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Effects of the biocide triclosan on multiple life stages of ferns *Onoclea sensibilis* and *Osmunda claytoniana*

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Abstract: The chemical triclosan is an antibacterial agent that was used in many consumer products, such as soaps, lotions, toothpaste, cosmetics and other personal care products. Triclosan passes through the processes of wastewater treatment plants and ultimately contaminates rivers and other waterways. The soil is also contaminated using wastewater sludge or biosolids as fertilizer on agricultural and reclamation land projects. Triclosan has been shown to inhibit seed germination, growth rate, and development in wide variety of plants including many crop plants. Aquatic algae are particularly sensitive to low levels of triclosan. The purpose of this experiment is to investigate the effects of triclosan on the life cycle of the sensitive fern (*Osmunda claytoniana*), and the royal fern (*Onoclea sensibilis*). The use of ferns in this study is important because they share a phylogenetic link between algae and higher plants. Ferns also have a distinct heteromorphic life cycle that lends itself to examining the effects of environmental chemicals on different aspects of plant development. Spores were germinated, grown to gametophyte stage and then allowed to produce sporophytes in the presence of concentrations of triclosan measured in contaminated agricultural lands. Triclosan was found to inhibit spore germination, gametophyte growth and alter sporophyte development.

Keywords: triclosan; gametophyte; sporophyte; Onoclea

Introduction

The chemical triclosan [5-chloro-2-(2,4-dichlorophenoxy)-phenol] is an antimicrobial agent that was used in many consumer products such as soaps, lotions, toothpaste, cosmetics and other personal care products (PCPs) (Cooney 2010). PCPs can act as organic pollutants due to their incomplete removal from sewage by traditional wastewater treatment plant methods and can be discharged into rivers, streams or deposited in the environment via sludge (Zarate et al, 2012). The concentration of triclosan in personal care products often ranges between 0.1% to 0.3%. Up to 0.2 to 2.7μg/L can end up in the environment through waste water (Amorim, et al, 2010). Triclosan can enter the soil through sludge-amendment or biosolids used for land application. Kinney et al. (2006) found an average of 10mg/kg of triclosan of biosolids from 9 different waste water treatment facilities. This would result in an application rate of 8900ug/m^2 if these samples were used for land augmentation. Cha and Cupples (2009) measured triclosan concentrations of approximately 0.4 ng/g of dry weight in biosolid amended agricultural soils. The persistence of triclosan seems to vary depending on conditions and detection methods. Once in the soil triclosan typically remains in the upper 10-20cm (Reiss et al. 2009). In a survey of 139 streams in 30 states, triclosan was among the seven most frequently detected organic chemical compounds (Lui, et al, 2008).

In the environment, triclosan has been found to be toxic to a wide variety of organisms including bacteria, algae and higher plants, invertebrates and earthworms (Ricart et al. 2010; Amorim et al. 2010). Amorim et al (2010) also found that green algae were

susceptible to triclosan at dosages similar to bacteria. Triclosan released into aquatic environments could decrease primary productivity if algae are killed. Other potential ecological problems associated with triclosan include decreased nutrient uptake, reduced competitive ability and increased potential for uprooting in plants (Zarate, et al, 2012). An et al. (2009) found that wheat (*Triticum aestivum*) seedlings were inhibited by 20 -25- mg/L of triclosan.

The Food and Drug Administration has ruled that triclosan and several other antiseptic additives as "ingredients not general recognized as safe and effective" as health care antiseptics (FDA. 2017). The FDA ruling on triclosan should result in the reduction of triclosan introduction into the environment, although some brands of toothpaste and other PCP still contain triclosan as an active ingredient.

Seedless vascular plants have not been studied for phytotoxicity in the presence of triclosan. Ferns have four life stages where exposed to triclosan could negatively impact growth and survival. The diploid sporophyte that may uptake triclosan through the roots similar to a higher plant. Haploid spores which germinates into a haploid gametophyte, both of which could absorb triclosan directly from contaminated soil. The gametophytes will produce haploid gametes, eggs and free-swimming haploid spermatozoids used for sexual reproduction, these could be exposed to triclosan in contaminated water. In this study we compared the influences of ecologically relevant concentrations (based on literature survey) of triclosan on the different life stages of ferns using *Onoclea sensibilis* (Sensitive fern) and *Osmunda claytoniana* (Interrupted fern) as model organisms.

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Materials and Methods

Fern spore collection

Spores were collected from the fertile fronds of *Onoclea sensibilis* from Milton WV. *Osmunda claytoniana* spores were collected in Greenbrier County WV. Collected spores were stored in small brown paper bags and allowed to air dry at least two weeks. Spores were separated from their sporangia and other debris by sifting onto white letter paper and removing non-spore material. Spores were then stored at 4ºC for up to two months until use.

Effects of triclosan on spore germination

Onoclea. sensibilis spores were sown onto 100mm Petri plates containing 25ml of Murashige and Skoog medium solidified with 1.5g/L Phytogel (Murashige and Skoog 1962). Plates were then incubated at room temperature under 12-hour full spectrum grow-light cycle. Triclosan powder (Sigma Aldrich >97% purity) was solubilized in a 55% polyethylene glycol 3350 and 20% ethanol solvent to 12 µg/L before dilution as described by Vandhana et al. (2010). Solubilized triclosan was added to the medium as it cooled to approximately 40ºC. A decimal dilution series of triclosan was created from 0.12ug/L to 0.00012 µg/L in the medium. Spores were considered germinated when a protonema was clearly visible growing from the spore. Germination or emergence effects were expressed as a ratio of germinated to non-germinated spores as modified from Ricart et al. (2010). Data from three replicates were combined for an average ratio at each triclosan concentration.

Effects of triclosan on gametophyte growth

To examine the effect of triclosan on gametophyte growth *O. sensibilis* spores were sown onto Murashige and Skoog medium (Murashige and Skoog 1962) and incubated at room temperature under 12-hour light cycle as previously described. The spores were allowed to germinate and grow until the majority of the gametophytes reached the cordate stage, approximately 10 days (Chiou and Farrar 1997). One ml of Triclosan solution was applied to gametophytes on each plate in concentrations of 0.40, 4.0, and 40.0mg/L. Petri plates were wrapped with Parafilm and allowed to grow an additional week under 12-hour light cycle as previously described. Width of individual gametophytes was measured using Leica DMF light microscope with attached Leica EC3 camera and Leica LAS EZ software.

Effects of triclosan on spermatozoid motility

To test the effects of triclosan on fern spermatozoids *O. claytoniana* was chosen over *O. sensibilis* due to the larger size of the gametophyte

and spermatozoids. Spores of *O. claytoniana* were sown onto 50mm Jiffy-7® peat pellets cut into 50mm diameter by 30mm tall sections after imbibing in distilled water. The sectioned peat pellets were placed onto 100mm plastic Petri dish covers which were covered with 250 ml glass beakers under a 12-hour photoperiod with artificial light. The gametophytes were then allowed to grow for approximately two months without chemical additives. Two or three of the 3-month-old *O. claytoniana* gametophytes were removed from the peat and placed in concave microscope slides with 1 mL of sterilized distilled water. After spermatozoids were released into the water, triclosan stock was added to a final concentration of a 20mg/L, 40 mg/L, or 60 mg/L. Motility was assessed with control solution of the polyethylene glycol and ethanol solvent without the addition of triclosan. Gametophytes were observed for 30 minutes under 400X magnification using a Leica DMF light microscope for the presence of spermatozoids. Motility was considered positive if sperm cells were swimming in the in with a corkscrew-like motion.

Effects of triclosan on sporophyte growth

To test the effects of triclosan on the sporophyte generation of the fern species *O. sensibilis*, spores were sown onto 50mm Jiffy-7® Peat Pellets as described above. The resulting gametophytes were grown covered with 250 ml glass beakers with a 12-hour photoperiod and artificial light without chemical additives. Plants were sub-irrigated with sterile distilled water. After approximately 30 days, the resulting gametophyte plants were then misted with distilled water. Individual gametophytes with a single primary sporophyte were transplanted five each onto 1/3 sections of peat pellets that had been saturated in 0, 300, or 600 mg triclosan/kg dry weight of peat and incubated under the same conditions as described above. After two and three weeks the number of living sporophytes emerging from each gametophyte was counted.

Statistical analysis.

Experiments were performed in triplicate and averages were calculated. A Shapiro-Wilk test of normality was performed on each dataset and averages from each experiment were compared by one-way ANOVA. A Tukey post-hoc was used when significance ($p<0.05$) was observed using Graphpad Prism 8.2.0 software. Data were plotted as means and error bars are ± standard error of mean for each point.

Results

Effects of triclosan on spore germination

Triclosan at 0.00012 µg/L appeared to have a slight, but not statistically significant, stimulatory effect on spore germination (Fig. 1). Concentrations of triclosan above 0.00012 µg/L showed significantly lower spore germination than the control with a 47 percent decrease in spore germination. This inhibition was not in a dose dependent manner. The observed inhibition of spore germination was not enhanced as the triclosan concentration increased up to 1000-fold than then control.

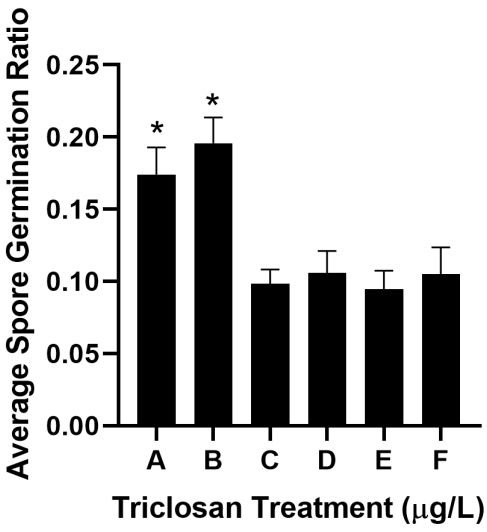

Figure 1. Effects of triclosan on *O. sensibilis* spore germination. Data are presented as average germinated/nongerminated ratio. n=15 per treatment. A=Control, B=0.00012µg/L, C=0.0012µg/L, D=0.012µg/L, E=0.12µg/L and F=1.2µg/L. *=$p<0.001$ from higher concentrations (C-F), but same as control (A). Bars indicate standard error.

Effects of triclosan on fern gametophyte growth

Onoclea. sensibilis gametophyte growth was inhibited on average by 13% when the ethanol:PEG

solvent was added to the growth medium. Addition of triclosan to the solvent mixture increased the inhibition another 16% (0.16mm average less growth) (Fig. 2). Increasing amounts of triclosan above 0.4 mg/l showed no significant difference as triclosan concentrations increased.

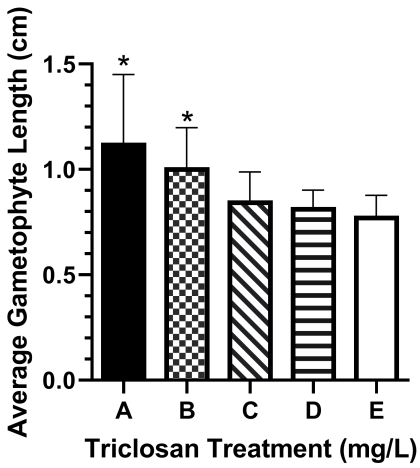

Figure 2. Effects of triclosan on *O. sensibilis* gametophyte growth. Growth measured as the widest part of the prothallus. n=30 per treatment. An ' indicates average after growth period. A=water control, B=solvent control, C= 0.4mg/L, D=4.0mg/L and E=40mg/L. An * represent difference in means from ANOVA and Tukey HSD P<0.001. Bars indicate standard error.

Effects of triclosan on fern spermatazoid movement

Triclosan was shown to inhibit the motility of spermatozoids at concentrations between seven and 13 mg/L (Table 1). The solvent control and 7 mg/L triclosan did not inhibit motility.

Table 1. Effects of triclosan on *O. claytoniana* spermatozoid movement.

Treatment	Solvent Control	7.0 mg/L	13 mg/L	20mg/L
Motility observed	yes	yes	no	no

Effects of triclosan on fern sporophyte growth

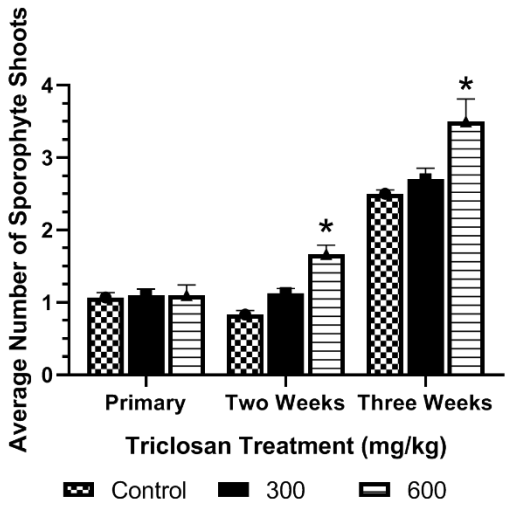

Figure 3. Effects of triclosan on *O. sensibilis* sporophyte growth. Average number of sporophytes produced from gametophytes exposed to 300mg/kg or 600mg/kg of triclosan per kg of soil. n=30 gametophytes for each treatment. * indicates significant different within each time interval. Bars indicate standard error

The effects of triclosan on the short-term growth of *O. sensibilis* sporophytes are summarized in Fig. 3. Sporophytes growing in the presence of 600mg/kg developed 40 percent more shoots than the control or plant growing with 400mg/kg triclosan. This was evident at two weeks with a 45% increase in sporophytes ($p < 0.001$) and after three weeks with a 25% difference ($p = 0.006$). Sporophyte length showed no significant difference at these triclosan concentrations (n=30).

Discussion

Experiments with different life stages of ferns showed that short-term acute exposure to triclosan influenced all stages of fern growth but the effects were different (positive or negative) depending on the life stage.

No evidence of overt lethality was observed, such as chlorosis or yellowing. Some reduction of growth or activities were observed at triclosan concentrations within the concentration ranges found in contaminated soils. For example, we found that the inhibitory dose of triclosan on *O. claytoniana* spermatozoids is between 7 mg/L and 13 mg/L. An et al. (2009) found that triclosan concentrations of 50

to 250 mg/L inhibited wheat seed germination by 25 to 60%. Amorin et al. (2010) showed that low levels of triclosan could decrease seed emergence rates, and higher concentrations (200 to 1000 mg/kg) could delay the time of emergence in both monocots and dicots, but delay rates were higher in *Triticum aestivum* compared to *Brassica rapa*.

In the present study, haploid fern spore emergence and gametophyte growth was inhibited by triclosan levels similar those reported by Lozano et al. 2010 and Walters et al. 2010 from agricultural lands treated with biosolids. The lack of linear relationship with the dosage of triclosan could be due to the concentration used in the current experiment was below the effective lethal dosage for the plant. If the low dosage was the case it would suggest that *O. sensibilis* may tolerate low doses of triclosan in the environment. An alternative explanation would be that the plant has a method of compensation such as a degradation pathway or an alternative pathway for the production of the fatty acids pathway normally inhibited by triclosan. Macheius et al. (2012) and Macheius et al. (2014) found that triclosan challenged callus cultures, roots of *Daucus carota* grown in contaminated soil and or horseradish hairy-root cultures could convert the triclosan into various metabolites such as methy-triclosan or different conjugates. Ferns may have similar mechanism that must be overcome before overt toxicity is evident. The evidence of metabolic conversion of triclosan may explain some of the variation in plant response to environmental triclosan.

The effect of triclosan on fern spermatozoids motility was less pronounced than that exhibited by microalgae. Microalgae have been negatively affected by as little as 5ug/L of triclosan (Ricart et al. 2010 and Roberts et al. 2014). Wilson et al. (2003) found that 0.15ug/L triclosan reduced levels of the motile green algae *Chlamydomonas* in environmental samples. Fern spermatozoids superficially resemble a motile algae cell but retained motility with triclosan concentration of 5 mg/L. These results suggest that these fern spermatozoids are more tolerant of triclosan than algae and more closely resemble higher plants in this regard.

The growth rate (increase in length) of young fern sporophytes was not inhibited by concentrations of triclosan found at ~~on~~ the upper spectrum found in biosolid amended soils. The explanation for the increase in sporophyte numbers with the addition of triclosan ~~is~~ was not directly apparent. Plants with mutations in the *MOD1* gene, which encodes the protein targeted by triclosan, have been found to have reduced fertility and increased sensitivity to temperature (Mou et al. 2000). Our results appear contrary to the observations in Mou et al. (2000).

The increased number of sporophytes could be due to enhancement of fertilization by reduction of surface tension thus allowing saturation of gametophytes from the substrate allowing multiple fertilization events. Ferns can exhibit polyembryony and embryonic selection can reduce the survival of less vigorous plants (Buchholz 1922). Mottier (1925) could produce multiple sporophytes in some prothalli of *Pteris longifolio* by multiple water additions on different days. The increased number of sporophytes could be due to enhancement of fertilization by reduction of surface tension. Reduction of surface tension may make it easier for spermatozoids to access the archegonia. This in turn could result in multiple fertilization events. Ravi (2016) showed that fern gametophytes experimentally manipulated with cytokinins can exhibit polyembryony. Therefore, another explanation is that triclosan could be influencing phytohormone metabolism and influencing the levels of cytokinins. An alternative explanation is that the triclosan concentration in the experiment was below the threshold level of toxicity. Reinhold et al. (2010) found sorption of triclosan by duckweed tissues could remove triclosan from aqueous solutions. Ferns may exhibit a similar ability to absorb or adsorb an unknown amount of triclosan.

To our knowledge, this is the first study to influence of triclosan on the fern species *Onoclea sensibilis* or *Osmunda claytoniana.* Our study found that ferns have a response to triclosan within the same dosage as higher plants. If triclosan does increase emergence rates of plants this could be potentially useful to increase germination of species hard to germinate in the lab. In addition, triclosan appeared to increase the number of sporphyotes produced from gametophytes in our work. Future work could be done to elucidate the mechanism of polyembryony in the presence of triclosan.

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