Huxley College of the Environment

An Evaluation of Environmental Influences on Tributyltin Toxicity Using the Macroalgae *Ulva lactuca*

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Abstract

Tributyltin (TBT), an active ingredient in many biocides, is often observed in waters surrounding busy seaports and in agriculture run-off. In aquatic systems, particular environmental factors affect and alter the speciation and sorption properties of contaminants like TBT. Few toxicity tests consider the use of common marine algae such as the chlorophyta, *Ulva lactuca*. In determining the influence of tributyltin on *U. lactuca*, a 2^3 full factorial design was used for the experimental set-up, data collection and analysis. The three environmental conditions that were varied were UV light, salinity, and temperature. The effects of these environmental factors on sporulation will be used to guide future work with respect to TBT toxicity. The factorial analysis suggests that salinity has the greatest effect on the toxicity of TBT.

Introduction

Scientists have been recommending for years the employment of standard methods for toxicity testing with marine macroalgae (Eklund and Kautsky, 2003). Currently, standardized methods for toxicity tests on the macroalgae genus, *Ulva*, do not exist. However, recent studies have utilized certain aspects of the *Ulva*'s life cycle as a measure of toxicity. Similar methods were applied in this experiment. This research is designed to address two data gaps. First, to develop a testing method with a macroalgae commonly found in Puget Sound and of importance as a primary producer in the trophic food web, and second, to identify the influence of 3 environmental factors on the bioavailability of tributyltin (TBT) on *Ulva lactuca*. Varying levels of UV light, temperature, and salinity were used in the treatment so that subsequent inferences can be made about how these three factors affect toxicity.

Due to the constant leaching of TBT from the hulls of ships, and fractions available in the sediment, TBT perpetuates in the aquatic environment and interacts with non-target organisms. In a natural environment, biomagnification and subsequent toxicity in higher trophic level organisms is partly a consequence of bioavailability to the lower trophic organisms, such as algae, as well as the diet of primary consumers (e.g., snacking turtles feed largely on *Ulva* and other

seaweeds). Bioavailability and toxicity of tin is highly dependent upon the chemical species of tin that is present (need a reference for this). Biodegradation of TBT usually involves the debutylation of TBT to dibutyltin (DBT) and monobutyltin (MBT) (Newman and McIntosh 1991). The half-lives of TBT have been shown to increase at decreasing light exposure and lower temperatures

Water quality greatly determines the chemical speciation of the contaminant. Salinity , for example,is a water quality factor that influences the sorption properties of TBT. Langston and Pope (1995) have modeled values of K_d at different salinities and have shown that at 0% salinity, K_d was at its highest at ~30,000 ($1\ Kg^{-1}$) and dropped down to around ~15,000 ($1\ Kg^{-1}$) at salinities 25% - 75%. TBT concentrations in water about doubled from 0% to 50% salinity (Langston and Pope, 1995). As salinities increase, competition among free ions in a solution is occurring.

Thain et al. (1987) have modeled the influence of light and temperature on the half-lives of TBT in a marine environment: in the presence of light, the $t_{1/2}$ is 3-8 days; in darkness it is 7-13 days; at 5°C, it is 60 days; and at 20°C, it is 13 days. The environmental factors varied in this experiment were based on this experiment. Toxicity tests were conducted for TBT in the presence and absence of laboratory delivered UV light, at high and low salinity, and at a high and low temperature.

Conditions that have been shown to support optimum growth of *Ulva* species in the laboratory are described by Taylor et al. (2001). These researchers found the greatest growth (measured as % grown/day) for *Ulva* occurred at temperatures ranging from 15° - 20°C and salinity ranging from 23.8% – 27.2%. Their results also indicated that at 10°C there was still growth of the *Ulva*. The fluorescent lighting conditions were modeled after Han et al. (2007) at 12:12 light:dark (LD), in which sporulation was an endpoint.

Sporulation has been used, in the past, as a measure of toxicity by Han et al (2003). They used *Ulva pertusa* as a means of measuring toxicity to several

stressors by evaluating sporulation. The stressors were varying degrees of photon irradiance, salinity, and temperature. In all cases, sporulation was minimal at low levels of irradiance, salinity, and temperature and increased as these environmental levels increased. Their test acceptability criterion was at least 75% sporulation in the negative control.

Han et al (2007) suggest having a quantitative value system of sporulation at 0%, 25%, 50%, 75%, and 100%. This method makes it easier for quick observations about the current level of effect in an organism, such as *Ulva* species. The area sporulated is easily identified, as the area that has lost the vegetative green color leaving the remaining algal tissue slightly transparent (figure 1).

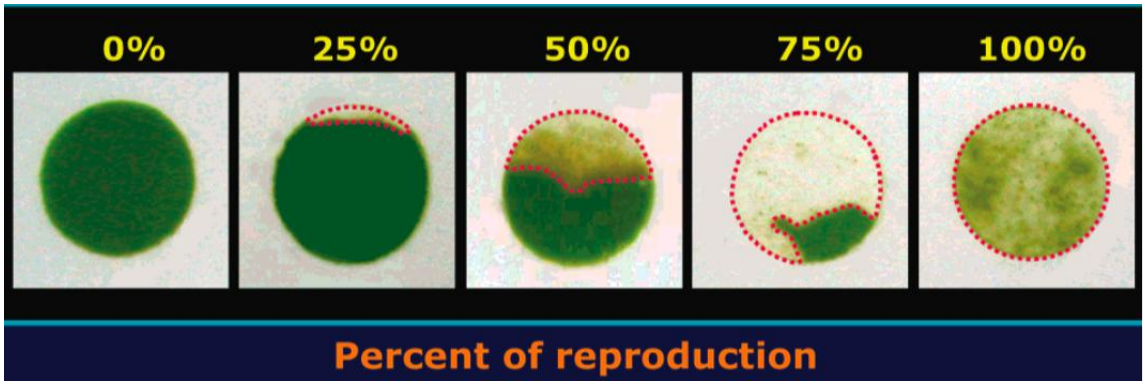

Figure 1. Images showing percent sporulation on algal disks (image from Han et al., 2007)

Methods

Ulva lactuca were obtained from Larrabee State Park, Washington, at low tide on May 7th, 2008. The field salinity was 30% and temperature was 13˚C. The algae was identified as *Ulva lactuca* by observing the characters as outlined by the phylogenetic resource at algaebase.org (Wilkes, 2005) and further verified by a local marine biologist (Dr. Gisèle Muller-Parker, a professor of Biology at Western Washington University). Following the procedure outlined by Naldi and Wheeler (1999) the algae were cleansed by rinsing with artificial seawater and, if necessary, gently scrubbed to remove sediment and epiphytes. The clean *Ulva* was then placed in seawater at 28% salinity and was continuously aerated. The

Ulva was acclimated to laboratory conditions in an environmental chamber at a 12:12 LD cycle and 17˚C for 2 weeks prior to treatment,.

All seawater in this experiment was made from Crystal Sea Forty Fathoms® salts dissolved in Nanopure® water. Prior to utilization, all glassware was acid washed and acetone washed. Salinities of 23% and 28% were prepared a day prior to day 0 of treatment exposure. Statistical calculations were performed by CETIS™ software.

Following the two weeks of incubation, 1100 (18 mm diameter) disks were cut with the tops of 10ml glass tubes as to achieve consistent sizes. Careful attention was taken to ensure each piece was cut away from the marginal and holdfast regions of the thallus so as to procure similar tissue types. The algal disks were then stored in the aquarium and aerated for another 48 hours prior to treatment at the same lighting and temperature regime as used during the laboratory acclimation 2-week period. Total incubation time from the time the algae was obtained to when the disks were exposed in the treatment was 2 weeks and 2 days (16-days).

To increase the solubility of TBT, and as outline by D'Agati et al (2006), the 250 ppm TBT stock solution contained 7% dimethylsulfoxide (DMSO) and was prepared with artificial seawater. The stock solution was used to make 5 concentrations of TBT for the toxicity exposures, each at the two salinities; the concentrations were 0.5, 1, 2, 4, and 8 ppb TBT. These concentrations were based on a range finding experiment in which the EC_{50} was 2 µg/L. All treatments were conducted in triplicate. The test chambers were 50 ml Kimax® glass beakers; each test chamber contained 30 ml of the appropriate TBT concentration at 23% salinity and at 28% salinity (figure 1). For a positive control, solutions of copper were made based on a range finding test in which the EC_{50} was 25 µg/L. The treatments for the positive control were performed in duplicate and at 28% salinity. Also, a DMSO solvent control was performed at 28% salinity, and at varying degrees of UV light and temperature.

After adding the correct volume of solution in the test chambers, five algal disks were added to each chamber. Careful attention was made to ensure that all disks used in this experiment had similar characteristics, such as color and toughness (qualitatively determined). The high temperature regimes were achieved by placing the test chambers in hot baths set at 22±2˚C. Figure 2 shows the experiment design in which water baths were used in the higher temperature exposures.

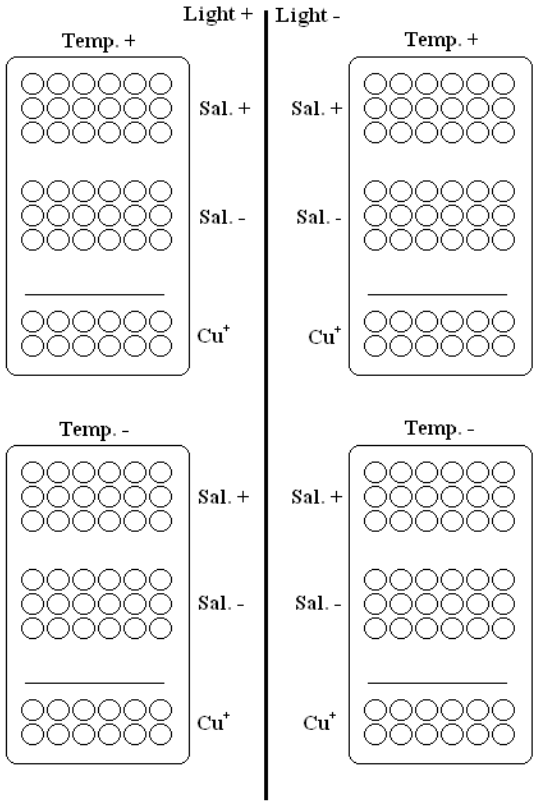

Figure 2. A diagram depicting an overhead view of the experimental design. High and low temperatures are represented by “Temp. +” and “Temp. -”, respectively. High and low salinities are represented by “Sal. +” and “Sal. –”, respectively. Exposure in the presence of UV light and without UV light are represented by “Light +” and “Light -”, respectively.

The lower temperature regimes were carefully regulated outside of water baths to maintain a temperature of 17±2˚C. As water warmed in lower temperature containers, it was replaced by refrigerated water at volumes and at increments that maintained the temperature regime within the ±2˚C range. Salinity was 23%

and 28% and in the presence of both fluorescent and UV lighting, photoperiods were 12:12 LD and 11:13 LD, respectively. The UV lighting was set at 11:13 LD so that work was not performed under hazardous conditions and that fluorescent lighting was available for physical observations.

Sporulation was classified as 0%, 25%, 50%, and 100% sporulated as outlined by Han et al (2007). Low sporulation is indicative of toxicity while higher levels of sporulation are indicative of cell viability and lower toxicity. The progression of sporulation across the treatment groups were observed and recorded every 24 hours for 10 days. Statistical analysis was then conducted to calculate EC_{50}s of each set of factors tested.

By performing a 2^3 full factorial analysis, inferences on the effects of the three environmental factors were weighted by the analysis to suggest how these factors influenced toxicity. Interactions between factors were also analyzed in the full factorial analysis. The factors with the most influence on toxicity were identified.

Results

Evaluation of EC_{50} values were initially calculated with two sporulation endpoints; (1) area sporulated per each disk using the % sporulated per disk as descibred above, or (2) the number of disks with any percentage of sporulation. Statistical analyses with the second endpoints were not conclusive.

Linear interpolation methods were used to calculate an EC_{20} for each treatment regime using CETIS. The one case in which the EC_{20}s were higher in the 28% salinity treatments was at high temperature and in the presence of UV light. Overall, most of the treatments had lower EC_{20}s in the 28% salinity solutions (Table 1).

Table 1. The 10 day EC_{50}s following treatment according to the varying salinities, UV light, and temperature.

Salinity	Light	Temp.	10 day EC_{20}
-	-	+	1.21
+	-	+	0.343
-	-	-	1.318
+	-	-	0.8883
-	+	-	1.169
+	+	-	0.2149
-	+	+	0.7487
+	+	+	1.305

Salinity and UV light had the most influence on the toxicity of TBT. When considering the EC_{20} as the measure of effect in the factorial analysis, both salinity and UV light increase toxicity. Interactions between factors

Table 1. 2^3 full factorial analysis showing the estimated effects of salinity, UV light, temperature, and interactions between factors on the toxicity of TBT parameterized by EC_{20}s.

f (factor)	Effect of *f* on toxicity
Salinity (S)	-0.423625
UV light (UV)	-0.080425
Temperature (T)	0.004125
S*UV	0.224725
S*T	0.268275
UV*T	0.330775
S*UV*T	0.486925

A cube plot displays the results obtained from each of the eight treatment groups (figure 3). The conditions of the resulting EC_{50}s are explained by their location on the cube's corners. The plus sign (+) represents the higher levels of factors and the minus sign (-) represents the lower levels of factors that were used in each treatment. The highest EC_{50} resides on the front, lower, left side of the cube where salinity was 23%, temperature was 17±2˚C, and there was no UV light

used. The lowest EC_{50} (the highest toxicity) was observed at high salinity (28%), the presence of UV light, and at the low temperature of 17±2˚C. This lowest EC_{50} resides on the front, top, right corner of the cube.

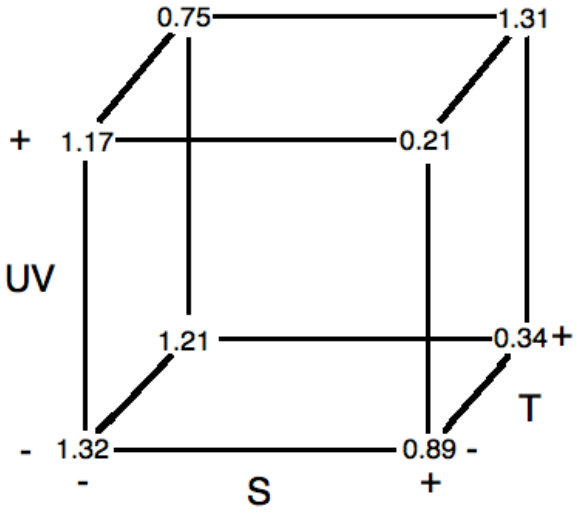

Figure 3. A cube plot of the eight treatment conditions used in the full factorial analysis and showing the calculated EC_{50}s that correspond to the different environmental factors.

The results of the factorial analysis describe whether or not an environmental condition had a significant effect on the toxicity of TBT. The interactions increase in a linear fashion, therefore it can be assumed that the interactions are random effects. The individual factors appear to be non-random (significant) effects due to the nature in which the values increase in a non-linear fashion. The resulting value of from the full factorial analysis indicated that salinity is the furthest from 0. This is an indication that salinity had the greatest effect when compared to the other factors in which temperature (0.004) had the least effect on toxicity. Interactions between factors may not have had a significant effect on toxicity.

Conclusion/Discussion

The negative control, and solvent negative control did not pass the acceptable criteria of 80% reproduction (sporulation in this case of *U. lactuca*) as outlined in

the ASTM's Special Technical Publications (STPs) for rhodophyta macroalgae toxicity testing (1983). There are a number of factors that may have inhibited the sporulation in the negative controls. By performing this experiment again, the causative factors lying behind the absence of sporulation may be better understood. Understanding the mechanisms that trigger sporulation could help determine at what point in the *Ulva*'s life cycle exposure should begin. Stress may be one of the main contributing factors of sporulation. Subsequent experimental tests could help to determine the effects of salinity, temperature, and UV light on sporulation alone. The applicability of this method in standardized toxicity testing is not at fruition.

Although the experiment failed the acceptable criteria, analysis of environmental influences was possible by observing sporulation. This was due to the concentration/response in all treatments. Also, some of the negative controls were close to passing the acceptable criteria, by extending the experimental period, an increase in sporulation would have been expected resulting in the acceptable criteria being achieved.

Other ways to measure toxicity on *Ulva* may be to evaluate the lateral growth. This method would be useful due to the growth characteristics in *Ulva lactuca*. The macroalgae grows by increasing the diameter only, which could be used as a measure of effect. In order to monitor growth, and as outlined by Taylor et al (2001), nutrients such as nitrogen and phosphorous would be added at specific quantities as to allow sufficient growth of up to 21% per day. This rapid growth rate can be used for short treatment chronic toxicity testing, enabling the elucidation of effective concentrations of any particular contaminant.

Measures of chlorophyll α and β ratios may be useful endpoints to investigate and may provide insight into the physiological responses in the presence of a stressor. Also, measurements of physiological responses can include changes enzyme activity as an endpoint. Further testing with *Ulva lactuca* will either help to develop or possibly discredit the likelihood of *Ulva* as an effective toxicological test organism. Major uncertainties exist due to genetic and life stage variations

of the field obtained algae. Efforts should be made in subsequent toxicity tests to utilize *Ulva* that has been cultured in a controlled setting so that life stages and genetic variability can be consistent.

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