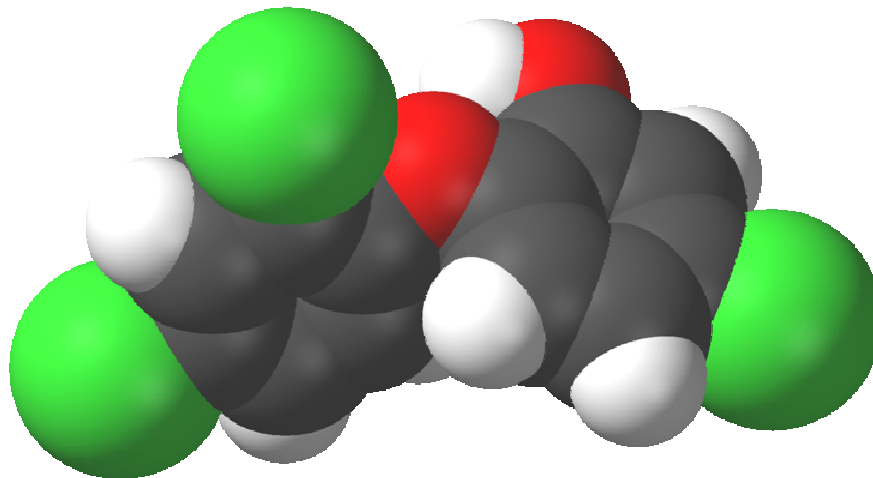


COMMON PHARMACEUTICALS in WASTE WATER
MAY NOT BE COMPLETELY REMEDIED BY
EXISTING TREATMENT FACILITIES
(TRICLOSAN and 17- β -ESTRADIOL, San Juan County, WA)



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Summary

Many residents of the San Juan Islands have recently questioned whether widely used prescription pharmaceuticals such as antibiotics, anti-depression drugs, medications for cardiovascular management, or hormone supplements, are removed from wastewater by existing on-site septic systems and sewage treatment facilities. San Juan County has implemented a take-back program for unused prescriptions to discourage disposal down toilets, sinks and drains. Medications are incompletely absorbed through the human gut, however, and many are excreted without modification of their molecular structure. Much of the medication we consume can be found in urine or in the fatty fraction of feces. We could find no data on the concentrations of pharmaceuticals in the islands' marine waters, or tests of the efficiency of local sewage treatment facilities in reducing drug loads.

To explore these issues, we acquired influent and effluent samples from the three sewage treatment plants in San Juan County, and tested them for two representative drugs using ELISA (enzyme-linked immunosorbent assay), which can be sensitive to parts-per-billion and, for some analytes, parts-per-trillion. We selected Triclosan (*aka* Irgasan), the most widely used over-the-counter antimicrobial in the United States, which is added to a broad range of soaps, shampoos, hand sanitizers, even baby clothing; and 17- β -Estradiol, a human steroid hormone, widely prescribed as a hormone supplement and contraceptive. We reasoned that these compounds should be among the most heavily consumed in local homes, and consequently be more concentrated in wastewater than other pharmaceuticals. Failure to detect significant concentrations of Triclosan or 17- β -Estradiol in sewage plant effluent would suggest that the concentrations of most or all other drugs is insignificant. At the same time, both Triclosan and 17- β -Estradiol are endocrine-disrupting compounds (EDCs) in the marine environment, with biological impacts at concentrations of one part-per-billion or less.

We found considerable differences in the influent concentrations and the remedial efficiency of island sewage treatment facilities. Between 26 and 96 percent of Triclosan was removed. Effluent concentrations were all on the order of 0.1 part-per-billion, which is below the level currently regarded as a threat, however. Influent and effluent Estradiol were generally one order of magnitude lower, confounded by adsorption to silt or sludge. While island treatment plants did not remove these compounds completely from sewage, then, they reduced concentrations to levels that are acceptable for now. Future laboratory studies of chronic exposure to EDCs may find that even 0.1 part-per-billion is a concern.

We also tested bacteria in influent wastewater for resistance to Triclosan and three common antibiotics (Ampicillin, Kanamycin, Tetracycline). Nearly all bacterial colonies were resistant to one or more antibiotics. One in seven were resistant to Triclosan.

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There is growing evidence of pharmaceutical contamination of wastewater at low but biologically effective concentrations, including human and veterinary drugs, natural and synthetic hormones, antibiotics, and antiseptics (Buxton and Kolpin 2002). Limited ability of existing wastewater treatment facilities to degrade or sequester these bioactive compounds is indicated by the fact that pharmaceuticals can be detected in natural waters downstream of urban areas where most if not all human waste has already been treated in some kind of central wastewater processing facilities.

Ecotoxicology data remain limited for most human drugs and hormones (Fent et al. 2006; Kummerer 2009). It is reasonable to assume that such compounds can affect the physiology of animals other than humans, however. Extremely low concentrations of hormones carry information to specialized receptors in our bodies from minute to minute, suggesting that equally low concentrations of hormones in aquatic ecosystems would be sufficient to affect molecular information pathways in aquatic animals such as fish and amphibians. Pharmaceuticals tend to resist conventional wastewater treatment methods (Stackelberg et al. 2004) and can be quite mobile in groundwater and resistant to bacterial metabolism (Heberer 2002). In some cases, partial metabolism of these molecules results in even more toxic configurations.

The most obvious vector for pharmaceuticals in wastewater is disposal of expired or unused medications down household toilets. But drugs are also regularly excreted in urine and faeces. Since the rate of uptake of drugs through the human gut is relatively low, drugs are prescribed at concentrations significantly greater than their target effective concentrations in blood. The surplus is excreted without being metabolized.

Most existing literature on drugs in wastewater were conducted in urban settings, where total pharmaceutical inputs to local waters would be expected to be considerable. Are pharmaceutical residues also a concern for relatively small, rural-residential settings such as the San Juan Islands? One factor may be the graying demography of the islands, which is probably associated with relatively high per capita use of medications. Another factor is discharge of the three islands' existing wastewater treatment plants directly into the marine environment, for example (at Lopez) into a significant sub-tidal eelgrass bed.

For the present study two "marker" compounds were selected for their widespread use, relative persistence, and ease of measurement at part-per-trillion levels with ELISA immunoassay techniques. Failure to detect these compounds in waste treatment effluent would render it unlikely that other pharmaceuticals are reaching the marine environment in appreciable concentrations.

17- β -estradiol is the most widely used estrogen in human medicine, and stands-in here for a variety of human hormones used as drugs. Because of its known bioactivity it has been the subject of considerable study in wastewater and sewage treatment facilities.

Naturally excreted by humans and other mammals at very low levels, estradiol loading is augmented in sewage by its widespread use for birth control. It is more likely to be found in measurable quantities in sewage than many other endocrine disrupting compounds, and so is a useful analyte for testing the efficiency of sewage treatment methods.

Naturally occurring bacteria can metabolize 17- β -estradiol completely under both aerobic and anaerobic conditions (Lee & Liu 2001). Efficiency of estradiol removal may vary considerably depending on the length of time that solids are retained in a wastewater treatment system and the methods used to strain solids from effluent (Khanal et al. 2006). Removal rates reported in the literature range from 0 to 90 percent in different treatment plants; moreover, estrogens may be retained in treatment sludge that is later released into the environment through aquatic discharge or agricultural use as manure (Comnalbert and Hernandez-Raquet 2010). Since estrogens can affect organisms at part-per-trillion levels, incomplete treatment can leave small but environmentally significant levels of estrogens in wastewater.

Triclosan is a chlorinated cyclic hydrocarbon widely added to consumer products as an antimicrobial agent. It can be found in soaps, deodorants, toothpaste, and lotions as well as clothing, bedding, and toys. At high concentrations triclosan can kill the bacteria in activated sludge, shutting down wastewater treatment plants. At lower concentrations it can have downstream toxic effects in aquatic habitats, with levels of biological effects ranging from as little as 500 parts-per-trillion for some algae to 500 parts-per-billion for some animals (Orvos et al. 2002; Capdevielle et al 2008). Triclosan is bioaccumulative, and like bisphenol-A, regarded as an endocrine disrupting compound in mammals (Crofton et al. 2007) and amphibians (Veldhoen et al. 2006).

Naturally occurring bacteria can degrade triclosan in aerated soils and waters over a period of months (McAvoy et al. 2002; Ying et al. 2007), but photolytic degradation of triclosan in water produces another chemical species of concern: 2,8 dichlorodibenzo-*p*-dioxin (DCDD), which is more stable than triclosan, especially in seawater (Aranami and Readman 2007). In activated sludge systems, triclosan may adsorb to solids (McAvoy et al. 2002), sequestered rather than degraded.

A complementary concern about triclosan in sewage is the potential for producing drug-resistant bacteria and discharging them into aquatic environments where they may infect humans. Triclosan appears to select broadly for hardiness. Some bacteria exposed to it not only develop triclosan tolerance, but are also more likely to develop resistance to a wide range of prescription antibiotics (Levy 2001). Triclosan loads too small to affect aquatic animals directly, may nonetheless promote hardier bacteria that pose a greater threat of infection to animals, including humans, through direct exposure (diving or swimming), or through the consumption of incompletely cooked fish or shellfish.

Testing effluent bacteria for drug resistance is less costly than measuring levels of antibiotics in wastewater: resistance is an indication that bacterial populations have been exposed to significant levels of drugs. Depending on the method of filtration of effluent, resistant strains may moreover be released continually into the environment. Very low concentrations of prescription antibiotics in wastewater can select for resistant bacterial strains in waste treatment facilities' sludge tanks, but pose little threat to human or animal health if retained in biosolids that are subsequently entombed in landfills or sterilized.

Methods

Samples of unfiltered influent and effluent were obtained from the Friday Harbor, Eastsound, and Lopez Village sewage treatment plants. Samples were drawn by sewage treatment personnel in Nalgene bottles, and immediately refrigerated. Sub-samples were drawn and filtered through 0.45-micron Acrodisc PTFE within 48 hours, and refrigerated in sterile pre-cleaned borosilicate vials for no more than 72 hours prior to use.

Estimation of contaminant loads was by enzyme-linked immunosorbent assays (or ELISA) using antibodies, standards, and controls supplied by Abraxis LLC (Warminster, PA) in a magnetic-particle format. Proprietary antibodies bound to ferrous particles are exposed to small aliquots of standards, controls, and samples and incubated. An enzyme-linked conjugate of the target analyte is then added to react with any antibodies that have not already reacted with target analyte in the standards, controls, or samples. A magnetic rack is used to separate the ferrous particles so that they can be washed, leaving only the bonded antibodies, and whatever target analyte or enzyme conjugate has reacted with the antibodies. A reagent is added to release chromophores from the conjugate molecules, so that the degree of color development, measured by visual spectrophotometry at 450 nm, is proportional to the ratio of conjugate to target analyte reacted with the antibody-coated particles exposed to each standard, control, and sample. Color development is therefore inversely proportional to the original concentration of the target analyte in each standard, control, and sample. Each batch of samples tested requires five different standards and controls for accurate calibration of results.

We processed two replicates of each standard, control and sample simultaneously. If results for both replicates were consistent—that is, within a 10 percent acceptable error window—they were combined and averaged. Any result that fell outside of this window was presumed to represent processing or measurement errors and was rejected.

Two of the three sewage treatment plants included in this study provided multiple influent and effluent samples from different holding and discharge tanks. Results for the multiple samples were consistent with each other, and have been averaged in this report.

Nominal limits of detection (LOD) are 25 parts-per-trillion (ppt) for triclosan and 2.5 parts-per-trillion (ppt) for 17- β -estradiol. The manufacturer of the antibodies reports no significant cross-reactions with non-target compounds.

Drug resistance is easily tested by streaking bacteria on nutrient media to which antibiotics or antimicrobial compounds have been added: only resistant strains, if present, will grow. We harvested bacteria from Eastsound sewage treatment plant influent, and cultured them initially on either untreated Luria Broth (LB), or LB doped with 1500 ppb triclosan. Colonies were then transferred to petri dishes of LB doped with either 150 ppb triclosan, 50 ppb ampicillin (a widely used broad spectrum antibiotic), 50 ppb kanamycin (a more recently developed antibiotic), or 15 ppb tetracycline (once widely used, now restricted due to side effects) and incubated. After incubation, each petri dish was scored for numbers of visible colonies.

As positive controls, we used colonies of *Escheria coli* OP50, a laboratory strain of bacteria with no known drug resistance; and as negative controls, nutrient dishes with no bacteria introduced, to ensure that our media, tools and drugs were initially sterile.

Results

Mean test results are shown in Table 1. Triclosan inputs varied considerably with the largest measured influent concentrations at Eastsound. Eastsound also appeared more successful at removing triclosan from wastewater. Residual levels of triclosan discharged into the environment, on the order of 100 parts-per-trillion, fell below the part-per-billion levels currently regarded as threats to aquatic organisms. However, some of the triclosan that disappeared during treatment probably degraded to DCDD, which was not measured independently. DCDD loads on the order of 1-2 parts-per-billion would be a concern.

Table 1: ELISA results in parts-per-trillion

<i>Analyte</i>	<i>Treatment plant</i>	<i>Influent</i>	<i>Effluent</i>	<i>% Change</i>
Triclosan	Lopez	588	110	-81
	Friday Harbor	209	155	-26
	Eastsound	1750	63	-96
17-β-Estradiol	Lopez	11.5	11.2	
	Friday Harbor	11.9	25.1	
	Eastsound (water)	2.7	5.2	
	Eastsound (solids)	21.0	2.2	-90

Estradiol effluent loads were the same *or greater* than influent loads, which seems counterintuitive. Estradiol and other steroidal hormones are lipophilic and adsorb to silt (Khanal et al. 2006). Filtered influent may underestimate estradiol because a significant portion of the analyte remains in the filter with particles. In that case, actual input levels could be several times greater than what was measured in the filtrate, and estradiol could accumulate in treatment plant sludge. Long residence in sludge may eventually degrade most estrogens, while flushing or cleaning of sludge tanks could briefly release estrogens into the environment.

To investigate the possibility that most estradiol is silt-bound, we re-tested both of the Eastsound samples after first diluting them 1:1 with Pestanal®-grade methanol, which should extract silt-bound lipophilic molecules, then filtering out particles over 0.45 μm in size. After extraction in methanol, Eastsound influent contained 21.0 parts-per-trillion of estradiol, which is to say nearly eight times the concentration previously measured. This indicates that roughly 87 percent of the estradiol in the sample had been adsorbed to silt. After extraction in methanol, Eastsound effluent contained 2.2 parts-per-trillion estradiol. This is close to the limit of detection for the ELISA method we used, and close to the 5.2 parts-per-trillion previously measured. It suggests that some degradation of estradiol may have occurred in the days between initial and follow-up testing of the Eastsound samples.

Drug resistance results are summarized in Table 2. Bacteria initially challenged with triclosan remained relatively resistant to this antimicrobial after a second exposure.

Table 2: Drug resistance in influent bacterial strains

		Percent of bacterial colonies later exhibiting resistance to:			
<i>Initial condition</i>	N	Triclosan	Ampicillin	Kanamycin	Tetracycline
Luria broth only	14	14	86	21	0
Triclosan added	20	35	55	5	5
	34	26	68	12	3

Of the triclosan-resistant bacterial colonies in the first data column in Table 2, 89 percent were *also* resistant to ampicillin, 22 percent were *also* resistant to kanamycin, and 11 percent (a single bacterial colony) were *also* resistant to tetracycline; 11 percent (a single colony) were resistant to *all* of the antibiotics. Only a single bacterial colony could be cultured from the Eastsound effluent, and it exhibited no antibiotic resistance.

Recommendations

It would be advisable to repeat these measurements on a large number of samples collected over several months of each treatment plant's operation, to determine whether the results shown in Table 1 are consistent over a wider range of operating conditions. We also recommend that each sample be divided into solids and liquid, and that the solids be extracted in methanol prior to testing.

Our results will best be understood in the context of differences in the design and operation of the three sewage treatment plants included in this exploratory study. Sludge adsorption is a major factor in the removal of estrogen from wastewater while breakdown of triclosan to DCDD is facilitated by exposure to light. Tank design, mixing and settling times, aeration and filtration can affect the fates of the analytes we studied. Degradation of triclosan to DCDD would also mean that the effluent triclosan loads in Table 1 may be misleadingly low.

Effluent concentrations of triclosan and 17- β -estradiol observed in this study were small but at threshold levels of concern. With a growing and aging human population in the islands, waste treatment facilities will face increasing influent concentrations of drugs that may reduce their biodegradation efficiency and even overwhelm the capacity of their bacterial sludge to survive over long periods. A two- to three-fold increase in the current influent concentrations of triclosan and estradiol would bring these compounds to known levels of biological effects on algae and fish.

It must be borne in mind that currently acceptable levels of EDCs in wastewater have are based on laboratory studies of a small number of animal models such as rainbow trout, *Daphnia*, and gammarid amphipods. Many organisms may be more sensitive than the ones employed thus far as laboratory models.

Laboratory studies generally expose test organisms to different concentrations or doses of a single EDC. If the threshold level of adverse effects for a single EDC is 1 part-per-billion, it is not unreasonable to suspect that very long exposure, or exposure to multiple EDCs that cumulatively attain 1 part-per-billion or more, could be as harmful.

Triclosan use results in bacterial resistance that increases with repeated exposures, and may be associated with multiple drug resistance (Table 2). This suggests that simpler chemical disinfectants such as ethanol (ethyl alcohol) may be more effective, in the long run, than triclosan. Our results also highlight the importance of removing bacteria from wastewater before it is released into the marine environment, where drug-resistant strains could affect humans eating infected raw or incompletely cooked seafood.

In any event, all three treatment plants studied were effective in removing bacteria from effluent. Multiple bacterial colonies were harvested from every influent sample that we cultured, but only a single colony could be cultured from our effluent samples.

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