

An experiment to identify levels of triclosan that are harmful to bacteria in Puget Sound.

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Running head: Triclosan effects on marine bacteria.

Non technical summary

Due to fast urban growth coastal marine environments are exposed to many substances used by people in daily activities. Triclosan is an example of an antibacterial substance added to personal care product, as soaps and deodorants, to keep people disease free. This increasingly common used antibacterial makes its way into Puget Sound through sewage outfalls. Bacteria, naturally living in the water, might become an accidental target of triclosan. During the period of 19 and 23 March 07, ten samples of water were collected in five locations throughout Puget Sound in order to investigate the effect of triclosan on the bacteria living in Puget Sound waters. Those Samples were exposed to different concentrations of the triclosan during one hour and then growth was calculated. There wasn't clear growth inhibition at most locations, and overall bacterial growth rate was very slow. This incongruity on results might be due to bacteria being already resistant to triclosan or triclosan effects went undetectable due to the slow growth rate and short incubation period. In other words, poisoning bacteria with something that doesn't kill them but only inhibit duplication (which is the case with triclosan) while they are barely duplicating jeopardized the measurements of the triclosan effects.

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Abstract

Triclosan is an antibacterial agent commonly used in household personal care products because of its high bactericidal performance against many bacteria including gram positives and gram negatives (Escalada et al. 2005). Antibacterial substances like triclosan are potentially toxic to the natural bacterial population in Puget Sound. This experiment focused on determining the concentrations at which these antibacterial substances become harmful to the bacterial population in Puget Sound. The research took place on board the *R/V Thomas G. Thompson* from 19 to 23 March 2007. Bacterial population samples were collected at two depths per location using Niskin bottles mounted to a CTD rosette. Samples were then incubated with tritium-labeled thymidine (to measure bacterial growth) and four different concentrations of triclosan (0.125, 1.250, 2.500 and 5.000 ng ml⁻¹). Growth rates were measured in the lab by counting the radioactive thymidine incorporated into bacterial DNA. By comparing growth rates of the samples with and without triclosan it was possible to see what might have been the beginning of inhibition in some of the experiments. However a clear trend of inhibition wasn't present in the samples. The lack of consistent results might be due the possibility that the effects of triclosan went undetectable due to the slow growth rate (which ranged from 0 to 30 pmol L⁻¹ hr⁻¹) and short incubation period. In other words, poisoning bacteria with a compound that doesn't kill them but only inhibit duplication, such as triclosan, while they are barely duplicating impaired the measurements of the triclosan effects.

Introduction

As the World's population increases and technologies advance, the stress put on the environment also increases. Surface waters in the coastal ocean become vulnerable to all stressors that arise from pollution due to rapid urban development (Beck 2005). Coastal estuarine ecosystems, like Puget Sound, are an example of an area subject to anthropogenic activities. During the last three decades emphasis has been put on the conventional environmental pollutants, at the same time the number of chemicals found in the aquatic environment has increased (Agüera et al. 2003). A new group of potential aquatic pollutants emerging are the pharmaceuticals and chemical compounds present in personal care products (Agüera et al. 2003). Triclosan is an antibacterial used by pharmaceutical and personal care products group that has attracted concern due to its presence in the environment and its potential impact in the ecosystem (Ying and Kookama 2007).

Human waste containing antibacterial substances designed to combat diseases enter the sewage treatment process eventually making its way into the environment, thus Puget Sound. Municipal wastewater treatment plants are a major source of pollutants for the environment because pharmaceutical and personal care products are not completely removed from treated sewage before being released into the environment (Coogan 2007). In addition, it is believed that contamination of surface waters is also due in part to sewage spills and leakage (Halden 2005).

Triclosan, 5-chloro-2-(2,4-dichlorophenoxy) phenol (Fig. 1), commercially known as Irgasan DP 300 or Irgacare MP due to its non-volatile attribute and relative solubility in water, has been often found in the aquatic environment (Aranami and

Readman 2007; Newton et al. 2005). A study, which used a variety of water samples from Baltimore, places triclosan in fifth place with respect to frequency of detection in the water (Halden and Paull 2005). A broader study conducted on 139 United States streams and rivers detected triclosan in 57.6% of them, a sign of its wide spreading ability (Latch et al. 2003). Triclosan is not only antibacterial but it also has antifungal and antiviral properties placing it in at a higher toxicity level (Newton 2005). In addition, “triclosan is acutely toxic to aquatic organisms” (Aranami and Readman 2007).

Antibiotics released into the aquatic environment are of concern for many reasons including their negative impact on important ecosystem bacteria (Costanzo 2005). Marine bacteria, naturally found in Puget Sound, become an accidental target and may be harmed by the antibacterial triclosan beyond recovery. Due to bacteria’s importance in the marine food chain and primary production, their impairment may have negative effects on the ecosystem. It is therefore, necessary to establish concrete parameters of toxicity for triclosan for Puget Sound. Furthermore, understanding the toxicity of triclosan enables a better management of triclosan use and disposal.

Methods

In order to determine the detrimental levels of triclosan to the growth of Puget Sound’s aquatic bacteria, the response of the bacterial communities were measured after exposure to different levels of triclosan. Bactericidal toxicology is usually concentration dependent; therefore to create an accurate profile of toxicology it was necessary to evaluate a broad range of exposure to the poison. Samples were collected using Niskin bottles attached to CTD, near the West Point (47°39.40’N 122°26.50’W) and Duwamish

Sewage (47°36.06'N 122°25.59'W) outfalls, in a location in the Main Basin (47°51'N 122°25'W), in central Hood Canal (47°32.80'N 123°00.50'W), and in one location near Dabob bay (47°43.75'N 122°52.00'W) (Fig 2). Outfall bacteria samples were chosen due to the likelihood of to pollution exposure. Central Hood Canal samples were suspected to have a more natural profile, in other words somewhere less changed by human activities. Samples were collected at two depths: near surface at about 5 m and close to bottom, which were: 20 m for stations 3 and 5 and 140 m for stations 1, 2 and 4. These locations and depths were chosen with the intention of creating an array of samples that could represent bacteria community in general for Puget Sound.

Bacterial growth rate was established measuring the incorporation of tritium-labeled thymidine into bacterial DNA, a method that is rapid and economical (Van Mooy et al 2004). Due to the radioactivity of tritium the samples were processed inside the radiation van when on board *R/V Thomas G. Thompson*. Samples of 1 ml of water were dispensed into the preloaded tubes and incubated at approximately 14°C (close to in situ temperature of the water) for one hour. The bacterial samples were exposed to four different levels of triclosan concentrations spanning two different orders of magnitude: 0.125, 1.250, 2.500 and 5.000 ng ml⁻¹, which were chosen based on concentrations used by Escalada et al. (2005). There were control groups and triplicates for all locations. All tubes were uniformly preloaded with 10 nmol of (H³) thymidine prepared as described on the Thymidine (H³) incorporation Microcentrifuge Method (TIMM) (pers. comm. K. Genter). Triclosan was obtained from Sigma-Aldrich laboratories under the name of Irgasan and dissolved with dimethylsulfoxide (DMSO also from Sigma-Aldrich laboratories) to create a solution of 494 ng/9.88 ml. The following concentration used on

the experiment was obtained by diluting the initial solution with distilled water. One ml of sample water was dispensed in all preloaded tubes. Each row of the triplicates is composed of a kill control and a zero concentration tubes, which did not have any triclosan, and the other tubes had increasing concentrations of triclosan (0.125, 1.250, 2.500 and 5.000 ng ml⁻¹). The water samples dispensed into the kill control tubes were exposed to the kill agent 50 µl trichloroacetic acid (TCA) 50% immediately to serve as bacterial background count.

In a University of Washington laboratory the following steps, also according to TIMM, were taken: First the samples were centrifuged for 10 min at 13000 rpm and the water was removed. After water was removed 1 ml TCA 5% was added to all tubes and centrifuged for 10 min again. After the TCA was centrifuged and removed 1 ml ethanol 80% was added and again centrifuged for 10 min. After the excess alcohol was removed the samples were left to dry overnight. The following day, ecolume was added to all tubes and after 48 hours they were put on the liquid scintillation counter. Growth was determined by conducting the radioassay using liquid scintillation counter 2250CA Tri-CARB running protocol # 9. Finally, responses from bacteria were calculated using averages of the counts and converting them to growth rates. These responses were compared for each concentration of triclosan at each site.

Results

The five stations were characterized by a temperature range of 8.11°C to 10.46°C. Oxygen levels vary around 7 ml L⁻¹ and 8 ml L⁻¹ range, with exclusion of station 2 deep that had oxygen level of 4.56 ml L⁻¹. The graphs represent bacterial growth rate

measurements at the different stations and depth versus the different concentrations of triclosan (Fig. 3). All five stations revealed different bacterial growth rate characteristics. The profiles also indicated great variation between shallow and deep on the same station. For example at station LS1 at 5.1 m depth, bacterial growth showed the biggest inhibition at the lowest triclosan concentration of 0.125 ng ml^{-1} . The station LS1 at 140.1 m showed no apparent growth due to high background counts (Fig 3).

Total growth rates ranged between -12.5 to $30.2 \text{ pmol L}^{-1} \text{ hr}^{-1}$. Negative growth rates are the result of high bacterial background counts in some stations and can be considered to be zero. The average bacterial growth rate for surface stations without triclosan was $5.36 \text{ pmol L}^{-1} \text{ hr}^{-1}$; and the average bacterial growth rate for deep stations without triclosan was $-0.98 \text{ pmol L}^{-1} \text{ hr}^{-1}$. Total average bacterial growth rate with triclosan was $3.4 \text{ pmol L}^{-1} \text{ hr}^{-1}$. The average surface bacterial growth rate with triclosan was $7.395 \text{ pmol L}^{-1} \text{ hr}^{-1}$; and the average bacterial growth rate for deep stations with triclosan was $-0.595 \text{ pmol L}^{-1} \text{ hr}^{-1}$. Overall, bacteria showed the most growth at the surface.

Discussion

Bacterial production has previously been measured and has shown that rates vary directly and indirectly with environmental physico-chemical parameters (Kirchman and Hoch 1988). Bacterial growth rates observed in Puget Sound ranged between 0 to $30.2 \text{ pmol L}^{-1} \text{ hr}^{-1}$ and when compared with growth rates observed in warmer areas, as in the Delaware Bay estuary which ranged between 150 and 230 pmol hr^{-1} (Kirchman and Hoch 1988; McManus et al. 2004), my observed Puget Sound bacterial growth rates are on average ten times slower. Previously documented slow bacterial growth rates have been

observed in Puget Sound (Van Mooy et al. 2004), lending credence to my observed slow measurements. Why are growth rates so slow in Puget Sound? Growth rates measured in coastal fjords of Vancouver Island during summer are two to ten times faster (Keil, pers. comm.), suggesting that slow growth is not ubiquitous to the Pacific Northwest.

One important environmental parameter is water temperature, which can directly influence duplication rates (McManus et al. 2004). Bacterial abundance and productivity seems to increase rapidly as temperature exceeds 15°C (McManus et al. 2004). During the period of the cruise the water temperature ranged between 7°C and 8°C which might account for the slow growth rates observed. Van Mooy et al. (2004) also measured bacterial growth rates in winter suggesting that the slow growth rates observed in Puget Sound might be phenomena due to sampling during the winter, rather than a regional specific attribute. Furthermore, measuring growth rate using incorporation of tritium-labeled thymidine into bacterial DNA accounts mostly for the increase in bacterial DNA, which is not always as much as actual cell division. In order to duplicate bacteria usually follow these steps before eventual cell division: “first increases RNA synthesis, followed by an increase in protein and finally increase in DNA” (Chin-Leo and Kirchman 1990). The actual variation on incorporating rates over time can be reflected in an unbalanced DNA synthesis prior to cell division (Chin-Leo and Kirchman 1990). The slow growth rates I measured make it difficult to assess the effects of a poison such as triclosan due to the experiment at the analytical limits of the method.

Forty samples from five locations were assessed for the impact of triclosan on thymidine incorporation of natural bacteria. In eighteen of the forty samples I observed the beginning of growth inhibition. Except for the surface water at station 1, there wasn't

a consistent trend of inhibition in the experiment. At station 1, however every level of added triclosan resulted in inhibition. With exception of station 1, why might there be no consistent impact from the triclosan? Sampling locations could have played a role in the different outcomes. During the same cruise, a fellow oceanography undergraduate experiment on the effects of the pesticide carbaryl on copepods at two locations in Puget Sound. She observed that her results varied, which also may have been due to sampling in different location (Fox, unpub. senior thesis 2007).

Another explanation for the variation observed in the results is the mode of action of triclosan. Triclosan is an antibacterial compound that targets fatty acid synthesis by inhibiting the enzyme enoyl reductase “and this inhibition has been described as being slow and competitive” (Escalada et al. 2005). Therefore, triclosan inhibits cell wall formation; consequently impairing bacterial duplication, but not necessarily causing death, while the incorporation of thymidine may still be occurring. When standard strains of bacteria normally used to test for chemical disinfectants and antiseptics were exposed to triclosan a lag phase of about two hours was observed in which there wasn’t growth (Escalada et al. 2005). Such lag phases can not be observed in my experiments due to the fact that incubation lasted only for one hour.

In addition, the bacterial compositions of the samples are not known, presenting another predicament to the clear measurement of triclosan effects. One recommendation for future studies is further investigation of bacterial growth rates and composition in Puget Sound. Concentrations of triclosan have been measured in many streams and estuaries throughout the country including the west coast (Halden and Paull 2005), but never before measured here. Professor Halden and Paull (2005) have found that

concentrations of triclosan ranged between 100 and 1000 ng L⁻¹ nationwide. Thereafter, not knowing how much triclosan is present in the waters of Puget Sound compromise the interpretation of the results from adding triclosan to the samples. Could the bacterial community of Puget Sound already be at its threshold? Further investigation should include the determination of the amount of triclosan in the water to evaluate how much the bacteria have already been exposed. Moreover, the outcome of a longer incubation might reveal the real efficacy of triclosan. In addition, to increase the accuracy of bacterial growth measurement tritium labeled leucine incorporation should be used in conjunction with (H³) thymidine incorporation to encompass most steps of cell duplication. (H³) Leucine is incorporated mainly in bacterial protein synthesis (Chin-Leo and Kirchman 1990).

Conclusion

Although it was not observed constantly in all stations, the inhibitory capacity of triclosan shows its initial effects in some of the experiments. With further investigation a trend will probably develop. As mentioned, the fact that triclosan interaction with bacterial cell is complex and not necessary lethal require accurate measurements. The slow growth rates observed in the samples; between 0 and 30.2 pmol L⁻¹ hr⁻¹ might explain why there was not a clear trend of inhibition on bacterial growth. It is indispensable that the effort to understand the mode of action and the toxicity of triclosan to the bacteria continue to be investigated.

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Figure Captions

Figure 1. Triclosan chemical structure (from Halden and Paull 2005).

Figure 2. General location of cruise showing CTD drops stations for water sampling throughout Puget Sound. LS1 is located at 47°51'N 122°25'W, LS2 located at 47°39.50'N 122°26.75'W, LS3 located at 47°39.40'N 122°26.50'W and LS4 located at 47°36.06'N 122°25.59'W and LS5 located at 47°43.75'N 122°52.00'W.

Figure 3 The graphs represent the bacterial growth rate ($\text{pmol L}^{-1} \text{hr}^{-1}$) measurements at the different stations and depth versus the different concentrations of triclosan (ng ml^{-1}). All five stations revealed different bacterial growth rates represented by the block sizes. A) Station 1 shallow (5.1 m). B) Station 1 deep (140.1 m). C) Station 2 shallow (5.2 m). D) Station 2 deep (139.8 m). E) Station 3 shallow (5.2 m). F) Station 3 deep (20.1 m). G) Station 4 shallow (4.8 m). H) Station 4 deep (140 m). I) Station 5 shallow (4.9 m). J) Station 5 deep (20.2 m).

Figure 1

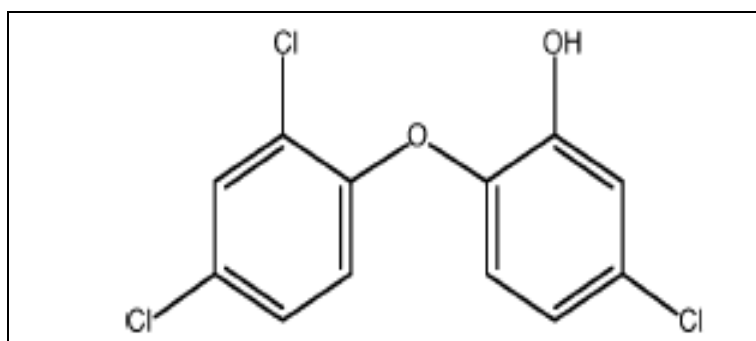


Figure 2

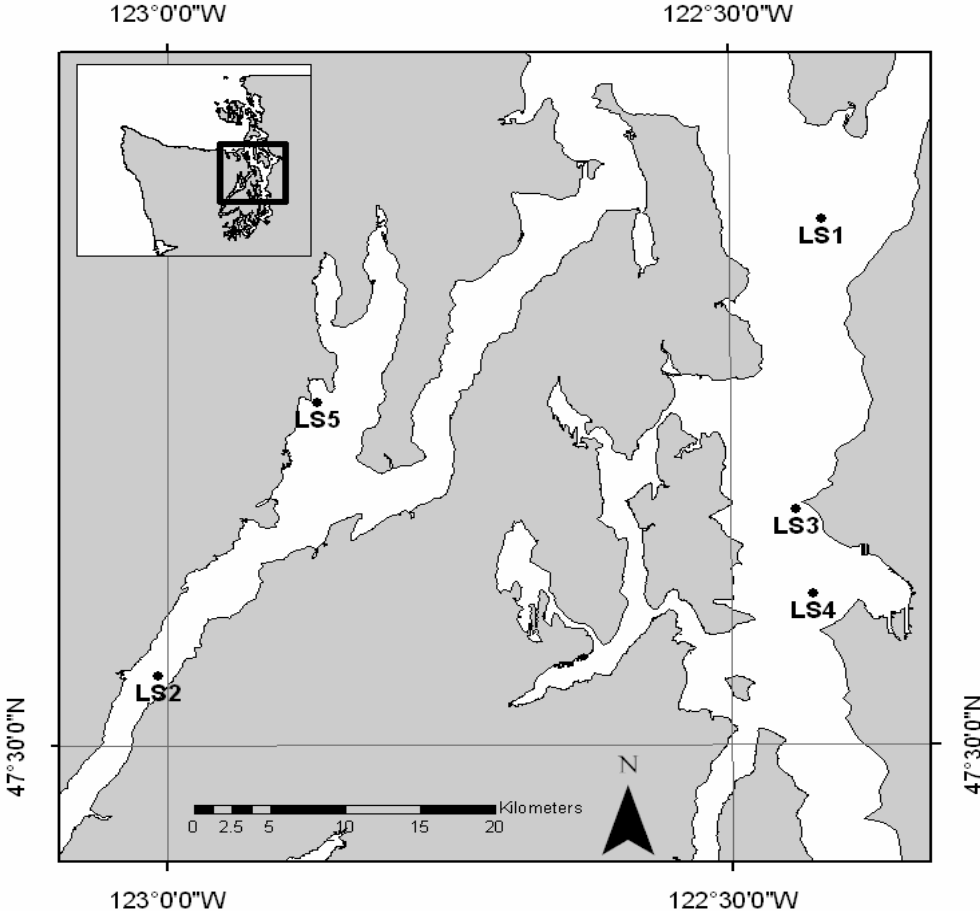


Figure 3

