DISSERTATION

PHYTOREMEDIATION OF TETRACYCLINE AND OXYTETRACYCLINE

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY NINAD P. GUJARATHI ENTITLED PHYTOREMEDIATION OF TETRACYCLINE AND OXYTETRACYCLINE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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Abstract of Dissertation

PHYTOREMEDIATION OF TETRACYCLINE AND OXYTETRACYCLINE

Tetracycline (TC) and oxytetracycline (OTC), when released to the environment through wastewater streams mainly from concentrated animal feeding operations (CAFO), may induce antibiotic resistance among several bacterial species. Phytoremediation involves the use of plants or plant-secreted metabolites in remediating pollutants from air, water and soil. If proven effective, phytoremediation may provide an inexpensive, efficient means for antibiotic remediation.

Two aquatic species, *Myriophyllum aquaticum* (parrot feather) and *Pistia stratiotes* (water lettuce), and hairy root cultures of *Helianthus annuus* (sunflower) are reported to have ability to remove TC and OTC from aqueous media. Root exudates from the three plant systems also exhibit significant antibiotic removal capability. Antibiotic modification is confirmed from the changes in ultraviolet (UV) absorption spectra of the modified antibiotics. Hairy root cultures of *H. annuus* are used as a model system for the experiments conducted to understand the mechanism of phytoremediation. OTC is used as the representative antibiotic for mechanistic experiments, as well as in bioreactor studies. Both *in vivo* and *in vitro* experiments using the hairy root cultures of *H. annuus* are used to demonstrate that reactive oxygen species (ROS) are secreted by the roots. The ROS inactivate OTC through oxidative modification. The rates of OTC oxidation by the

hairy root cultures are enhanced by elicitation of ROS using salicylic acid (SA) and methyl jasmonate (MeJA). Modification of OTC results in oxidation product(s) devoid of anti-microbial activity.

When used to treat wastewater from a dairy cow operation, which was spiked with OTC, root exudates significantly reduce OTC as well as biological oxygen demand (BOD) concentrations. The effects of varying physiological conditions, such as pH, temperature and aeration, on the OTC remediating activity of the root exudates are reported. The ROS-mediated modification of OTC by the root exudates is superior under aerobic conditions.

A novel, integrated bioreactor system is designed for effective removal of OTC from water, under continuous-flow conditions. The design consists of a pond-microcosm for growth of *P. stratiotes*, coupled to a continuous flow reactor for contacting ROS and OTC. The reactor configuration with continuous-stirring gives more efficient OTC removal than a plug-flow configuration.

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Chapter I. Introduction and Background

Introduction

The objective of this doctoral study is to determine the applicability of phytoremediation in removing antibiotic growth promoters, tetracycline (TC) and oxytetracycline (OTC), from water. The later part of this chapter provides the background literature for the antibiotic-pollution problem, earlier studies on removal of the antibiotics, and finally phytoremediation as a possible solution to the problem.

The second chapter addresses the use of the two aquatic species, *Myriophyllum aquaticum* and *Pistia stratiotes*, in removing TC and OTC from the respective plant growth media. Further, confirmation of the modification of the antibiotics by the root exudates is provided with the aid of UV-absorption spectra of the antibiotic species. The third chapter demonstrates the use of *Helianthus annuus* hairy root cultures as a model system to study the mechanism of TC and OTC remediation. The results from the chapter suggest the reported TC/OTC remediation is not directly enzyme-catalyzed, and the possibility of reactive oxygen species (ROS) in the antibiotic modification process.

The fourth chapter provides supporting evidence for the oxidative modification of OTC by the ROS produced in the nutrient medium of *H. annuus* hairy root cultures. The supporting evidence is provided by the enhancement of the OTC-modification activity on

treatment of the root cultures with ROS-elicitors, SA and MeJA. Assays of the antioxidant enzymes, ascorbate peroxidase, catalase, and guaiacol peroxidase, further support the involvement of the ROS. The fifth chapter describes the effects of variation in physiological conditions on the OTC modifying activity of the root exudates.

The sixth chapter explores the possibility of using an integrated bioreactor system in removing OTC from water in a continuous-flow operation. The design consists of a pond-microcosm coupled to a bioreactor. The pond-microcosm, containing live *P. stratiotes*, acts as a source of the root exudates. The bioreactor provides contact between the root exudates and the antibiotics. The performance of two reactor configurations, plug-flow and continuous-stirred, are discussed. The seventh chapter demonstrates the applicability of the root exudates in removing OTC from wastewater samples, collected from a dairy cow operation in Northern Colorado.

Lastly, the conclusions from this doctoral study are provided along with some recommendations for future work. An appendix section follows the conclusions and recommendations section.

Background

Antibiotic growth promoters in livestock operations: Usage, pollution and harmful effects

In the United States, and some parts of Europe, antibiotics are used in large quantities in confined livestock (e.g. swine, poultry and cattle) operations as growth promoters and

feed efficiency promoters [De Liguoro et al., 2003; and Meyer et al., 2000]. Antibiotics are also used in aquaculture for fish production, where they are directly added to the surface water. These antibiotic growth promoters (AGPs) are given to the animals at sub-therapeutic levels. Of 50 million pounds of antibiotics produced in the United States annually approximately 40% are used as AGP [Meyer et al., 2000]. Tetracycline antibiotics are the most widely used class of antibiotics [De Liguoro et al., 2003; Meyer et al. 2000; and Winckler and Grafe, 2001]. The growth promoting responses are generally associated with improved nitrogen and protein metabolism. The feed efficiency is increased by the inhibition of the microbial population in the intestines of the animals. Four mechanisms have been proposed for the growth promoting activity of these AGP: (1) inhibition of mild, sub-clinical infections caused by the microorganisms; (2) reduction of growth-depressing metabolites produced by the microorganism; (3) reduction of microbial destruction of essential nutrients; and (4) enhanced efficiency of absorption and utilization of nutrients through the thinner intestinal wall associated with antibiotic-fed animals [Gaskins et al., 2002; and Visek, 1978].

In humans and animals from 30 to 90% of an administered antibiotic dose is excreted with the urine as active substance [Halling-Sorensen et al., 1998]. Antibiotics released by the animals enter the environment through two main sources. First, the runoff or leakage, emerging from the livestock operations, that eventually ends up in the environment (in a natural water body or soil). Second, the land application of incompletely matured manure; farmers use manure as an organic fertilizer due to its high nutrient content. In a typical livestock operation, the wastewater runoff is collected in a lagoon. Meyer et al.

(2000) report tetracyclines at concentrations up to 0.8 mg/L in hog lagoons. Kolpin et al. (2002) report tetracycline concentrations as high as 0.7 μg/L in water samples taken from rivers across the United States. Although the concentration of the antibiotics found in the rivers are considerably lower than that found in the lagoons, it still means that these antibiotics are persistent enough to be exposed to the environment for considerably long period of time. Tetracyclines are also found in pig manure in concentrations up to 4 mg/L [Thiele-Bruhn, 2003]. Antibiotics are commonly found in sediments of fish farm ponds at concentrations in the range of 0.5 to 4 mg/L [Thiele-Bruhn, 2003]. Thus antibiotics are found in water, soil and sediments, making their exposure to the microorganisms inevitable.

While documenting the occurrence of AGP in the environment, it is important to determine their usage and persistence in a typical livestock operation. In the experiments conducted by De Liguoro et al. (2003) calves were treated with 60 mg/(kg body weight)/day of oxytetracycline (OTC) for 5 days. This dosage level was administered to 50 calves, with an average body weight of 70 kg at the start of the treatment. The fecal matter collected on the first day after the end of the treatment was reported to contain OTC at approximately 871 mg/kg. Though the OTC concentrations decreased with the maturing time for the manure, it took 5 months for the OTC concentrations to decrease to less than 1 mg/kg. In a parallel experiment, where the calves were treated with 20 mg/(kg body weight/day of tylosin (TYL) for 5 days, the TYL concentrations decreased rapidly. The concentrations of the two antibiotics in different matrices (applicable in a livestock operation) are tabulated in Table 1. The antibiotic concentrations listed in Table 1 are

Table 1. Concentrations of OTC and TYL in different waste matrices [De Liguoro et al., 2003]

Matrix	OTC (mg/kg)	Tylosin (mg/kg)
Faeces (day 0)	871.7	115.5
Bedding (day 0)	366.8	32.8
Bedding (day 10)	160.8	5.4
Manure (day 30)	19.0	0.1
Manure (day 45)	8.8	BDL
Manure (day 60)	7.9	BDL
Manure (day 75)	11.9	BDL
Manure (day 105)	6.4	BDL
Manure (day 135)	2.1	BDL
Matured manure (after 5 months)	0.8	BDL

* Bedding refers to the heap of faeces accumulated together to prepare the manure.

** BDL- below detection limit

based on unit mass of the respective matrix (e.g. faeces, bedding or manure). Thus, OTC appears to be more persistent than tylosin, and therefore can be found at high concentrations in wastes generated by livestock operations.

The presence of antibiotics in the environment often results in long-term exposure of these antibiotics to microbes, plants and the animals in the ecosystem. Antibiotics in the environment are suspected to induce antibiotic resistance in bacterial strains, which may cause severe health problems due to the ineffectiveness of antibiotic drugs. Antibiotic resistant strains of *Salmonella, Campylobacter, Escherichia colli* and *Listeria* are suspected or known to exist [EPA, 2001]. In the 2001 EPA report on the CAFO operations two cases of the possible existence of antibiotic resistance bacteria were cited. First, an antibiotic-resistant strain of the bacterium *Clostridium perfringens* was detected in the ground water below plots of land treated with swine manure, while it was nearly absent beneath unmanured plots. Second, a 12-year-old boy in Nebraska was reportedly infected with a strain of *Salmonella* that was resistant to 13 anti-microbial agents. The cause of the illness is believed to be the exposure to cattle at his family ranch. Thus, if uncontrolled, the emergence of more and more antibiotic resistant bacteria can potentially cost millions of dollars in research for discovering new antibiotics.

The input of antibiotics in wastewater may not be the only pathway for the development of antibiotic resistant species of microorganisms. Antibiotic resistant microorganisms, present in manure, are suspected to spread the resistance to other species [Thiele-Bruhn, 2003]; manure generated at livestock operations is generally used in land applications.

The induction of antibiotic resistance in pathogens, after transfer of resistance genes from nonpathogenic to pathogenic microorganisms, has been proposed as a possibility. Mobile genetic elements conferring antibiotic resistance have been readily obtained from microbial communities of environmental habitats [Smalla and Sobecky, 2002]. Thus, land application of matured manure, with little or no antibiotics in it, can still provoke the dissemination of antibiotic resistance in natural habitats.

Although no major toxic effect on plants has been reported, the accumulation of these antibiotics in edible aquatic foods is a cause of concern. The tetracycline concentration in red rock crabs (*Cancer productus*), collected after tetracycline application to the water, exceeded the US FDA limit of 0.1 mg/L [Thiele-Bruhn, 2003]. There is also a possibility of transfer of antibiotic resistance genes to humans, through the food chain. Similarly, if the release of antibiotics (or antibiotic-resistant microorganisms) is uncontrolled, the fauna populations of natural ecosystems may become susceptible to epidemics.

Therapeutic treatment of livestock with antibiotics can also be a significant source of antibiotics in the environment because of higher dosage levels. Extensive use of antibiotics by humans, and the subsequent discharge to the environment through municipal wastewater is another source of antibiotic pollution.

Alternative methods for treatment of antibiotic-contaminated water

Since the EPA has not yet regulated antibiotic compounds in the United States, currently there are no established methods for their removal. Most of the contemporary research

pertaining to antibiotics in the environment is focused on establishing antibiotics as pollutants and studying their effects on the microbial pollution. There are very few references in the literature that address treatment of antibiotic-contaminated water. Of the papers that discuss this topic, a majority of them aim at treating the antibiotics in conventional wastewater treatment plants. Larsen et al. (2004) provides a review of different physical, chemical and biological processes that can be potentially applied to avoid pharmaceutical compounds from entering the aquatic environment.

Physical treatment of antibiotic-contaminated water by sorption to activated carbon, reverse osmosis, and coagulation has been explored as possible solution [Adams et al., 2002]. Physical treatments cannot be a final solution for treatment of the antibiotic-contaminated water, because they would only change the matrix, in which the antibiotic species might be present, the biological activity of the antibiotics would be unaltered. The only purpose of the physical treatments is to concentrate the antibiotics in a confined medium, which prevents environmental exposure. Sorption of organic compounds to activated carbon is commonly used in drinking water treatment plants. Here the contaminant gets adsorbed on the activated carbon through hydrophobic interactions. Adams et al. (2002) observed good removal for several antibiotic species (e.g. carbadox, sulfamerazine, trimethoprim etc.), but all of the studied antibiotics are reasonably hydrophobic in nature, with log $K_{ow} > 0$, where K_{ow} is the octanol-water partition coefficient. The octanol-water partition coefficient is the ratio of the concentration of a compound in octanol to that in water, which would result when the compound is added to a 50:50 octanol-water mixture. Effective removal of polar antibiotics in such processes

can be difficult [Hirsh et al., 1999] because they pass through the hydrophobic beds. Reverse osmosis involves the pressure-driven diffusion of water from a dilute to a concentrated solution across a semi-permeable membrane, which allows only the passage of water. Reverse osmosis has proven effective in removing antibiotics from water [Adams et al., 2002], but it is an expensive process that is generally used only at drinking water treatment plants; it is not feasible for wastewater treatment and again simply concentrates the problem. Coagulation is the process by which chemicals (coagulants) added to water bring about physical agglomeration, followed by flocculation or sedimentation, of colloidal particles. Coagulation with metal salts (like aluminum sulfate and ferrous sulfate) gave no significant removal of the antibiotics [Adams et al., 2002].

Almost all antibiotics used in livestock operations are organic compounds with aromatic or benzyl moieties in their structures (e.g. tetracyclines, sulfonamides, quinolones, and other macrolides). Such compounds usually have unsaturated carbon-carbon double bonds and/or other relatively unstable hydroxyl (OH) groups, attached to the ring structures. These functional groups can act as sites for oxidation by various chemical, biological and electrochemical processes. Ozonation and chlorination reactions provide good antibiotic removal in quick time [Adams et al., 2002; Balcioglu and Otker, 2003; and Larsen et al., 2004]. Balcioglu and Otker (2003) report significant antibiotic removal on treatment with hydrogen peroxide and ozone. Hofl et al. (1997) demonstrate high COD removal for pharmaceutical wastewater using advanced oxidation processes such as treatment with Fenton's reagent (Fe^{2+} + hydrogen peroxide); ozonation in the presence of hydrogen peroxide; and ultraviolet irradiation in the presence of hydrogen peroxide. All

of these processes are known to produce reactive radicals or reactive oxygen species that eventually oxidize the substrates. Weichgrebe et al. (2004) report the use of electrochemical oxidation for successful removal of tetracyclines from wastewater, and evidence, though inconclusive, on the inhibition of antimicrobial properties of tetracycline is also presented.

Studies on biodegradation of veterinary antibiotics, Olaquindox (OLA), metronidazole (MET), tylosin (TYL) and oxytetracycline (OTC), are reported elsewhere [Ingerslev et al., 2001]. At concentrations between 0.05 and 5 mg/L the ranges of half-lives, assuming first-order kinetics, for aerobic degradation of the four antibiotics are between four and eight days (OLA), 9.5-40 days (TYL), 14-104 days (MET) and 42-46 days (OTC). The degradation rate increases with the increased microbial activity from addition of either 1 g/L of sediment or 3 g/L of activated sludge from a wastewater treatment facility. The biodegradation is found to be significantly slower in the absence of oxygen.

Phytoremediation

Phytoremediation is the use of vegetation to contain, sequester, remove, modify or degrade inorganic and organic contaminants in soils, sediments, surface waters and groundwater [Tsao, 2003]. Plants can be used to treat systems containing many types of contaminants, including petroleum hydrocarbons, chlorinated solvents, pesticides, metals, radio-nuclides, explosives, and excess nutrients. Certain species of herbs, shrubs, and trees can concentrate specific organic compounds and heavy metals at levels much greater than can the average spectrum of plants [Ouyang, 2002]. Those particular plant

systems use a variety of processes that may collectively contribute to the overall level of remediation. These processes include: (i) modifying the physical and chemical properties of contaminated soils; (ii) releasing root exudates, that may conjugate with or degrade the pollutant; (iii) improving aeration by releasing oxygen directly to the root zone, as well as increasing the porosity of the upper soil zones; (iv) intercepting and retarding the movement of chemicals; (v) effecting co-metabolic microbial and plant enzymatic transformations of recalcitrant chemicals; and (vi) decreasing vertical and lateral migration of pollutants to ground water by extracting available water and reversing the hydraulic gradient [Susarla et al., 2002].

Some of the factors affecting chemical uptake and distribution of the contaminants within living plants include: (a) physical and chemical properties of the compound (e.g. water solubility, vapor pressure, molecular weight, and octanol-water partition coefficient, *K*ow); (b) environmental characteristics (e.g. temperature, pH, organic matter, and soil moisture content); (c) plant characteristics (e.g. type of root system, and type of enzymes).

Based on the biological mechanisms adopted by plants to remediate the contaminants, phytoremediation is classified either as phytoextraction, phytoaccumulation, phytostabilization, phytotransformation, phytovolatilization or rhizodegradation [Macek et al., 2000; Meagher, 2003; Salt et al., 1995; and Susarla et al., 2002]. Phytoextraction refers to the physical removal of the contaminant from the soil or wastewater by the use of live plant or plant systems. If the plant purely accumulates the contaminant in its

tissues or cell walls, with little or no degradation, then the process is termed as phytoaccumulation. Phytostabilization refers to the minimization of migration of contaminants in the soil; the plant takes advantage of the ability of the roots to alter soil environmental conditions such as pH and moisture. In some cases plant roots exudate (secrete) chemicals that cause the precipitation of compounds. There have been some suggestions that plant roots are negatively charged and thus attract the positively charged metal ions to effect phytostabilization of the toxic metals. Phytotransformation deals with the degradation of the organic waste into smaller, simpler and less toxic compounds. In phytovolatilization, the organic pollutants are broken down into volatile compounds, which are either released in the environment or are used up by the plant. Lastly, rhizodegradation generally deals with the complex interaction between the roots, contaminant and soil microorganisms. The plant modifies the geological environment in the rhizosphere and supplements chemicals sufficient to sustain the microorganisms that assimilate, metabolize or react with the contaminants.

There are some distinct advantages associated with phytoremediation that can make it a good choice for certain types of contaminant remediation. Phytoremediation, usually, does not involve high capital or operating costs. Phytoremediation is solar driven so the energy requirements are low. Plants are usually non-specific when dealing with toxic metals or organic compounds so a greater number of pollutants can be remediated by plants, while pure culture microbiological remediation systems usually are specific with respect to the type of contaminants. One major advantage associated with phytoremediation is that it can be applied to remediate heavy metals, toxic elements and

radioactive materials, which cannot be easily achieved using microbiological systems. In microbial systems sudden variations in the influx of the contaminants, or the nature of the contaminants can kill or destabilize the functional microbiota while plant are able to withstand temporary changes in the physiological conditions. One more striking feature about phytoremediation is the capacity to reduce very low concentrations of a pollutant to nearly zero.

Phytoremediation is relatively new and there are many aspects to be studied before it can be universally applied. To start with, longer times are required than the other available technologies to remediate the same amount of contaminant. Due to the possible toxic effects, there is an upper limit on the concentration of the pollutant that can be treated. In the case of phytoaccumulation, the contaminants may find a way back in to the ecosphere through litter fall or plant death. Accumulation may occur in fuel woods and upon combustion be released to the environment [Macek et al., 2000]. High concentrations of hazardous contaminants may prove to be toxic to the plant. Cycling of hazardous contaminants to the other plants and animals that acquire nutrients from these plants may occur. Phytoremediation is generally restricted to shallow groundwater, soils and sediments. [Fedoroff and Cohen, 1999; Ouyang, 2002; Salt et al., 1995; and Susarla et al., 2002]. While the ability of the rhizosphere to alter the conditions is beneficial, the result may be harmful, such as an increase in the solubility levels of a particular contaminant [Macek et al., 2000]. Lastly, this technology is still in initial stage of development. Research needs to be conducted before applicability of such systems can be questioned and/or accepted.

As of now, one hope of phytoremediation is to turn abandoned non-productive lands into green and fertile ones at very low costs. It is being increasingly considered as a polishing step for wastewater treatments. The cost effectiveness of this method actually drives the growth of this industry, for instance, other in-situ and ex-situ methods of remediation cost anywhere between $10-$300 per cubic meter of contaminated land, while phytoremediation can cost as low as $0.05 per cubic meter of the land [Watanabe 1997]. The market potential has also been increasing in the last few years; in 1998 the United States phytoremediation market was worth between $16.5 -$ 29.5 million, in 2000 it went up to $55-$103 million and by the end of 2005 it is expected to reach a figure anywhere between $214-$370 million [Flathman and Lanza, 1998]. The industry seems to be on a rise and analysts are quiet positive about it. In some specific cases of contaminants like heavy metals and highly toxic compounds, where other methods fail, phytoremediation prevails. There is a need to accelerate the research work in this field, and more funds should be made available. With the EPA restrictions getting stringent, one can project a prospering phytoremediation industry.

Previous work done on phytoremediation of antibiotics

Azolla filiculoides, an aquatic fern, has shown capability to remove the antibiotic sulphadimethoxine from an aqueous phase [Forni et al., 2001; and Forni et al., 2002]. In the experiments carried out by Forni et al. (2002), the removal observed was ranging from 55.7% to 86.3%, for the treatments with 50 and 450 mg/L drug in the media, respectively, in a 5-week period. The sulphadimethoxine remediation activity, partitioned on the basis of the processes found to be involved, is summarized in Figure 1. T50, T150,

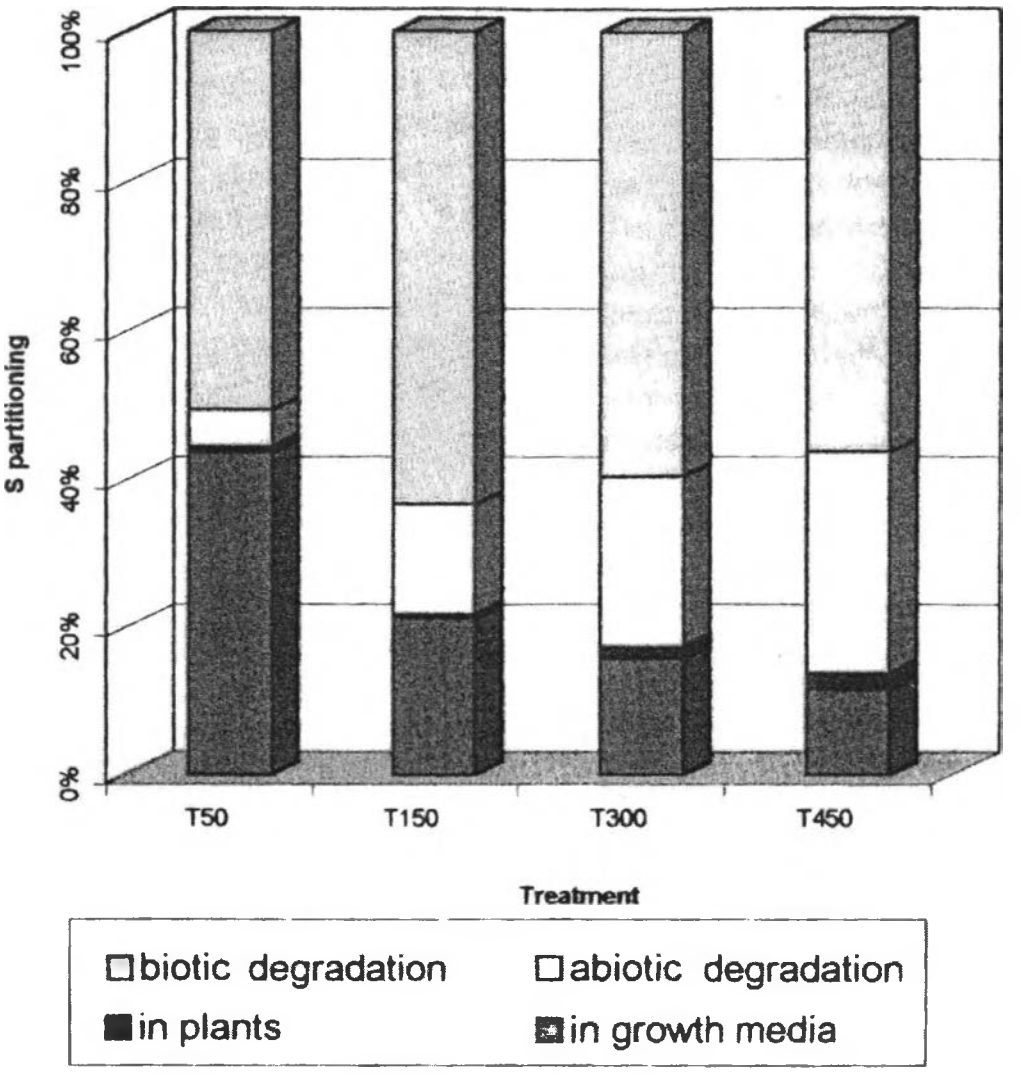

Figure 1. Suphadimethoxine fate in *Azolla filiculoides* [Forni et al. 2002]

T300 and T450 represent the treatment concentrations of 50, 150, 300 and 450 mg/L, respectively. As shown in Figure 1, most of the antibiotic remediation activity is attributed to biotic degradation, either in the growth medium or inside the plant body. The authors do not define the distinction between phytodegradation (degradation due to the plant-secreted compounds) and rhizodegradation (degradation assisted by microbial activity in the growth medium). The source for abiotic degradation is also not mentioned. The plants take up a very small fraction (up to 3%) of the antibiotics. A significant fraction of the antibiotics, for all the treatment concentrations, is left in the growth medium. *Lythrum salicaria*, a perennial weed found in freshwater marshes, has also shown capability of taking up sulphadimethoxine [Forni et al., 2001].

Plant systems used

The plants/plant systems studied to remediate TC and OTC are:

1. Hairy root cultures of *Helianthus annuus* (sunflower), grown in an aseptic Gamborg B5 plant medium supplemented with sucrose.
2. *Myriophyllum aquaticum* (parrot feather) and *Pistia stratiotes* (water lettuce) grown in Hoagland's basal salt medium.

In addition to the three plant systems for this antibiotic-phytoremediation, the late Dr. Rajiv Bhadra in the Department of Chemical Engineering at Colorado State University initiated this project using *Eichhornia crassipes* (water hyacinth) as well. The project was allotted funds a couple of weeks after his sad demise, at which point Professor James C. Linden kindly agreed to guide me through this project as the advisor of my doctoral

studies. There is not much information about the ideas that initiated the design of this project by Dr. Bhadra. Also, it would be worth mentioning that according to the project proposal submitted by Dr. Bhadra, his initial understanding was that the plant systems accumulate the two antibiotics in their tissues. But, as the later sections of this dissertation would illustrate, the *H. annuus* root cultures and the aquatic plants oxidize the antibiotic through the action of the reactive oxygen species that are secreted in the nutrient media.

H. annuus is a proven accumulator for several elements and nutrients [Madejon et al., 2003]. *P. stratiotes* (See Figure 2) and *M. aquaticum* (See Figure 3) have been used for uptake and accumulation of trace metals [Qian et al., 1999]. *M. aquaticum* is also reported for phytodegradation of TNT and other organic pollutants [Hughes et al., 1997; and Susarla et al., 1999]. Further discussion on the two aquatic species is provided in Chapter II, while that on the hairy root cultures is provided in Chapter III.

Once it was shown that the above plant systems remediated TC and OTC from the nutrient media, *H. annuus* root cultures (See Figure 4) were primarily used as the model system to study the mechanism of phytoremediation involved. Transformation of plants using *Agrobacterium rhizogenes* produces the hairy roots (Giri and Narasu, 2000; and Hamill and Lidget, 1997). Hairy roots are usually maintained in plant growth medium supplemented with sucrose. Hairy root cultures provide excellent models for studying pollutant/root interactions.

Figure 2. *P. stratiotes* (water lettuce).

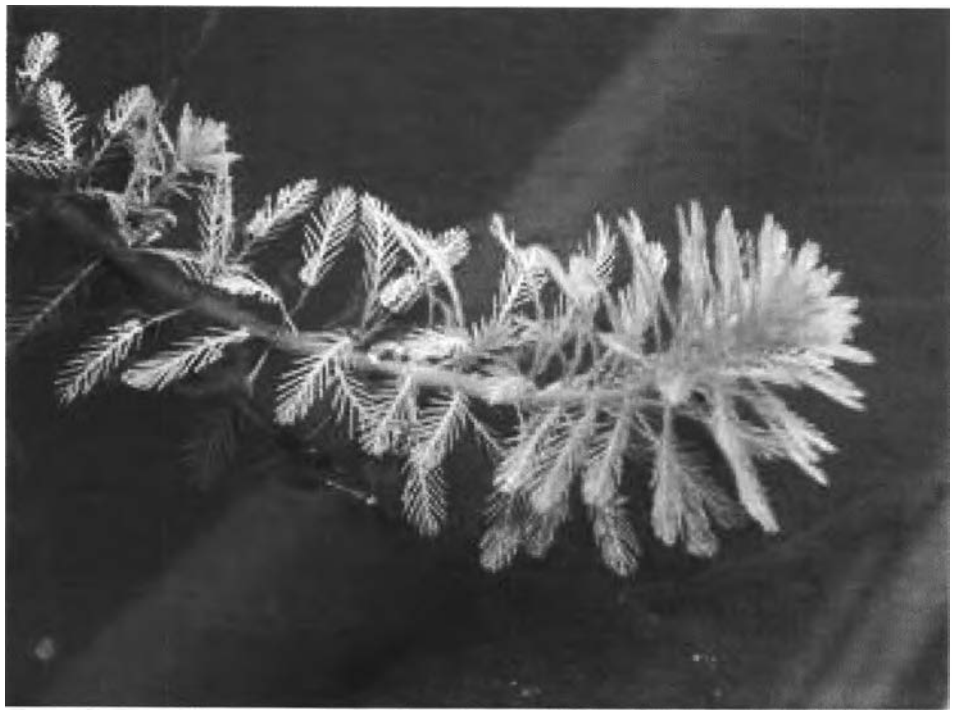

Figure 3. *M. aquaticum* (parrot feather).

Figure 4. *H. annuus* root culture in a 250-mL Erlenmeyer flask.

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Chapter II. Phytoremediation Potential of *Myriophyllum aquaticum* and *Pistia stratiotes* to Modify Antibiotic Growth Promoters, Tetracycline and Oxytetracycline, in Aqueous Wastewater Systems

Reference: Gujarathi, N. P.; Haney, B. J.; and Linden, J. C. P*hytoremediation potential of* Myriophyllum aquaticum *and* Pistia stratiotes *to remove antibiotic growth promoters, tetracycline and oxytetracycline, from aqueous systems.* **International Journal of Phytoremediation** (In press, 2005)

Abstract

Antibiotics are frequently used in the United States as feed efficiency promoters and medicines for livestock destined for human consumption. These antibiotics are released into the environment through the run-off and wastewater streams from animal feedlots and land applications of manure. The exposure of microorganisms to these antibiotics has reportedly resulted in the development of resistant species of microorganisms, which in turn can lead to human health hazards. Phytoremediation of these antibiotics can be a useful tool for countering this problem. The aquatic plants, *Myriophyllum aquaticum* (parrot feather) and *Pistia stratiotes* (water lettuce), were used for studying phytoremediation of tetracycline (TC) and oxytetracycline (OTC) from aqueous media. TC and OTC are two of the most commonly used tetracyclines in veterinary medicine. *M. aquaticum* and *P. stratiotes* gave high antibiotic modification rates of both antibiotics.

Kinetic analyses dismiss direct enzyme catalysis; the modification rates decreased with increasing OTC concentrations. Sterile, cell-free root exudates (filtered through 0.2 μm membranes) from both species also exhibited comparable antibiotic modification rates. The involvement of root-secreted metabolites in antibiotic modification is suggested. The changes in the UV absorbance spectra of OTC during treatment with the root exudates confirmed the modification.

Key terms: Antibiotic pollution, root exudates, UV spectra, aquatic plants.

Introduction

Antibiotics used as antibiotic growth promoters (AGP) increase the efficiency of animal growth by inhibiting microorganisms and their growth-depressing metabolites that are often present in the gastrointestinal tracts of the animals. Another important effect is the enhanced uptake and use of nutrients through the thinner intestinal wall associated with antibiotic-fed animals [Gaskins et al., 2002]. Of 50 million pounds of antibiotics produced in the United States annually, approximately 40% are used as AGP or feed efficiency promoters [Meyer et al., 2000]. In humans and animals, from 30 to 90% of the antibiotics are excreted in the urine as active substances [Halling-Sorensen et al., 1998]. Antibiotics added to animal feeds are eventually released to the environment through run-off streams from the feedlots or leakage streams from storage structures. Use of animal manure as fertilizer also results in a substantial exposure of the antibiotics to the environment. Meyer et al. (2000) reported tetracyclines at concentrations up to 0.8 mg/L in hog lagoons. Kolpin et al. (2002) reported tetracycline concentrations as high as 0.7

μg/L in water samples taken from rivers across the United States. Although the concentrations of the antibiotics found in the rivers were considerably lower than those found in the lagoons, it still meant that these antibiotics were persistent enough to be exposed to the environment for considerably long period of time. Tetracyclines have also been found in pig manure in concentrations up to 4 mg/L [Thiele-Bruhn, 2003]. Antibiotics are commonly found in marine sediments underneath fish farms at concentrations in the range of 0.5 to 4 mg/L [Thiele-Bruhn, 2003]. Thus antibiotics are found in water, soil and sediments, making their exposure to the microorganisms inevitable.

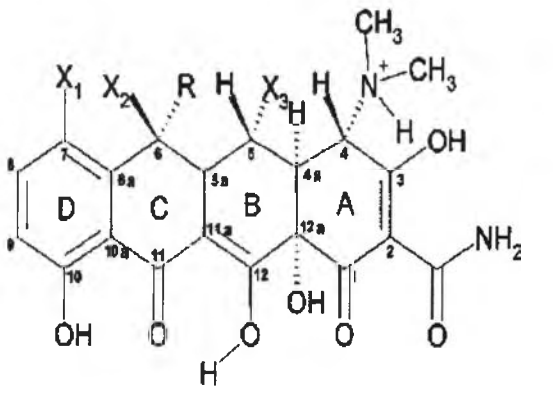
The presence of antibiotics in the environment often results in long-term exposure of these antibiotics to microbes, plants and animals in the ecosystem. Although these antibiotics are found at low concentrations in lakes and rivers (< 1 mg/L) [Halling-Sorensen et al., 1998], the effect on microbial populations is a cause of concern. Antibiotics in the environment are suspected to induce antibiotic resistance in bacteria, which may cause severe health problems due to increasing ineffectiveness of antibiotic drugs. Antibiotic resistant bacterial strains of *Salmonella, Campylobacter, Escherichia coli, Clostridia* and *Listeria* are suspected or known to exist [EPA, 2001]. In the 2001 EPA report on concentrated animal feeding operations (CAFO), two cases of the possible existence of antibiotic resistant bacteria were cited. First, an antibiotic-resistant strain of *Clostridium perfringens* was detected in the ground water below plots of land treated with swine manure, while it was nearly absent beneath unmanured fields. Second, a 12-year-old boy in Nebraska was reportedly infected with a strain of *Salmonella* that was

resistant to 13 anti-microbial agents. The cause of the illness is believed to be the exposure to cattle on the family ranch. Thus, if uncontrolled, the emergence of more and more antibiotic resistant bacteria can potentially cost millions of dollars for medical treatment.

Some of the AGP, e.g. tylosin, degrade naturally in a period of time [De Liguoro et al., 2003]; these are not a major cause of concern. For antibiotics that are fairly persistent in nature, e.g. the family of tetracyclines, means of removal or modification need to be determined. Adams et al. (2002) suggest employing carbon sorption and oxidation with ozone or chlorine species at the surface water treatment plant for reducing effective concentrations of some antibiotics. The deployment of such treatment plants at farm locations may be beyond the feasible limits of both individual farmers and government agencies that regulate run-off pollution. According to Adams et al. (2002), the conventional water treatment processes, coagulation/flocculation/sedimentation with alum or ferric salts, ultraviolet radiation, and water softening result in little or no removal of the studied antibiotics. Reverse osmosis (RO), which was found to be effective in removing the antibiotics, results in a concentrated waste stream that needs further treatment due to the higher concentrations of the antibiotics. Most of the antibiotic removal in wastewater treatment plants is brought about by adsorption on the surface of activated sludge through hydrophobic interactions; effective elimination of polar antibiotics is difficult.

Phytoremediation is the use of vegetation to contain, sequester, remove, modify or degrade inorganic and organic contaminants in soils, sediments, surface waters and groundwater [Tsao, 2003]. Plants can be used to treat systems containing many types of contaminants, including petroleum hydrocarbons, chlorinated solvents, pesticides, metals, radionuclides, explosives, and excess nutrients. Certain species of herbs, shrubs, and trees can concentrate specific organic compounds and heavy metals at levels much greater than can the average spectrum of plants [Ouyang, 2002]. Those particular plant systems use a variety of processes that may collectively contribute to the overall level of remediation. These processes include: (i) modifying the physical and chemical properties of contaminated soils; (ii) releasing root exudates, that may conjugate with or degrade the pollutant; (iii) improving aeration by releasing oxygen directly to the root zone, as well as increasing the porosity of the upper soil zones; (iv) intercepting and retarding the movement of chemicals; (v) effecting co-metabolic microbial and plant enzymatic transformations of recalcitrant chemicals; and (vi) decreasing vertical and lateral migration of pollutants to ground water by extracting available water and reversing the hydraulic gradient [Susarla et al., 2002]. *Azolla filiculoides*, an aquatic fern, has shown capability to remove sulphadimethoxine from an aqueous phase [Forni et al., 2001]. In experiments carried out by Forni et al. [2002], the observed antibiotic removal ranged from 56% to 86% in a 5-week period, for aqueous treatments containing 50 and 450 mg/L of drug, respectively.

TC and OTC are fairly hydrophilic in nature with log K_{ow} (octanol-water partition coefficient) values of –1.19 and –1.22 and aqueous solubilities of 1700 and 1000 mg/L,



	X_1	X_2	R	X_3
Tetracycline	H	OH	CH_3	H
Minocycline	$N(Me)_2$	H	H	H
Oxytetracycline	H	OH	CH_3	OH
Doxycycline	H	H	CH_3	OH
Methacycline	H	$=CH_2$		OH

Figure 1. General chemical structure of tetracycline derivatives [Leypold et al., 2003].

respectively [Tolls, 2001]. The general structure of tetracycline derivatives is shown in Figure 1. High performance liquid chromatography (HPLC), coupled with UV detection, is most commonly used for determining the concentrations of tetracycline antibiotics in water. The UV absorption spectra of tetracyclines (See Figure 2 for the spectrum for OTC) are characterized by two maxima, one at around 270 nm and another at about 360 nm. The maximum at 270 nm is attributed to the UV absorption by the so called A chromophore, while the maximum at 360 nm is from the UV absorbing BCD chromophore [Aly et al., 2002; Leypold et al., 2003; Schmitt and Schneider, 2000]. The A chromophore consists of the diketone moiety, amide group and a hydroxyl group in the A-ring. The BCD chromophore involves the phenyl D ring and the □-hydroxyketo system extending across the carbonyl group and the ketone group, in the C and B rings, respectively.

M. aquaticum is a submerged, oxygenating species; while *P. stratiotes* is a floating, non-oxygenating species. Both have been used for uptake and accumulation of trace metals [Qian et al., 1999]. *M. aquaticum* is also known for phytodegradation of TNT and other organic pollutants [Hughes et al., 1997; Susarla et al., 1999]. These aquatic plants are used in this study to determine rate coefficients for the modification of TC and OTC by both living plant and cell free root exudates systems.

Materials and Methods

Plant growth conditions

Fifteen *M. aquaticum* propagules were purchased from a garden shop and transferred to an aquatic tank filled to 5 cm depth with 100% strength Hoagland's modified basal salt mixture [Phytotechnology Laboratories, Shawnee Mission, KS] in water, hereafter referred to as Hoagland's medium. These propagules were maintained for over a month in open aquatic tanks at room temperature with exposure to light from a north-facing window until they propagated to produce sufficient plant material required for the experiments.

Similarly, *P. stratiotes* were maintained in aquatic tanks containing 25% strength Hoagland's medium for over a month. Leaves of *P. stratiotes* that were transferred to 100% strength Hoagland's medium wilted badly and turned yellow. Both the plant species were transferred to the experimental flasks or jars 8-10 days before the start of the experiments to acclimatize the plants to fresh medium. Rhizospheric compounds were thereby allowed to accumulate in the medium before the addition of the antibiotics. For *M. aquaticum* the period of 8-10 days was important to allow the propagules to produce roots; the *P. stratiotes* propagules were already rooted. *M. aquaticum* propagules were cut to approximately 30 cm in length and transferred to 250-mL Erlenmeyer flasks containing 125 mL of freshly made 100% strength Hoagland's medium. The *P. stratiotes* propagules were transferred to 800-mL mason jars containing 300 mL of 25% strength Hoagland's medium.

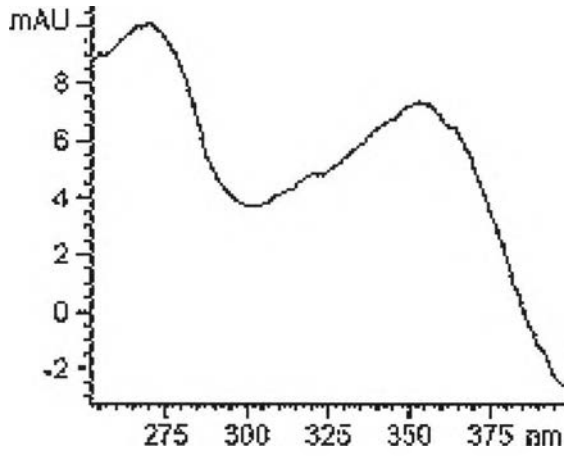


Figure 2. UV absorption spectrum of OTC dissolved in RO water.

Experimental conditions

All antibiotic-plant interactions described in this study were conducted under static conditions at room temperature (~25°C) and fluorescent lighting [General Electric F40PL Gro and Sho Plant Light, Cleveland, OH] using aqueous solutions of the antibiotics. Stock solutions of TC and OTC [Sigma-Aldrich, St. Louis, MO] were prepared and added to the aqueous systems consisting of either the plants or the plant-derived root exudates. All the experiments described in this study involved the addition of either TC or OTC, but not both, to the aqueous systems. As described earlier, the stock solutions were added to the plants 8-10 days after they were transferred to the experimental flasks/jars. Triplicates for each concentration were used to account for error due to variability. Control experiments were conducted to check if the antibiotics degraded both in light and in dark due to photolysis or natural degradation, respectively. For the control experiments, the antibiotics were added to 100% strength Hoagland's medium, without plants. The light control was placed under artificial plant lighting the same fluorescent lighting as the plant. The dark controls were placed in an enclosure completely devoid of light, but at the same temperature as experimental conditions.

Growth media from the two plant systems was filtered through 0.2 μm membrane filters (Millipore, Bedford, MA) to prepare solutions of root exudates. The media were removed from the plants after variable periods of growth, both in the presence and absence of TC and OTC. Filtration ensured no live cells and/or cell debris, or microorganisms would interfere in exudate/antibiotic interaction studies. Experiments involving the study of

interactions between the root exudates from the two plant species and the antibiotics were carried under similar conditions as those involving live plants.

Sampling

The 0^{th} day samples were taken 20 minutes after addition of the antibiotics; this was done in order to allow the antibiotics to mix uniformly in the liquid medium. The 0^{th} day samples represented the initial concentrations of the antibiotic for the remediation studies. A 1 mL micro-pipettor was used to withdraw about 0.9 ml of the sample from the flasks/jars. For the first two days, samples were taken after every 12 hours; thereafter samples were taken after intervals of one day or more. Preliminary experiments had shown that maximum remediation activity took place in the first two days after the addition of the antibiotics. The samples were collected in 1.5-mL Eppendorf centrifuge tubes and immediately frozen in order to inhibit further antibiotic degradation.

Sample preparation for HPLC

After thawing, samples were mixed to eliminate concentration gradients in the tube that result due to freezing. The Eppendorf tubes containing these samples were then centrifuged in a micro-centrifuge [Brinkman Instruments Inc., Westbury, NY] for 10 minutes. The supernatant from the centrifuged samples was then filtered through a 0.45 μm centrifuge tube filter [Millipore, Billerica, MA] and transferred to a 250 μL silanized inert insert [Agilent Technologies, Wilmington, DE] before placement in an HPLC auto-sampler sample vial [Agilent Technologies, Wilmington, DE].

HPLC method

A HPLC system [Hewlett Packard 1050 series] with a photodiode array detector was used with a 250 x 4.6 mm C-18 column [Phenomenex, Torrance, CA] for analyzing the antibiotic concentrations in the samples. The mobile phase consisted of a 20 mM sodium phosphate buffer solution (solvent A) and 2-propanol (solvent B). The pH of both the solvents, A and B, was maintained at pH 2.5 by adding either trifluoroacetic acid or formic acid at 0.1% concentration by volume. Before each sample injection, the column was preconditioned with the solvent A to ensure optimum antibiotic adsorption on to the column. An 82-18 ratio of solvent A to B was used to elute the antibiotics from the column. A combined flow rate of 1 mL/minute of the solvents was used. The concentrations of TC and OTC were determined by measuring their UV absorbance at 365 and 355 nm, respectively. The retention time for the two antibiotics was found be in the range of 5.9 minutes. Periodically, slight changes in the retention times of the antibiotics were encountered, probably due to increase in the pressure drop across the column. The increase in the pressure drop, across the column, resulted mostly due to the build up of various root-secreted compounds that accompanied the antibiotics in the samples. The detection limit for the method was 0.2 mg/L of TC and OTC. Note that the same HPLC method was used for both TC and OTC because the two antibiotics were never used simultaneously for any experiment.

UV spectra of OTC

The diode array in the UV detector of the HPLC system enabled the acquisition of spectral data for the antibiotic samples. Spectrophotometer scans showing the UV spectra

of the samples were confounded by the high density of root-secreted compounds in the samples. The chromatographic separation of the antibiotic species from the samples ensured that a UV-spectrum, specific to the antibiotic (modified or unmodified), was obtained. The change in the characteristics of the UV spectra for the samples was used as the basis to investigate the modification of the antibiotics. All UV spectra for OTC reported in this study were obtained for the antibiotic species that were chromatographically separated from their respective solutions, either in RO water or in root exudates.

Results and Discussions

Control experiments

The control experiments, both light and dark controls, showed no significant levels of antibiotic degradation. The results for OTC are shown in Figure 3, and those for TC are shown in Figure 4. The increase in the antibiotic concentrations was observed due to evaporation from the flasks/jars. The flasks were refilled with RO water to the initial level following sampling on day 3 for OTC and day 4 for TC. These experiments showed that there was little or no antibiotic degradation (natural or by light) in four to six days under the conditions of the experiments.

Antibiotic modification by *P. stratiotes*

Live *P. stratiotes* and root exudates *P. stratiotes*, both gave almost complete modification of OTC (Figure 5) and TC (Figure 6) from the liquid medium. Since, 0.2 μm filtered, cell-free root exudates derived from the plants gave comparable antibiotic modification

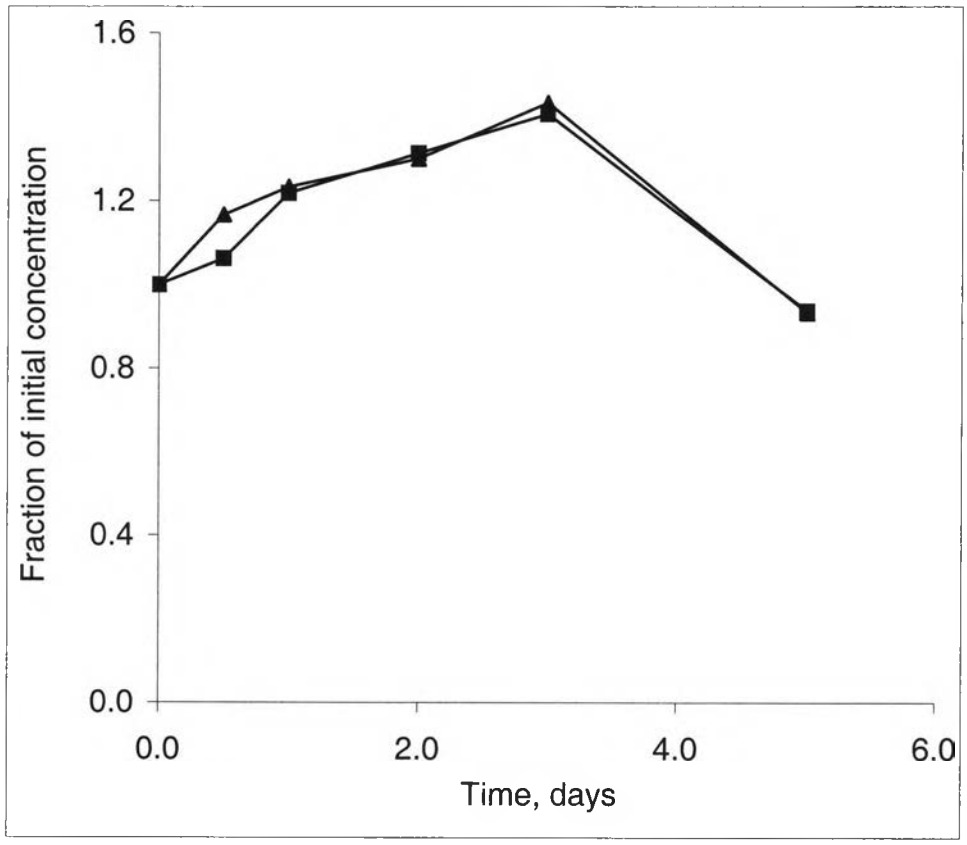


Figure 3. OTC exposed to Hoagland's medium, under light (□) and in dark (□).

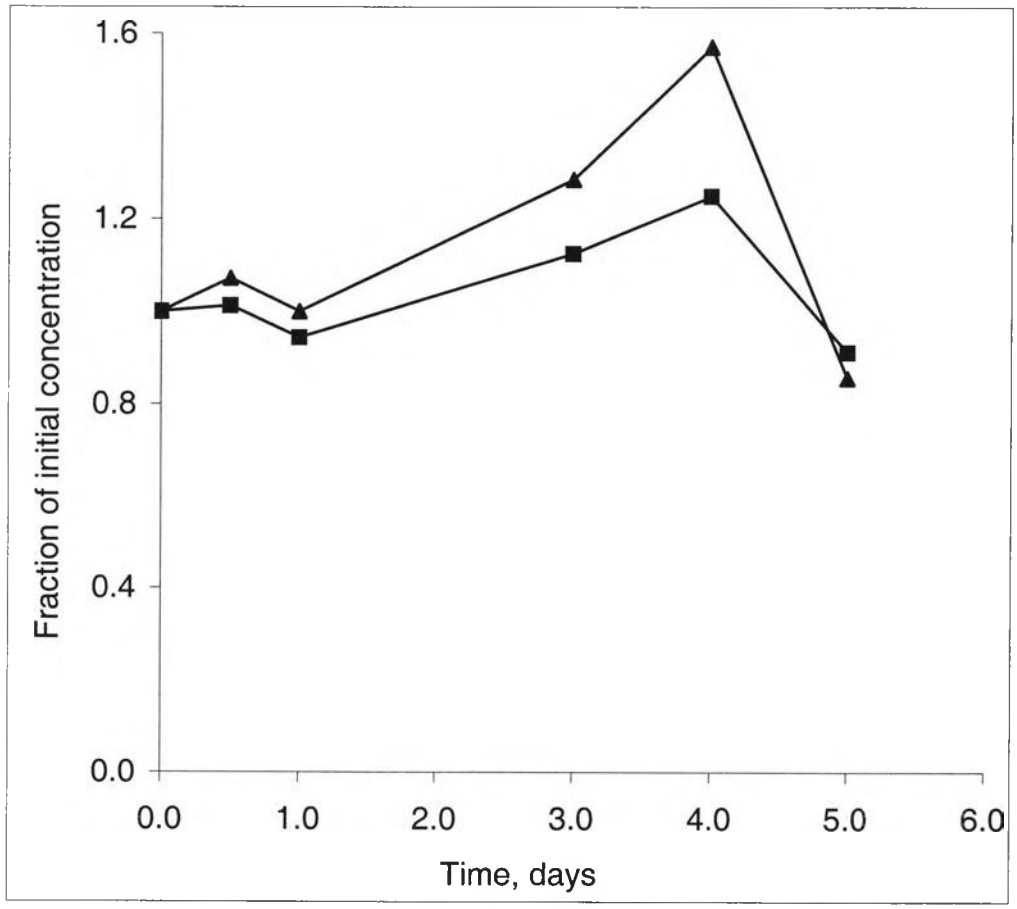


Figure 4. TC exposed to Hoagland's medium, under light (□) and in dark (□).

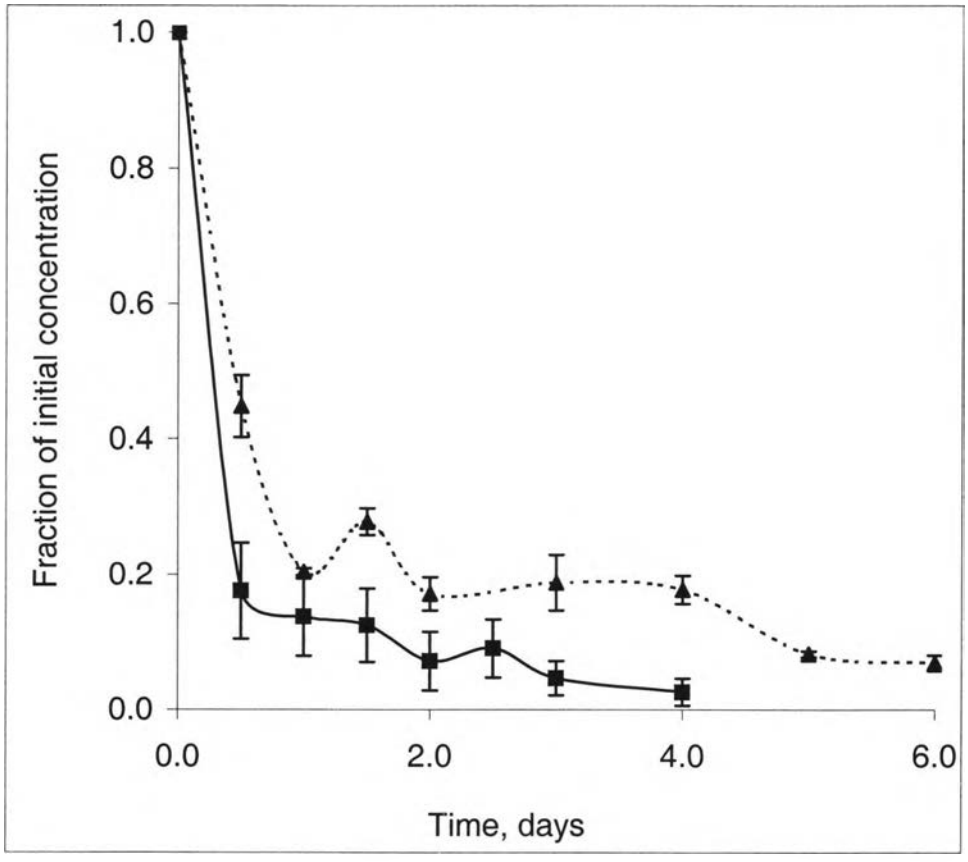


Figure 5. OTC disappearance from the liquid medium on exposure to *P. stratiotes*, live plants (□) and root exudates (□), at 5 mg/L treatment concentrations.

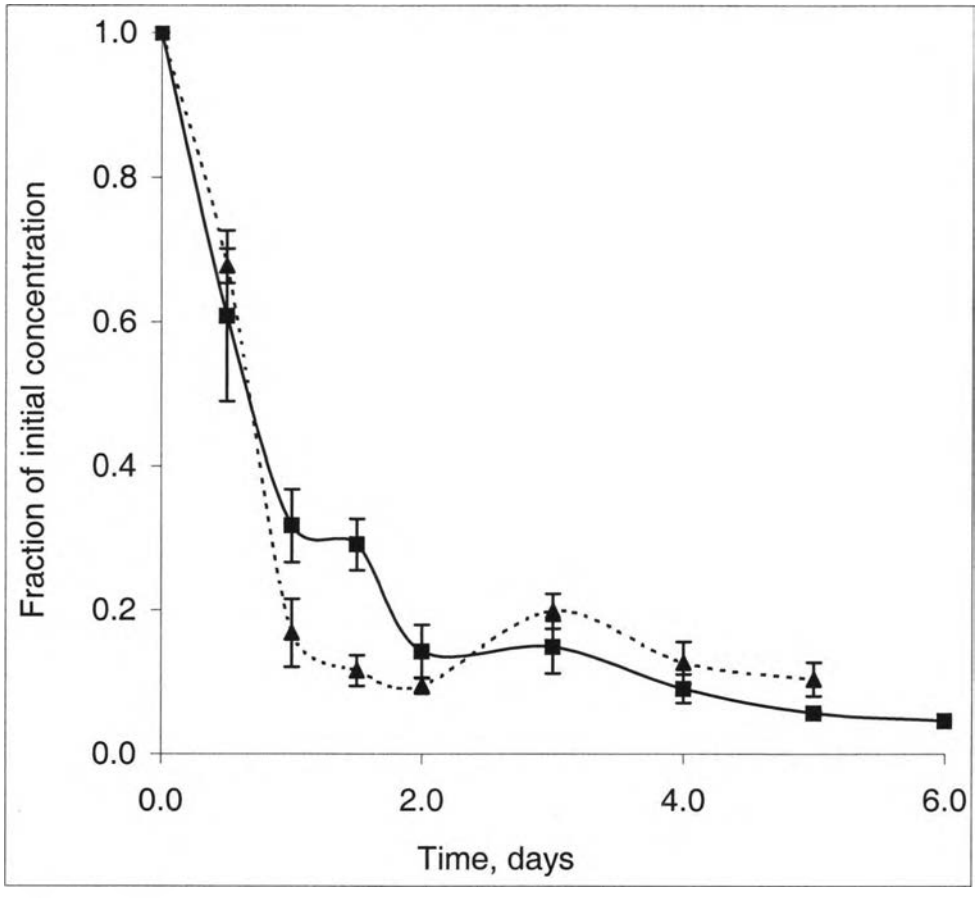


Figure 6. TC disappearance from the liquid medium on exposure to *P. stratiotes*, live plants (□) and root exudates (□), at 5 mg/L treatment concentrations.

rates, the uptake of the antibiotics by the plants or adsorption to the root surface were ruled out; likewise involvement of microorganisms in antibiotic modification is eliminated. This observation also suggests that some component of the root exudates in the liquid medium might have a more significant effect on antibiotic modification than did the total plant biomass. Similar results were obtained for other antibiotic concentrations (1.0, 2.5, 7.5 and 10.0 mg/L). Exponential decay, characterized by a first-order curve fit, suitably represented the antibiotic concentration changes vs. time data.

Figures 5 and 6 show that most of the antibiotic modification occurred during the first 24 hours after antibiotic addition, after which the disappearance rate decreased. Such phenomena may be due to the involvement of root-secreted enzyme(s)/metabolite(s) that may be involved in antibiotic degradation/modification. The higher availability of these compounds at the start of the experiment (and the consequent decreasing availability on subsequent interactions with antibiotic molecules) would result in a decrease in modification rate with time. The first order rate coefficients obtained for the five treatment concentrations of OTC declined with increasing concentration (Figure 7). Similar results were obtained for TC (data not shown). The decrease in the rate coefficient value at higher initial antibiotic concentrations suggests the involvement of metabolites/radicals that might be present in limiting concentrations.

Antibiotic modification by *M. aquaticum*

Live plants from *M. aquaticum* gave almost complete modification of OTC (Figure 8) and TC (Figure 9) within 15 days. The experiments with filtered root exudates from *M.*

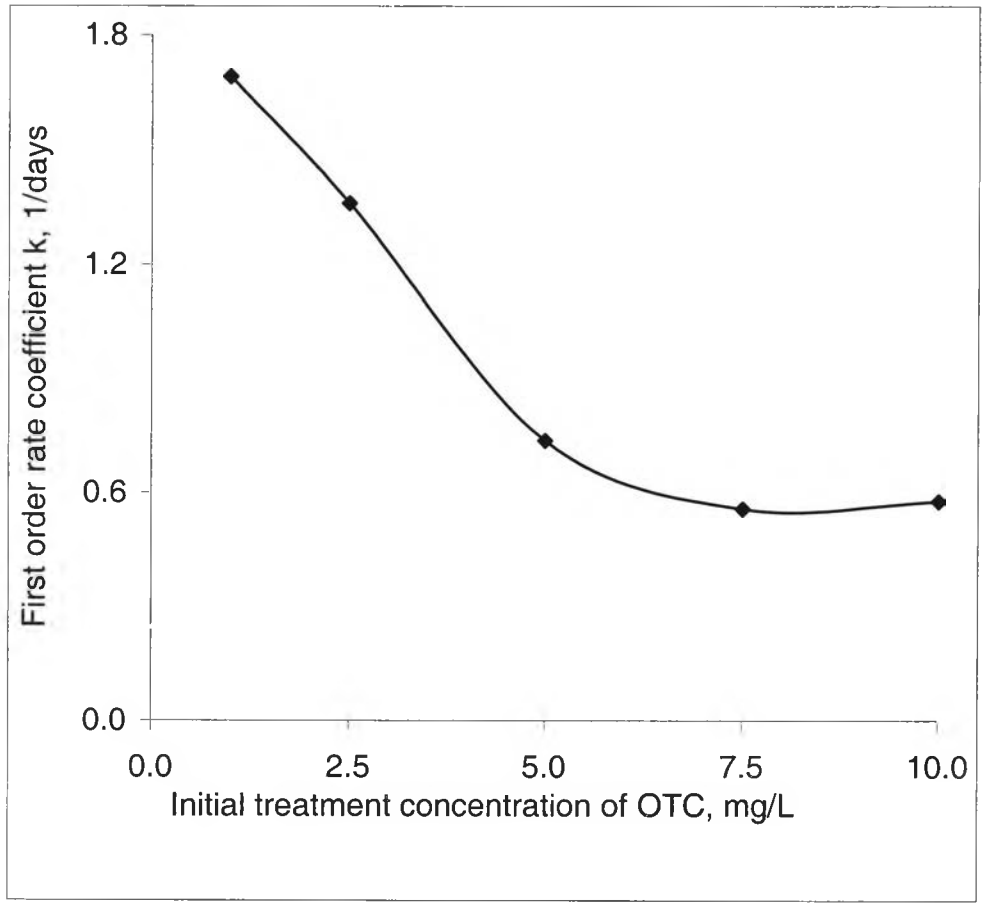


Figure 7. First-order rate coefficient vs. treatment OTC concentration for the *P. stratiotes*-OTC interaction.

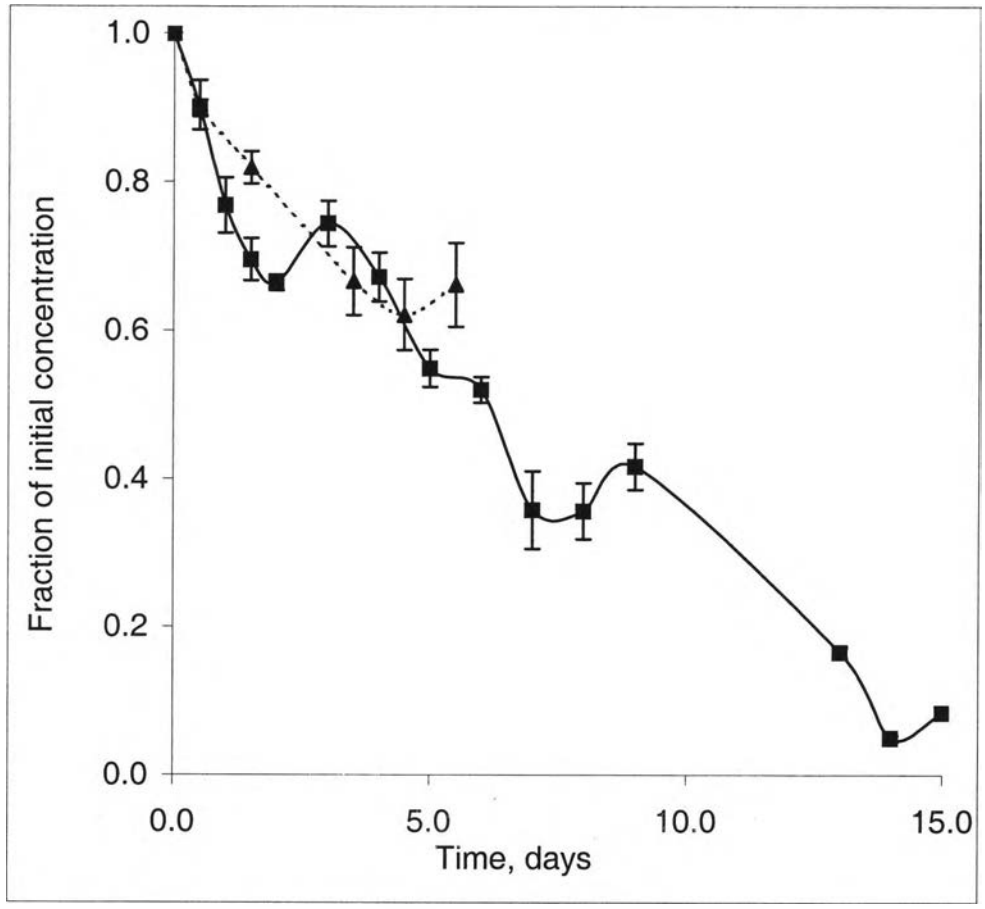


Figure 8. OTC disappearance from the liquid medium on exposure to *M. aquaticum*, live plants (□) and root exudates (□), at 5 mg/L treatment concentrations.

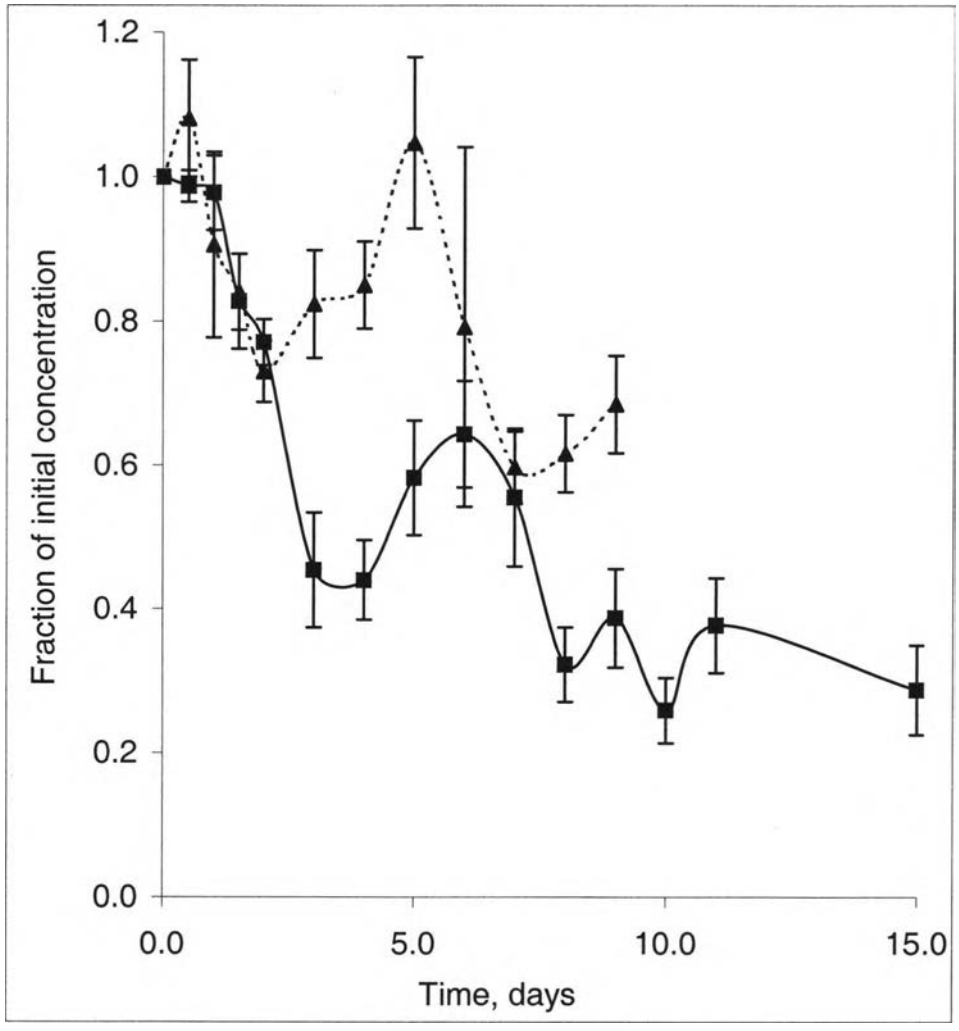


Figure 9. TC disappearance from the liquid medium on exposure to *M. aquaticum*, live plants (□) and root exudates (□), at 5 mg/L treatment concentrations.

aquaticum, although carried out for a shorter time period, gave antibiotic disappearance rates (~ 0.08 day^{-1}), which were less than that achieved with the live plants (~ 0.17 day^{-1}). A slightly greater extent of antibiotic modification was observed in the initial period compared to the later stages. The differences in extents of modification were significantly less than those observed for *P. stratiotes*. This might be due to the fact that *P. stratiotes* has a higher root density compared to *M. aquaticum.* Production of significantly higher levels of metabolite(s) may result in higher initial rates of, and overall, antibiotic modification. The first order rate coefficient showed a similar relationship to concentration of the antibiotic (Figure 10) as that observed for *P. stratiotes*.

UV absorption spectra

Figure 11 compares the UV absorbance spectra for OTC exposed to RO water with the spectra for OTC exposed to the cell-free, 0.2 μm filtered, root exudates from *P. stratiotes*. The retention times of 36 and 96 hours represent the time that OTC was exposed to either RO water or the root exudates. OTC exposed to RO water retained its characteristic UV absorption spectrum (Compare with the spectrum in Figure 2), even after a retention time of 96 hours. For OTC exposed to the root exudates, the UV absorption in the 360 nm-range decreased significantly within the first 36-hour interval, and by the end of 96 hours the absorption at 360 nm went to zero. Although the absorption in the 270 nm-range decreased, the extent of decrease was not as great as in the 360 nm-range. Since the absorbance in 360 nm-range is due to the BCD chromophore, the disappearance in the absorbance in this range can be attributed to the modification of the functional groups in the BCD chromophore by components of the cell-free root exudates.

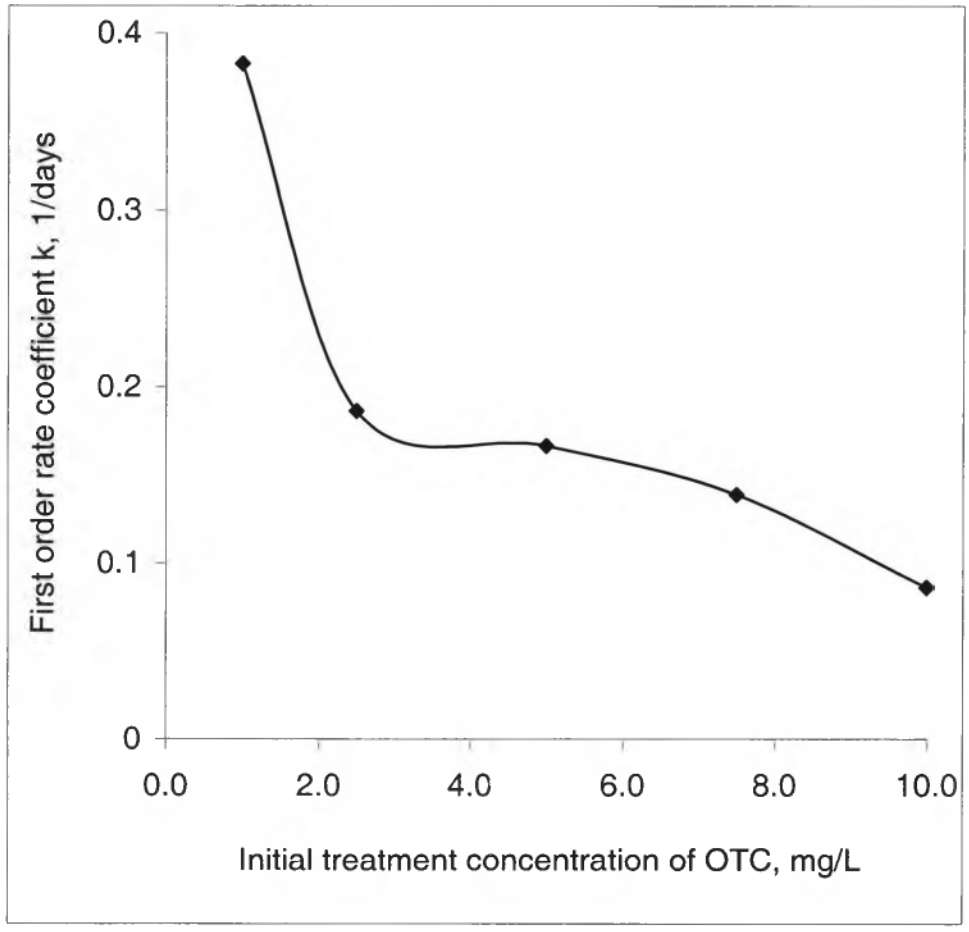


Figure 10. First-order rate coefficient vs. treatment OTC concentration for the *M. aquaticum*-OTC interaction.

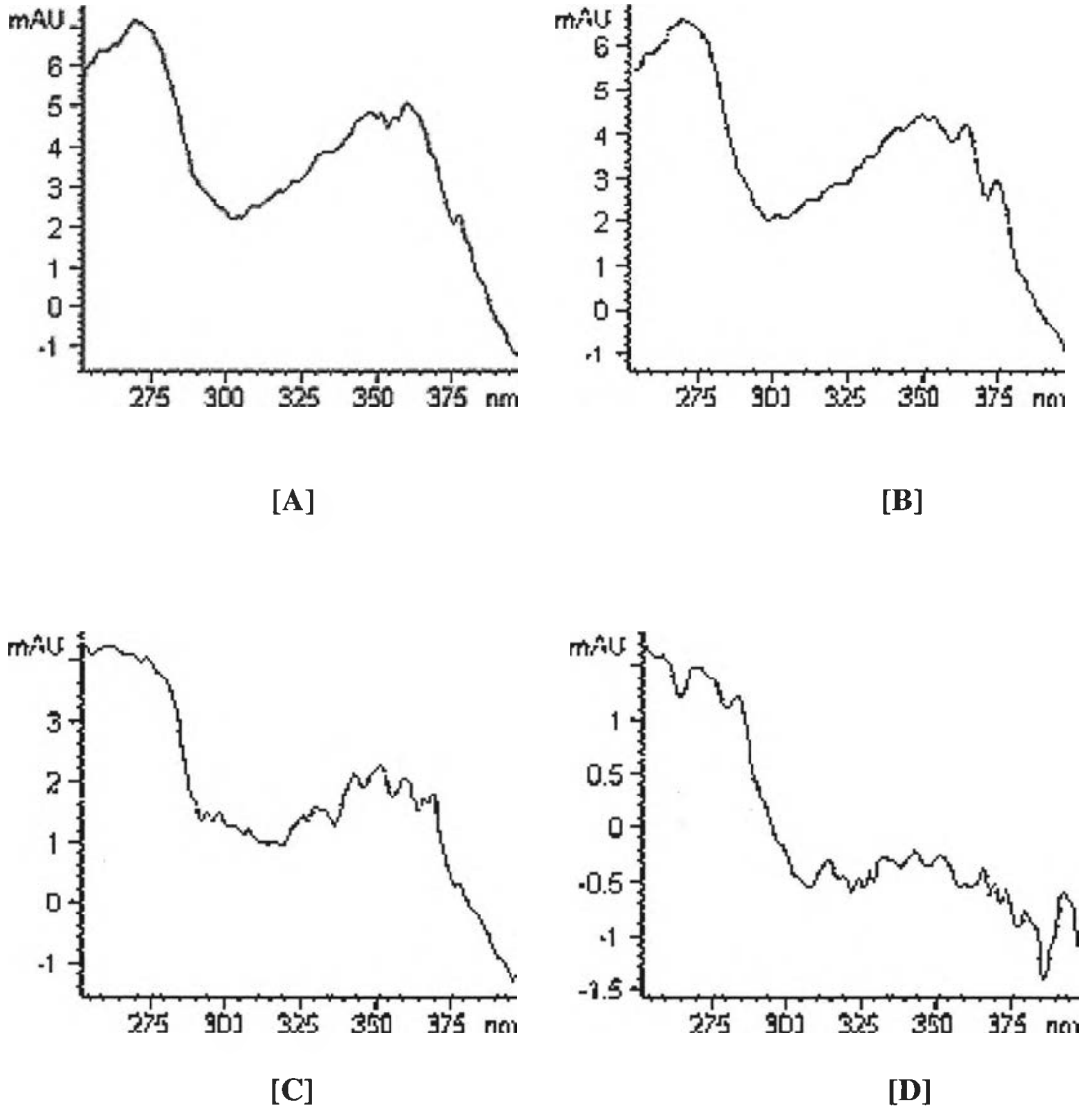


Figure 11. UV absorption spectra of: [A] OTC in RO water, with a retention time of 36 hours; [B] OTC in RO water, with a retention time of 96 hours; [C] OTC in root exudates from *P. stratiotes*, with a retention time of 36 hours; [D] OTC in root exudates from *P. stratiotes*, with a retention time of 96 hours. The concentration of OTC at 0 hours was 5 mg/L for both, the RO water and root exudates of *P. stratiotes.*

Conclusions

M. aquaticum and *P. stratiotes* gave almost complete OTC and TC modification, with *P. stratiotes* taking up to 6 days and *M. aquaticum* as long as 15 days. *P. stratiotes* with distinct physical advantages like longer and denser root systems seems to have a greater potential for phytoremediation of OTC and TC. Comparable levels of antibiotic modification by filtered root exudates from these two species suggest the involvement of root-secreted enzyme(s)/metabolite(s) in degrading/transforming the antibiotics. The rate of antibiotic modification decreased with increasing initial concentration of the antibiotics, suggesting that the modifying compound might be present in the root exudates in limiting concentrations.

According to Michaelis-Menten kinetics for enzyme-catalyzed reactions, the rate of the reaction increases hyperbolically with increasing substrate concentrations (Bailey and Ollis, 1986). However, we observed a decrease in antibiotic disappearance rates with increasing OTC (the substrate) treatment concentration, which suggests the observed disappearance is not enzyme catalyzed. Instead interaction of the antibiotic with root-secreted metabolites/radicals that are present at limiting concentrations is a possibility. Increasing the treatment concentration of the antibiotic results in diminished availability of such metabolites/radicals, and consequently reduces antibiotic modification rates.

The modification of the antibiotics was confirmed from the changes observed in their UV absorption spectra. The UV spectrum of the modified OTC, characterized by no absorbance in the near UV range (around 360 nm), suggested modification at the BCD

chromophore. Peroxidases from several plant species have been known to oxidize recalcitrant polycyclic aromatic hydrocarbons (PAHs) [Adler et al., 1994; Criquet et al., 2000; De Araujo, 2002; Miksaniva et al., 2001; and Strycharz and Shetty, 2002]. Peroxidases are widely distributed among higher plants, and are known to catalyze oxidation reactions by hydrogen peroxide. Hydrogen peroxide has been shown to be involved in bioremediation of toluene and o-xylene from groundwater [Mohammed and Allayla, 2000]. Hydrogen peroxide radicals, commonly found in the rhizophere of plants, are currently being investigated as the possible oxidizing agents involved in the modification of TC and OTC. Preliminary experiments (results not shown) have shown that hydrogen peroxide, when added to an aqueous solution of OTC in RO water, gave significant reduction in the UV absorbance of the OTC solution in the near UV range (~ 360nm). Thus oxidative modification of the two antibiotics by the root exudates, predominantly at the BCD chromophore, is a possibility. Other oxidoreductases e.g. tyrosinase and laccases, which are also found in several plant species, are known to be involved in transformations of PAHs [Criquet et al., 2000]. Further experiments, involving the above mentioned plants enzymes/radicals, need to be conducted to determine the exact mechanism of TC/OTC modification by the root exudates of *M. aquaticum* and *P. stratiotes*. The observation that plant secreted compounds, rather than the whole plant, are involved in antibiotic modification, might be helpful in developing remediation systems such as controlled wetlands, hydroponics or flow-through treatment systems.

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Chapter III. Hairy roots of *Helianthus annuus*: A Model System to Study Phytoremediation of Tetracycline and Oxytetracycline

Reference: Gujarathi, N. P.; Haney, B. J.; Park, H. J.; Wickramasinghe, S. R.; and Linden, J. C. *Hairy roots of* Helianthus annuus*: A model system to study phytoremediation of tetracycline and oxytetracycline*. **Biotechnology Progress** (In press, 2005).

Abstract

The release of antibiotics to the environment has to be controlled due to serious threats to human health. Hairy root cultures of *Helianthus annuus* (sunflower), along with their inherent rhizospheric activity, provide a fast growing, microbe-free environment for understanding plant-pollutant interactions. The root system catalyzes rapid disappearance of tetracycline (TC) and oxytetracycline (OTC) from aqueous media, which suggests roots have potential for phytoremediation of the two antibiotics *in vivo*. In addition, *in vitro* modifications of the two antibiotics by filtered, cell- and microbe-free, root exudates suggest involvement of root-secreted compounds. The modification is confirmed from changes observed in UV spectra of exudate-treated OTC. Modification appears to be more dominant at the BCD chromophore of the antibiotic molecule. Kinetic analyses dismiss direct enzyme catalysis; the modification rates decrease with increasing OTC concentrations. The rates increase with increasing age of cultures, from which root

exudates are prepared. The decrease in modification rates upon addition of the antioxidant ascorbic acid (AA), suggests involvement of reactive oxygen species (ROS) in the antibiotic modification process.

Introduction

Transformation of plants using *Agrobacterium rhizogenes* produces the hairy roots [Giri and Narasu, 2000; and Hamill and Lidget, 1997]. Hairy roots are maintained in plant growth medium supplemented with sucrose. Hairy root cultures provide excellent models for studying pollutant/root interactions. The distinct advantages of using hairy roots for phytoremediation studies are:

i. Availability of an aseptic environment, free of microbial interactions;

ii. Exposure of the pollutant-containing medium to dense root concentrations per unit volume;

iii. Presence of enhanced levels of root exudates/enzymes;

iv. Availability of a homogeneous root exudates medium for studying the biochemical interactions with the pollutant, as well as with externally added enzymes, cofactors or reagents;

v. The biochemical resemblance of hairy root cultures to the roots of the host plant [De Araujo et al., 2002]; and

vi. The genetic and biochemical stability of hairy root cultures [Flores and Curtis, 1992].

According to Strycharz and Shetty (2002), plants transformed with *A. rhizogenes*, show potential for phytoremediation of aromatic compounds. Since our hairy roots cultures of

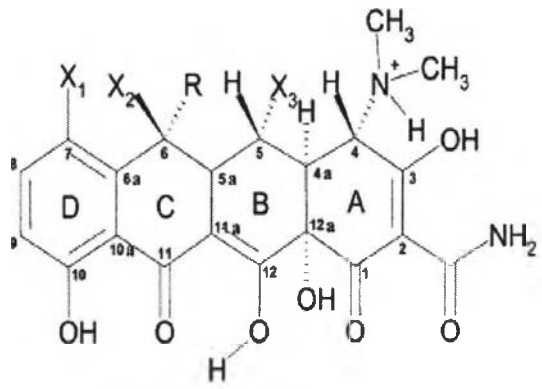
H. annuus reach densities of ~ 250 gm/liter on fresh weight basis, the system has potential as an effective remediation model system, as demonstrated with other hairy root cultures [Agostini et al., 2003; De Araujo et al., 2002; and Shanks and Morgan, 1999].

Antibiotics used as antibiotic growth promoters (AGP) in the livestock industry increase the efficiency of growth by inhibiting microorganisms and growth-depressing metabolites present in the gastrointestinal tracts of the animals [Gaskins et al., 2002]. Of 50 million pounds of antibiotics produced in the United States annually, approximately 40% are used as AGP or food efficiency promoters [Meyer et al., 2000]. In humans and animals, from 30 to 90% of the antibiotics are excreted in the urine as active substances [Halling-Sorensen, 1998]. Antibiotics added to animal feeds are eventually released to the environment through run-off from concentrated animal feeding operations (CAFO) or leakage from storage structures. Use of animal manure as fertilizer also results in a substantial exposure of the antibiotics to the environment. In the United States, tetracycline antibiotics are found at concentrations ranging from 0.1 to 0.8 mg/L in lagoons and rivers [Meyer et al., 2000]. Antibiotic resistance in bacteria may cause severe health problems due to increasing ineffectiveness of antibiotic drugs.

Phytoremediation is the use of vegetation to contain, sequester, remove, modify or degrade inorganic and organic contaminants in soils, sediments, surface waters and groundwater [Tsao, 2003]. Plants are used in remediation of many types of contaminants, including petroleum hydrocarbons, chlorinated solvents, pesticides, metals, radionuclides, explosives, and excess nutrients. Peroxidases from several plant species oxidize

recalcitrant polycyclic aromatic hydrocarbons (PAHs) and other phenolic compounds [Adler et al., 1994; Criquet et al., 2000; De Araujo et al., 2002; Fang and Barcelona, 2003; Miksanova et al., 2001; and Strycharz and Shetty, 2002]. Hydrogen peroxide is involved in bioremediation of toluene and o-xylene from groundwater [Mohammed and Allayla, 2000]. Plant derived enzymes, such as xanthine oxidase, superoxide dismutase and ascorbate peroxidase, are involved in oxidation of 2, 4-dichlorophenoxyacetic acid, an herbicide, through the action of ROS generated by the plant [Romero-Puertas et al., 2004]. Peroxidases are widely distributed among higher plants and catalyze oxidation reactions by hydrogen peroxide. Oxygen, hydroxyl radical and hydrogen peroxide are known to oxidize tetracyclines [Gilio and Cilento, 1993; and Lau et al., 2004].

The redox system in plants, involving the formation of ROS, which is activated under stress conditions, is well documented for *H. annuus* [Jouili and El Ferjani, 2003; Liste and Alexander, 1999; Magwa et al., 1993; Ranieri et al., 2000; Ranieri et al., 2003a; Ranieri et al., 2003b; Sauerbon et al., 2002; Zhang and Kirkham, 1996a; and Zhang and Kirkham, 1996a]. Since hairy roots retain the original biochemical characteristics of the plant [De Araujo et al., 2002], hairy root cultures of *H. annuus* prove to be an effective model for oxidative remediation of tetracycline pollutants. The general structure of tetracycline derivatives is shown in Figure 1, the oxidative modification of which will be demonstrated by the results, which follow.



	X_1	X_2	R	X_3
Tetracycline	H	OH	CH_3	H
Minocycline	$N(Me)_2$	H	H	H
Oxytetracycline	H	OH	CH_3	OH
Doxycycline	H	H	CH_3	OH
Methacycline	H	$=CH_2$		OH

Figure 1. The general structure for tetracyclines [Leypold, 2003].

Materials & Methods

Culturing and propagating *Helianthus annuus* hairy root cultures

Hairy root cultures of *H. annuus*, which were produced by infecting seedlings with *Agrobacterium rhizogenes* ATCC 15834, were acquired from the late Dr. Rajiv Bhadra, whose work initiated these studies. Records retrieved from Dr. Bhadra's work indicate seeds of the sunflower cultivar 'mammoth' were purchased from Burpee, Warminster, PA germinated in January 2001 and used for transformation. The root cultures were maintained in Gamborg's B-5 basal salt mixture [Phytotechnology Laboratories, Shawnee Mission, KS], supplemented with 3% sucrose (w/w) [Sigma-Aldrich, St. Louis, MO] and Gamborg's B5 vitamin solution [Phytotechnology Laboratories, Shawnee Mission, KS]. The medium was prepared using reverse osmosis (RO) water; the pH of the solution was adjusted to 5.7 by adding potassium hydroxide [Fisher-Scientific, Fairlawn, NJ]. The medium was then transferred to the 250-ml Erlenmeyer flasks (75 ml per flask) and autoclaved for 20 minutes. After cooling, vitamin solution was added to the autoclaved medium at 1 mg/L final concentration by passing it through a sterile 0.2 μm syringe-filter [Millipore, Bedford, MA]. Four or five growing root tips, about 30 to 40 mm in length, were transferred from a previously existing culture to the fresh medium. The flasks were placed on a shaker table set at 110 rpm at room temperature in the dark. Transfers were conducted every two or three weeks, depending on the required age of the root exudates for specific experiments.

Preparation of the root exudates

Preparation of root exudates of a specific age involved growing the hairy roots in B5 medium for the required time. For root exudates of age 5 weeks, fresh medium was added after 3 weeks; while for root exudates of age 7 weeks, fresh medium was added after the 3^{rd} and 5^{th} weeks to replenish the 75 mL initial volume. Once the required age was attained, roots were separated from medium, and the medium was filtered through a 0.2 μm filter membrane [Millipore, Bedford, MA] to remove microorganisms, root cells or cell debris from the root exudates. After filtration the root exudates (filtrate) were refrigerated until use in the experiment.

Experimental conditions and setup

The antibiotic-plant interactions described in this study were carried out in aqueous medium. Stock solutions of TC and OTC [Sigma-Aldrich, St. Louis, MO] were prepared by dissolution in RO water. For *in vivo* experiments, stock solutions were added to the root cultures on the 7^{th} or 8^{th} day after the cultures were transferred. Before addition, the antibiotic stock solutions were filter-sterilized through 0.2 μm syringe filters [Millipore, Medford, MA]. Five test concentrations of the antibiotics: 1, 2.5, 5, 7.5 and 10 mg/L, were studied in triplicate. Control experiments were conducted to determine the natural degradation of the two antibiotics in sterile RO water and growth medium in the absence of the plant catalysts (live roots or the root exudates). Experiments involving the study of *in vitro* interactions between root exudates from *H. annuus* hairy root cultures and the antibiotics were carried under conditions similar to that with the *in vivo* experiments involving the root cultures. Since OTC and TC have very similar structures (See Figure

1), most of the *in vitro* experiments were conducted only with OTC. To the experiments, which were performed to study the effect of root exudate age on the OTC modification rates, initial concentrations of 5 mg/L OTC were added to root exudates of age 3, 5 and 7 weeks that were acquired from 3, 5 and 7 week old cultures, respectively.

Because of the presence of various reducing substrates and enzymes (i.e. catalase, peroxidase and superoxide dismutase) in biological systems, addition of an antioxidant would reduce the rate of OTC disappearance and would prove the presence of ROS. Ascorbic acid (AA), an electron donor (standard 1-electron reduction potential of 282 mV), is commonly used, experimentally, as an antioxidant for detoxification of ROS [Perez et al., 2002; Ranieri et al., 2000; and Shankaran et al., 2001]. In aqueous solutions, AA reacts with ROS to produce oxidation products, such as monodehydroascorbate, dehydroascorbic acid and 2,3-diketogulonic acid [Davey et al., 2000]. L-ascorbic acid [Fisher-Scientific, Fairlawn, NJ] was added to the root exudates, prior to OTC addition. Concentrations of AA studied were 0.01, 0.05, 0.1, and 0.5 mM. As a control, OTC was added to root exudates without AA. In all cases, an initial concentration of 5 mg/L OTC was used.

Sampling and analysis

After addition of the antibiotic, the flasks were transferred to the shaker table for about 20 minutes to distribute the antibiotic uniformly in the root exudates preparations and hairy root cultures. The flasks were sampled in a laminar flow hood. The 0^{th} day samples represented the initial concentrations of the antibiotic for the remediation studies.

Samples were then taken at time intervals indicated in the graphs and immediately frozen for later analysis. High performance liquid chromatography (HPLC) was used for analyzing antibiotic concentrations in the growth media. After thawing, the samples were centrifuged in a micro-centrifuge [Brinkman Instruments Inc., Westbury, NY] for 10 minutes. The supernatant from the centrifuged samples was then filtered through a 0.45 μm centrifuge tube filter [Millipore, Medford, MA], transferred to a 250 μL silanized inert insert [Agilent Technologies, Wilmington, DE] and placed in a HPLC auto-sampler sample vial [Agilent Technologies, Wilmington, DE].

The HPLC system with photodiode array detector [Hewlett Packard 1050 series] was used with a 250 x 4.6 mm C-18 column [Phenomenex, Torrance, CA] for analyzing antibiotic concentrations in the samples. The mobile phase consisted of 20 mM sodium phosphate [Phytotechnology Laboratories, Shawnee Mission, KS] (solvent A) and 2-propanol [Fisher-Scientific, Fairlawn, NJ] (solvent B). The pH of both the solvents, A and B, was adjusted to 2.5 by adding either trifluoroacetic acid [Aldrich, Milwaukee, WI] or formic acid [Aldrich, Milwaukee, WI] at 0.1% concentration by volume. Before each sample injection, the column was preconditioned with solvent A to ensure optimum hydrophobic interaction between the antibiotic and the column packing. An 82/18 ratio of solvent A to B was used to elute the antibiotics from the column at combined flow rates of 1 mL/minute. The concentrations of TC and OTC were determined by measuring their UV absorbance at 365 and 355 nm, respectively. The retention time for both antibiotics was on the order of 5.9 minutes. Periodically, slight changes in the retention times were encountered, possibly due to an increase in the pressure drop across the column. The

detection limit of the method was 0.2 mg/L of both TC and OTC. The same HPLC method was used for both, because the two antibiotics were never used simultaneously in a given experiment.

UV spectra of OTC

The diode array in the UV detector of the HPLC system enabled acquisition of spectral data for the antibiotic samples. Changes in the characteristic UV spectra for the samples were used as the basis for investigating modification of the antibiotics. The spectra for OTC reported in this study were of chromatographically separated species, which insured no spectral interference from other compounds in the root exudates.

Results and Discussion

***In vivo* experiments**

The growth curve for the root cultures of *H. annuus* is shown in Figure 2 with two growth phases that are characterized by specific growth rates of 0.28 and 0.63 day^{-1}. The specific growth rates of 0.28 and 0.63 day^{-1} were determined over the respective time frames of 2 to 8 days and 8 to 10 days using the relationship:

$$W_t/W_0 = e^{\mu.t} \qquad [I]$$

Where,

W_0 and W_t represent the root biomasses (fresh weight basis) at initial and final times of the respective time frames in t days, respectively, and μ is the specific growth rate of the hairy root culture, in day^{-1}. Because in our experiments antibiotics are added on day 8 after culture transfer, exposure to roots is during the stage of maximum growth (see

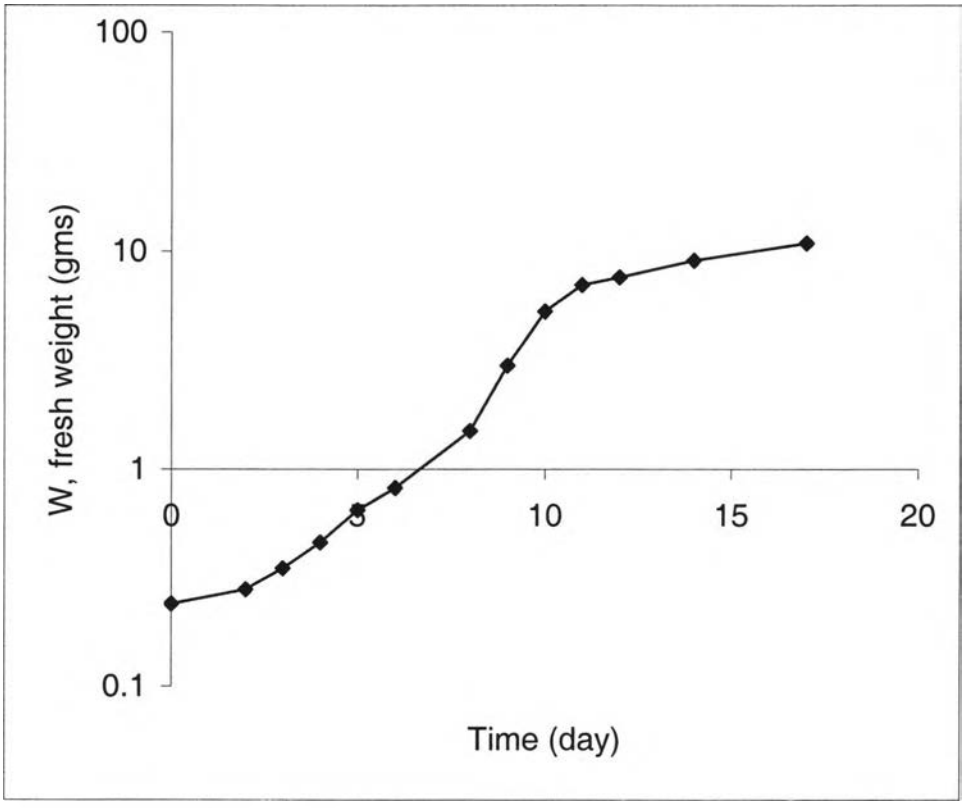


Figure 2. Growth curve for *H. annuus* hairy roots.

Figure 2). We have modeled hairy root growth by adopting a population balance approach elsewhere [Han et al., 2004]. This model accounts for the linear growth of root-branches by cell division as well as exponential growth due to formation of new branches on the already existing root-branches. Since the linear growth phase contributes little to the increase in the biomass, we fit an exponential function to the hairy root growth data (as depicted in equation I). The dependence of modification rates on amount of root biomass is not discussed further, because most of the modification activity appears to occur in the rhizosphere.

There is little TC or OTC degradation in sterile growth medium, as illustrated in Figure 3. The initial increase in the concentration of the two antibiotics might be due to evaporation of water from the growth medium. The controls exhibit no measurable antibiotic degradation in the first two days of the experiment. Approximately 20% of the antibiotics are degraded in the time frame of the experiment (5 days). Photodegradation is a slow process, as Halling-Sorensen et al. (2003) reported approximately 90% of an initial amount of OTC disappeared over a period of 100 days exposure to light.

Equation [II] is used for quantitation of the observed OTC disappearance, which is discussed in the following sections, only as a fit to the data.

$$C_t = C_0.e^{-k.t} \qquad [II]$$

Where,

C_0 and C_t are the concentrations of the antibiotic at time 0 and t, respectively, and

k is the first order rate coefficient for the process. Exponential decay does not necessarily

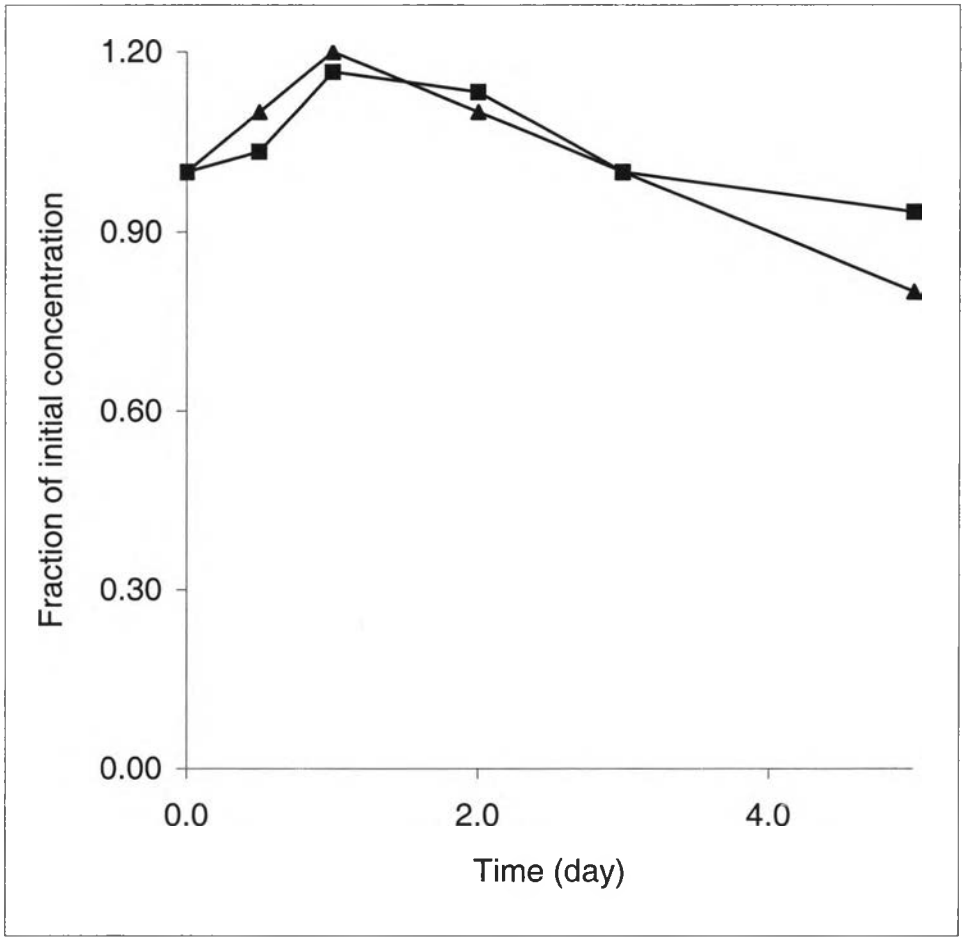


Figure 3. Natural degradation of OTC (□) and TC (□) in the growth medium.

model the actual reactions kinetics for the reported OTC-hairy roots/root exudates interactions. The first order rate coefficient value, k, is used to represent the OTC disappearance rates.

Mixed descriptions of *in vivo* and *in vitro* experiments

Complete disappearance of OTC is observed within six days of addition to the hairy root cultures. The disappearance curve shown in Figure 4 is for the 5-mg/L-treatment concentration. A first order OTC disappearance rate coefficient of 0.7 day^{-1} is obtained for this case. When OTC is added to 0.2 μm-filtered, cell- and microbe-free, root exudates from *H. annuus* hairy roots, 95% removal is observed in seven days of addition (Figure 4). The treatment concentration of 5 mg/L of OTC is again represented with the first order rate coefficient of 0.44 day^{-1}. This *in vitro* observation is in the absence of the living cells, or cell debris, and rules out the possibility of physical adsorption or uptake of OTC by the roots; it also suggests involvement of root-secreted enzymes or metabolites. For both, live roots and root exudates, most of the OTC disappearance is observed within two days of addition, after which the disappearance rate decreases and may be due to limiting concentrations of either OTC and/or root-secreted compounds. From several experiments involving live roots, the first order disappearance rates are plotted against the treatment concentration in Figure 5. The OTC disappearance rate, k, decreases with an increase in treatment concentrations of OTC < 5 mg/L and levels off for concentrations > 5 mg/L. Thus, OTC is not the limiting reactant in the interaction. According to Michaelis-Menten kinetics for enzyme-catalyzed reactions, the rate of the reaction increases hyperbolically with increasing substrate concentrations [Bailey and

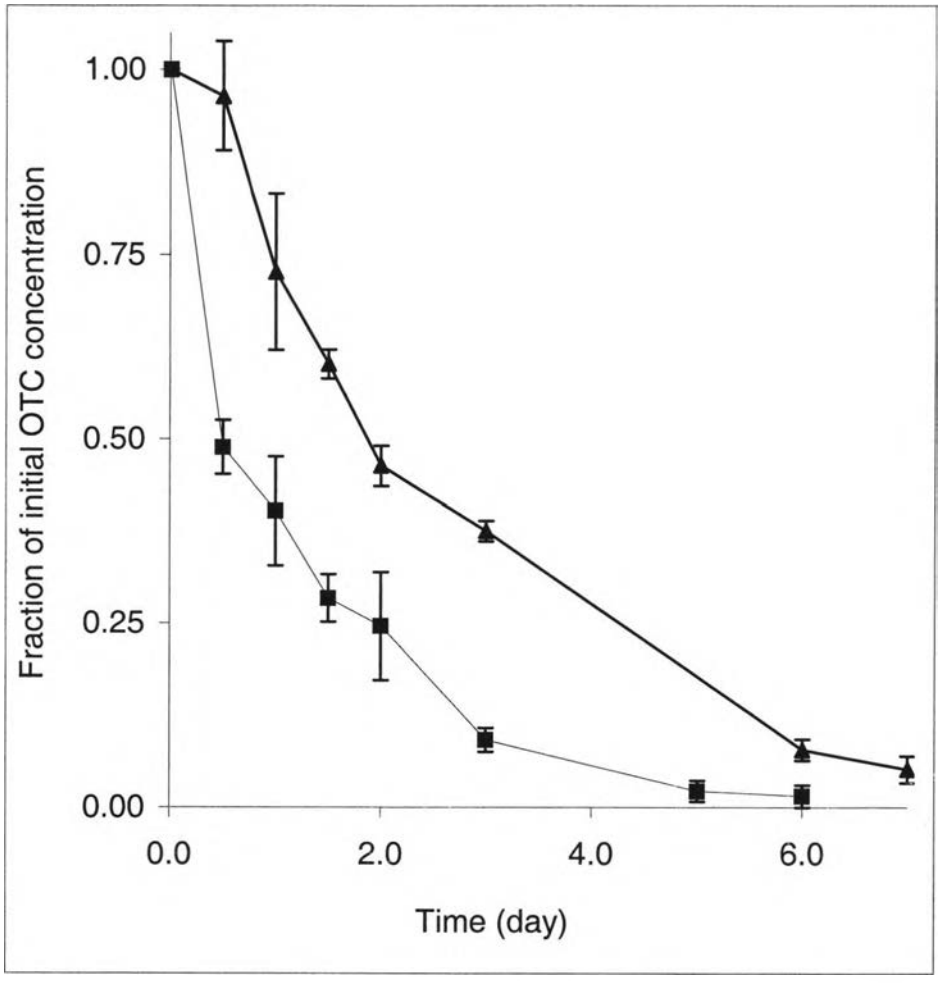


Figure 4. OTC disappearance on exposure to the hairy roots (□) and filtered root exudates (□) from *H. annuus* at 5 mg/L initial treatment concentrations.

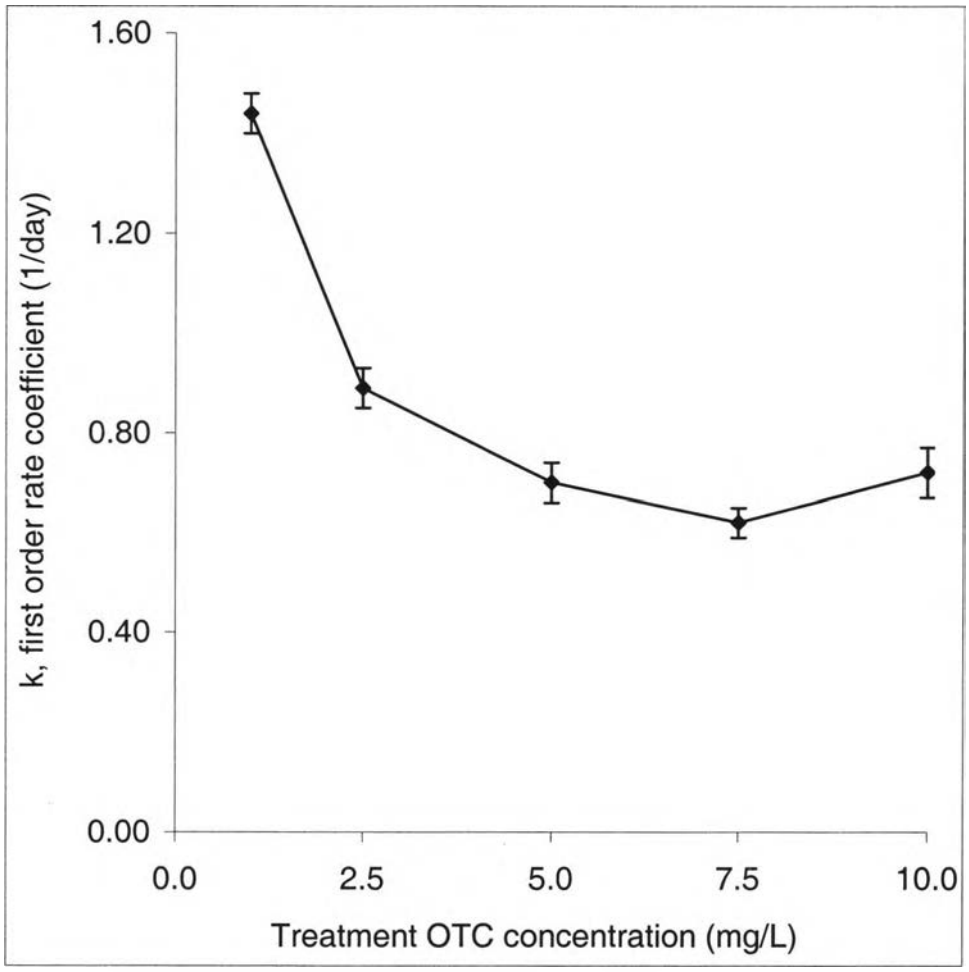


Figure 5. Effect of treatment concentration of OTC on the observed OTC disappearance rates.

Ollis, 1986]. However, we observed a decrease in OTC disappearance rates with increasing OTC (the substrate) treatment concentration, which suggests the observed disappearance is not enzyme catalyzed. Instead interaction of OTC with root-secreted metabolites/radicals that are present at limiting concentrations is a possibility. Increasing the treatment concentration of OTC results in diminished availability of such metabolites/radicals, and consequently reduces OTC disappearance rates. The treatment concentration relationship of TC has a similar effect on disappearance rates (data not shown).

Disappearance of TC upon *in vivo* and *in vitro* exposure to live roots and root exudates at 5 mg/L initial concentration is represented in Figure 6. The nature of the disappearance curves for TC are similar to that for OTC, although the extent of TC disappearance is less than that observed for OTC. This difference might be due to variations in experimental conditions, such as extent of growth of the root cultures or age of the root exudates used, or differences in reaction kinetics between OTC and TC with the active catalytic species in the rhizosphere or exudate preparations. TC and OTC are moderately hydrophilic with log K_{ow} (octanol-water partition coefficient) values of –1.19 and –1.22 and aqueous solubilities of 1700 and 1000 mg/L, respectively [Tolls, 2001]. The UV absorption spectrum for OTC is shown in Figure 7, part A (parts B, C and D are discussed later in this section). The maximum at 270 nm is attributed to the UV absorption by the A chromophore, while the maximum at 360 nm is from the UV absorbing BCD chromophore [Leypold et al, 2003; Ally et al., 2002; and Schmitt and Schneider, 2000]. The A chromophore consists of the diketone moiety, amide group and a hydroxyl group

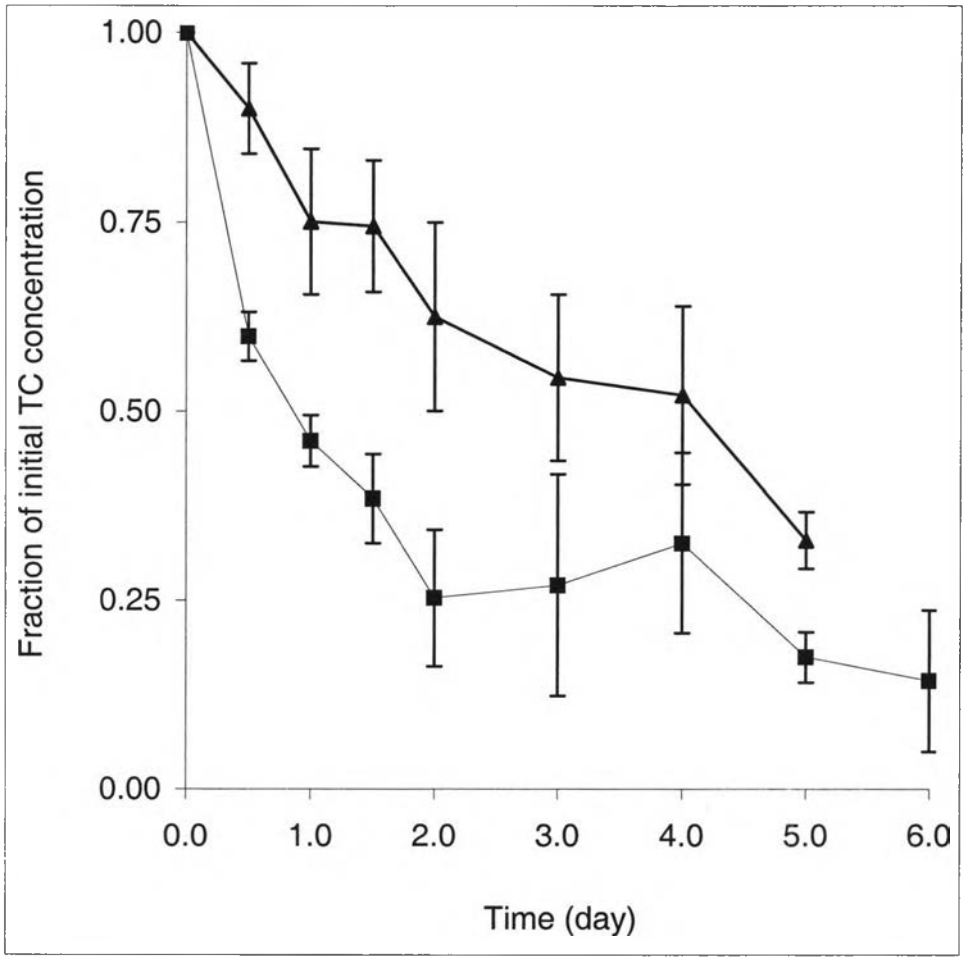


Figure 6. TC disappearance on exposure to the hairy roots (□) and filtered root exudates (□) from *H. annuus* at 5 mg/L initial treatment concentrations.

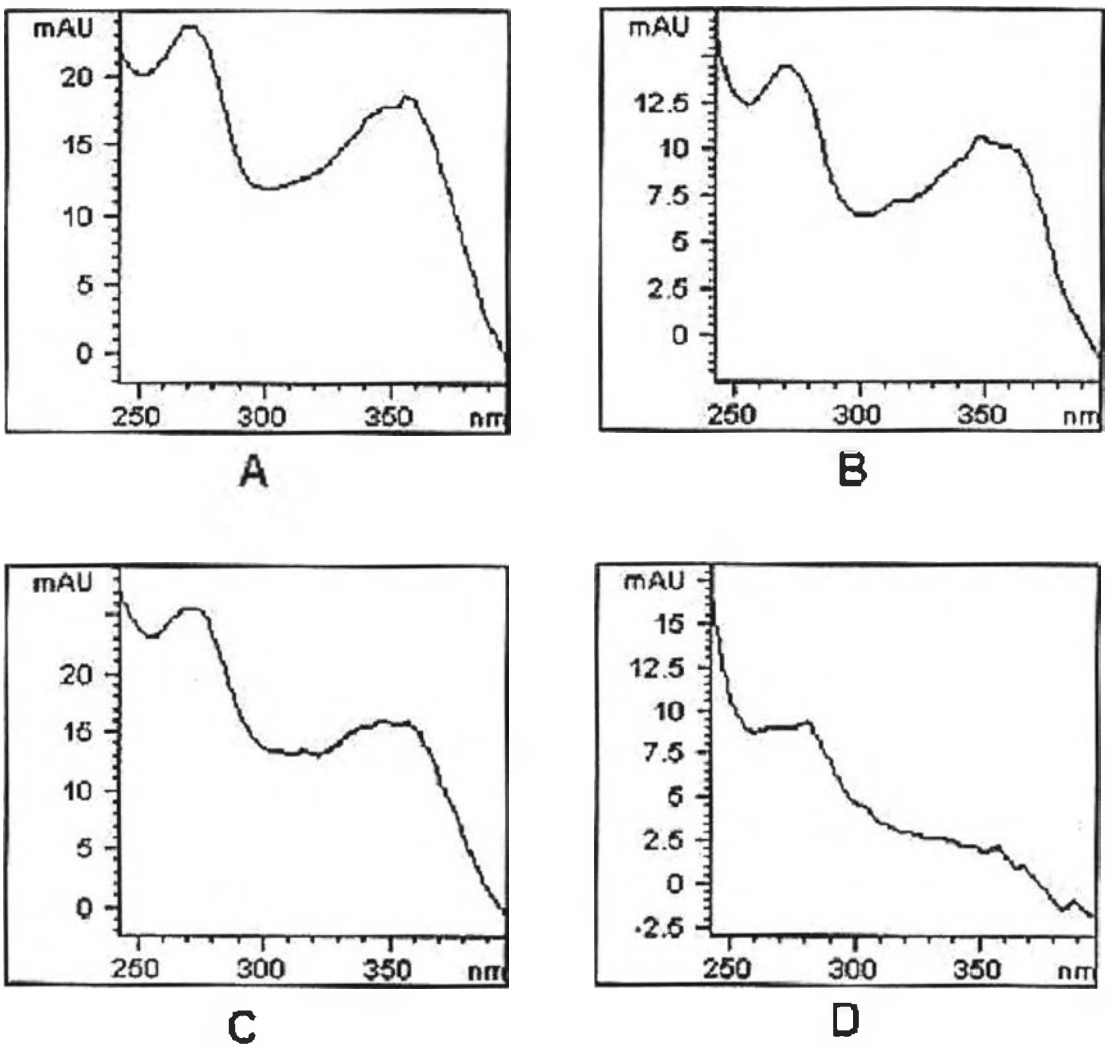


Figure 7. UV absorption spectra for OTC. A- OTC in RO water, after 0 days. B- OTC in RO water, after 6 days. C- OTC in root exudates of *H. annuus*, after 0 days. D- OTC in root exudates of *H. annuus*, after 6 days.

in the A-ring. The BCD chromophore involves the phenyl D ring and the □-hydroxyketo system extending across the carbonyl group and the ketone group, in the C and B rings, respectively.

In vitro mechanistic experiments

The UV absorbance spectra for OTC exposed to RO water are compared with the spectra for OTC exposed to the cell-free, 0.2 μm filtered, root exudates in Figure 7. OTC exposed to RO water under the same conditions as the root exudates experiments retains the characteristic UV absorption spectrum (compare 7-A and 7-B), even after 6 days. The decrease in the UV absorbance at the two absorption maxima, 270 and 355 nm, is about 40%. The UV absorption maxima of OTC exposed to the root exudates for six days, decrease by 65% at 270 nm and by 90% at 355 nm. Since the absorption maximum at 355 nm is due to the BCD chromophore, changes in absorbance at this wavelength can be attributed to modification of functional groups in the BCD chromophore by components of the root exudates. The phenolic group in the D-ring and the enol group in the B-ring provide possible sites of oxidation [Gilio and Cilento, 1993]; therefore oxidation of the BCD chromophore at these sites is a strong possibility. The A-ring has only one possible site of oxidation, which is the OH group [Gilio and Cilento, 1993]. Thus, in the case of limited availability of oxidants, the modification at the BCD chromophore may be expected to exceed that at the A chromophore, depending on the reduction potentials of functional groups at the two locations. Oxidation of an OTC molecule, at the above-mentioned sites, is expected to yield quinone derivatives of OTC [Lau et al., 2004]. Although, no spectral data were obtained for the interactions of the root exudates with

TC, similar results are expected because both OTC and TC have similar UV absorbing chromophores [Jouili and El Ferjani, 2003; Liste and Alexander, 1999; and Magwa et al., 1993].

The age of the root exudates, i.e. the time the roots are maintained in the medium, has a significant effect on the rate of OTC modification. The first order rate coefficients for OTC modification by the root exudates of age 3, 5 and 7 weeks obtained are 0.34, 0.87, and 1.22 day^{-1}, respectively (Figure 8). This observation fortifies the case for oxidative modification of the antibiotic by root-secreted compound(s), since the concentration of such compound(s) is expected to increase with culture age.

Addition of the antioxidant, AA, causes the rate of modification of OTC by the root exudates to decrease compared to the modification rate in the absence of AA. First order rate coefficients obtained for OTC modification at the four concentrations of AA (0.01, 0.05, 0.1, and 0.5 mM) are graphed in Figure 9. The rate of modification progressively decreases with increasing AA concentration. No OTC modification is observed for the root exudates treated with 0.5 mM AA, when using 5 mg/L OTC, which implicates involvement of ROS in OTC modification. Addition of salicylic acid, another strong ROS scavenger, gives similar results in preliminary experiments (data not shown).

Conclusions

Hairy root cultures of *H. annuus* are used for studying high rates of modification of TC and OTC. Filtered, cell- and microbe-free, root exudates from the hairy root cultures give

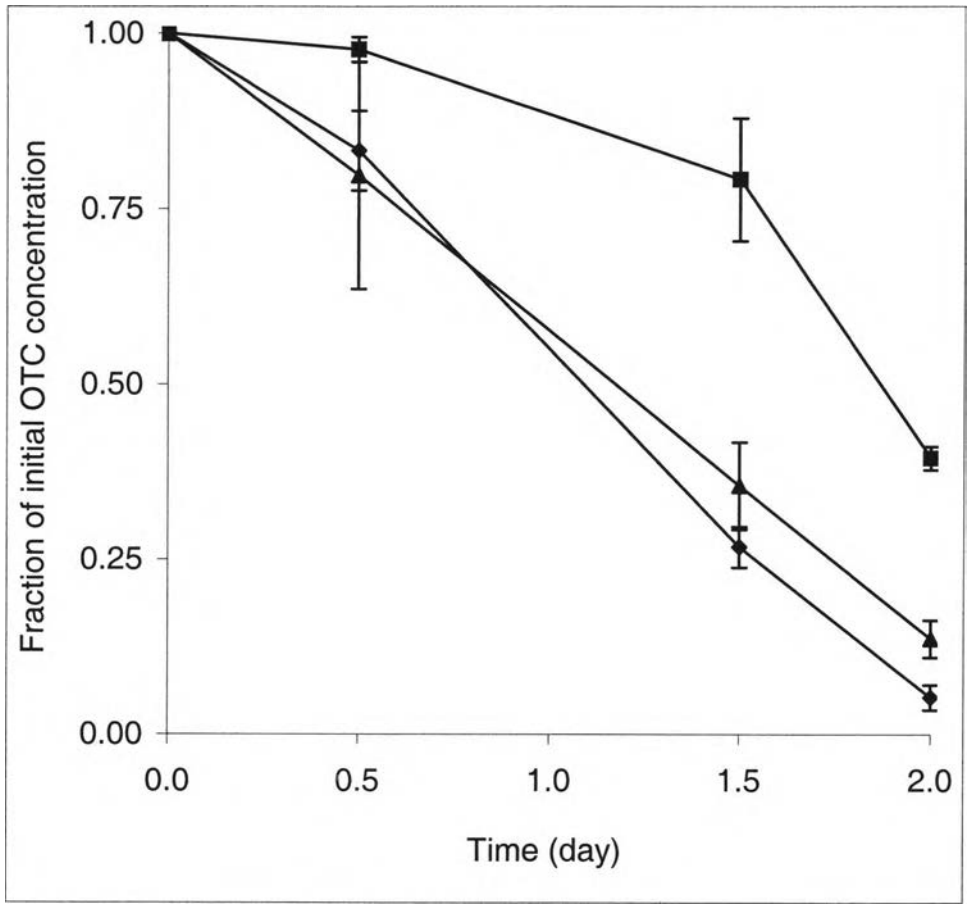


Figure 8. Comparison of OTC modification rates by root exudates of age 3 weeks (□), 5 weeks (□) and 7 weeks (□).

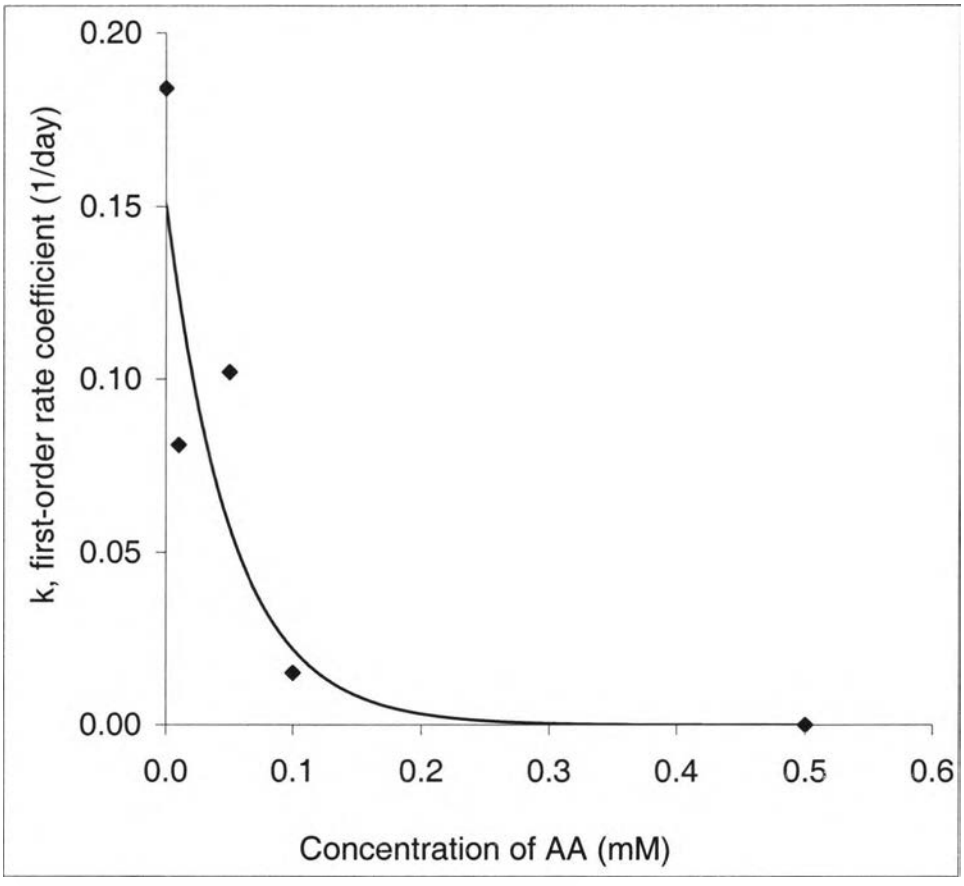


Figure 9. Effect of increasing concentration of AA on the OTC modification rate.

rates of antibiotic removal comparable to *in vivo* systems. Comparison of the UV spectra of freshly prepared antibiotics with spectra of the root exudate-interacted antibiotics suggests modification of these antibiotics by oxidative processes. Since the aromatic ring is a dominant part of the BCD chromophore, an analogy can be drawn with oxidation of PAHs by ROS. The production of ROS by plants is well documented. AA, a well-studied antioxidant, inhibits the antibiotic modification process by the root exudates with greater inhibition at higher antioxidant concentrations. Thus, root exudates appear to oxidize TC and OTC through the action of ROS. Although the OTC modification rates decrease with increasing OTC concentrations, the rates increase with increasing age (culture time) of the root exudates. Thus ROS appear to be the limiting reactant in this antibiotic modification reaction.

Plants respond to stress situations, such as pathogen attack, nutritional deficiency and other biotic and abiotic stresses by rapid production of ROS [Bolwell et al., 1995]. This response, commonly a part of hypersensitive response, leads to induction of plant defense enzymes, cross-linking of cell walls, and localized cell death. In plants, the production of ROS is usually initiated by the enzymatic reduction of oxygen or water. Enzymes, such as NADPH-dependant oxidase, xanthine oxidase, cell wall peroxidase, oxalate oxidase and amine oxidase, utilize reduced-substrates (NADPH, xanthine, oxalic acid and other amine substrates) for reduction of water and oxygen to form hydrogen peroxide and superoxide ion, respectively [Apel and Hirt, 2004; and Bolwell et al., 1995]. Superoxide dismutase produces hydrogen peroxide and molecular oxygen by breaking down superoxide ions. Sometimes, the defense system involving ROS is activated on the plant

exposure to harmful chemicals [Becana et al., 1998]. Thus, plants that produce enhanced levels of ROS on exposure to certain environmental contaminants have the capacity to oxidize those contaminants.

Overall, this study suggests a functional option for abating antibiotic pollution. Since the observed phytoremediation of the two antibiotics is carried out in the aqueous phase, the hairy roots (or the root exudates by themselves) have the potential for treating antibiotic contaminated wastewater that emerges from animal feedlots and municipal waste treatment facilities. Further studies are needed on the mechanism involved in antibiotic modification and the identification of the subsequent reaction products.

Acknowledgements

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Chapter IV. Oxytetracycline Oxidation by Reactive Oxygen Species Produced in the Nutrient Medium of *Helianthus annuus* Hairy Root Cultures

Reference: Gujarathi, N. P.; and Linden, J. C. *Oxytetracycline oxidation by reactive oxygen species produced in the nutrient medium of* Helianthus annuus *hairy roots cultures*. Submitted to **Biotechnology and Bioengineering** (2005).

Abstract

When subjected to stress, plants produce reactive oxygen species (ROS) as a part of the hypersensitive response. The oxidative response is also used to degrade organic pollutants. Hairy roots of *Helianthus annuus* (sunflower) are shown to oxidize oxytetracycline (OTC) through the action of the ROS released in to the nutrient medium by the hairy root cultures. Methyl jasmonate (MeJA) and salicylic acid (SA) are shown to elicit ROS activity in the hairy root cultures. The activities of the antioxidant enzymes, ascorbate peroxidase (APX), catalase (CAT), and guaiacol peroxidase (GPX), are reported for the root cultures treated with increasing concentrations of MeJA and SA. A bioassay, with *Enterococcus hirae* as the test microorganism, is used to demonstrate the root-catalyzed oxidation process results in conversion of OTC into product(s) devoid of antibiotic activity.

Keywords

Phytoremediation, oxidative burst, elicitor, methyl jasmonate, salicylic acid, antioxidant enzymes

Introduction

In the United States, and some parts of Europe, antibiotics are used in large quantities in confined livestock (e.g. swine, poultry and cattle) operations as growth promoters and feed efficiency promoters [De Liguoro et al., 2003; and Meyer et al., 2000]. Antibiotics are also used in aquaculture for fish production, where they are directly added to the surface water. These antibiotic growth promoters (AGPs) are given to the animals at sub-therapeutic levels. In humans and animals, around 30 to 90% of an administered antibiotic dose is excreted with the urine as active substance [Halling-Sorensen et al., 1998]. Meyer et al. (2000) report tetracyclines at concentrations up to 0.8 mg/L in hog lagoons. Kolpin et al. (2002) report tetracycline concentrations as high as 0.7 μg/L in water samples taken from rivers across the United States. Tetracyclines are also found in pig manure in concentrations up to 4 mg/L [Thiele-Bruhn, 2003]. Antibiotics are commonly found in sediments of fishponds at concentrations in the range of 0.5 to 4 mg/L [Thiele-Bruhn, 2003]. Thus antibiotics are found in water, soil and sediments, making their exposure to the microorganisms inevitable. Antibiotic resistant strains of *Salmonella, Campylobacter, Escherichia colli* and *Listeria* are suspected or known to exist [EPA, 2001]. Antibiotic resistant microorganisms, present in manure, are suspected to spread the resistance to other species [Thiele-Bruhn, 2003]. Mobile genetic elements conferring antibiotic resistance have been readily obtained from microbial communities

of environmental habitats [Smalla and Sobecky, 2002]. Thus there is a need to control and/or prevent the environmental release of antibiotics from livestock operations. Tetracycline antibiotics are among the most widely used class of antibiotics for livestock operations [De Liguoro et al., 2003; Meyer et al. 2000; and Winckler and Grafe, 2001].

The research focus of our laboratory is to study the phytoremediation of tetracycline antibiotics (tetracycline and OTC) from water and to develop strategies for effective field applications of the technology. In our previous research communications, we have reported the phytoremediation potentials of two aquatic plant species (Gujarathi et al. 5005a, In press) and the hairy root cultures of *Helianthus annuus* (Gujarathi et al. 2005b, In press) in remediating the two antibiotics. In Gujarathi et al. (b), we have suggested oxidative modification of OTC through the activity of ROS produced by *H. annuus* roots in the nutrient medium. The suggestion was based on the observed inhibition of the OTC modification activity of the *H. annuus* root exudates on addition of the antioxidant, ascorbic acid. Here, we extend the earlier work and, through various elicitation experiments aided by enzyme assays, confirm that ROS indeed are involved in the OTC modification process. We show that known ROS elicitors, MeJA and SA, increase the rates of OTC modification (oxidation) by the hairy roots of *H. annuus*.

In living organisms, ROS are usually produced as byproducts of aerobic metabolic processes such as respiration and photosynthesis; superoxide, hydrogen peroxide and hydroxyl radicals are produced during the reduction of oxygen to water (Apel and Hirt, 2004; and Vranova, et al., 2002). ROS are also involved in plant defense and signal

transduction systems. Plants respond to biotic and abiotic stresses by rapid production of ROS [Apel and Hirt, 2004]. This response, commonly a part of the hypersensitive response, leads to induction of plant defense enzymes, cross-linking of cell walls, and localized cell death. The abiotic stresses also include plant exposure to organic pollutants. For example, 2,4-dichlorophenoxyacetic acid (2,4-D), an herbicide, is oxidized by ROS-mediated enzymatic systems in *Pistium sativum* (pea) [Romero-Puertas et al., 2004]. Thus the available redox system in plants can potentially be put to use in detoxifying a number of environmental contaminants. In plants, the production of ROS is usually initiated by the enzymatic reduction of oxygen. Enzymes, such as NADPH-dependant oxidase, xanthine oxidase, cell wall peroxidase, oxalate oxidase and amine oxidase, utilize reduced-substrates (NADPH, xanthine, oxalic acid and other amine substrates) for reduction of oxygen to form superoxide ion, and subsequently hydrogen peroxide [Apel and Hirt, 2004; and Bolwell et al., 1995]. Hydrogen peroxide, if not detoxified, is converted to the highly reactive hydroxyl radical, by Fenton's reaction through the catalytic action of transition metals such as the ferrous ion.

Plants protect themselves from the toxic ROS by activating their antioxidant defenses that involve, both, non-enzymatic and enzymatic mechanisms. The non-enzymatic antioxidants include cellular redox buffers such as ascorbate and glutathione, as well as tocopherol, flavonoids, alkaloids, and carotenoids [Apel and Hirt, 2004]. The enzymatic antioxidants include enzymes such as APX, GPX, CAT superoxide dismutase (SOD), and glutathione reductase (GR) [Apel and Hirt, 2004; Zhang and Kirkham, 1996a; and Zhang and Kirkham, 1996b]. APX and GPX scavenge hydrogen peroxide by utilizing it for

oxidation of ascorbate and guaiacol, respectively. CAT breaks down hydrogen peroxide to give water and oxygen; SOD breaks down superoxide ions to form hydrogen peroxide and oxygen. Usually, with an increase in the ROS activity of the plant, the activity of these enzymes increases in a compensatory fashion. Plant peroxidases are also known to produce ROS through oxidation of Fe^{2+} to Fe^{3+} and the concomitant reduction of oxygen, or through the transfer of electron from reducing agents, such as NADH, to oxygen [Kawano 2003; and Minibaeva and Gordon, 2003]. Thus the ROS activity in plant systems can also be studied by assaying for the activity of the antioxidant enzymes native to that plant species.

The ROS production in plants can be increased through plant treatment with various elicitors. As mentioned earlier, ROS are produced as a result of stress; therefore compounds that 'mimic' stress situations can be effectively used as ROS elicitors. SA is known to elicit the formation of ROS in certain plant species and its ROS elicitation activity has been demonstrated [Custers et al., 2004; Kawano, 2003; Minibaeva and Gordon, 2003; and Minibayeva et al., 2003]. SA elicits ROS activity by inhibiting CAT activity and thus resulting in hydrogen peroxide accumulation, but not formation [Minibaeva and Gordon, 2003]. SA is known to, first, facilitate secretion of peroxidases in to the growth medium and then react with the peroxidase solution to generate SA free radicals that go on to form superoxide radicals [Minibaeva and Gordon, 2003; and Minibayeva et al., 2003]. But SA is also known to be effective in scavenging ROS [Gheldof et al., 2002; and Shankaran et al., 2001]. SA reacts with hydroxyl radical to give oxidation products such as 2,5-dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid, and

catechol [Owen et al., 2000]. The review by Kawano (2003) discusses the role of SA in the inactivation of peroxidases that produce ROS in tobacco cell cultures. Therefore, depending on reaction conditions, SA can act as either an antioxidant and/or as an elicitor of ROS.

According to Garrido et al. (2003), MeJA induces oxidative bursts in *H. annuus* roots because it is a signal molecule involved in the naturally occurring signaling cascade in the defense system of the plant. MeJA induces hydrogen peroxide generation through activating NADPH oxidase activities at the outer face of the plasma membrane. NADPH is suggested to be involved in the generation of superoxide radicals through the reduction of molecular oxygen. The superoxide radicals are dismutated by SOD to generate hydrogen peroxide. We will show that MeJA, as well as SA, elicit ROS formation in hairy root cultures of *H. annuus*.

Materials & Methods

Culturing and propagating *Helianthus annuus* hairy root cultures

Hairy root cultures of *H. annuus*, which were produced by infecting seedlings with *Agrobacterium rhizogenes* ATCC 15834, were acquired from the late Dr. Rajiv Bhadra, whose work initiated these studies. Records retrieved from Dr. Bhadra's work indicate seeds of the sunflower cultivar 'mammoth' were purchased from Burpee, Warminster, PA germinated in January 2001 and used for transformation. The root cultures were maintained in Gamborg's B-5 basal salt mixture [Phytotechnology Laboratories, Shawnee Mission, KS], supplemented with 3% sucrose (w/w) [Sigma-Aldrich, St. Louis,

MO] and Gamborg's B5 vitamin solution [Phytotechnology Laboratories, Shawnee Mission, KS]. The medium was prepared using reverse osmosis (RO) water; the pH of the solution was adjusted to 5.7 by adding potassium hydroxide [Fisher-Scientific, Fairlawn, NJ]. The medium was then transferred to the 250-mL Erlenmeyer flasks (75 mL per flask) and autoclaved for 20 minutes. After cooling, vitamin solution was added to the autoclaved medium at 1 mg/L final concentration by passing it through a sterile 0.2 μm syringe-filter [Millipore, Bedford, MA]. Four or five growing root tips, about 30 to 40 mm in length, were transferred from a previously existing culture to the fresh medium. The flasks were placed on a shaker table set at 110 rpm at room temperature in the dark. Transfers were usually conducted every three weeks.

Preparation of the root exudates

The root exudates from *H. annuus* were used in the experiment that was carried out to see if SA would act as an antioxidant in the absence of live roots. Root exudates of age 5 weeks were used for this experiment. Preparation of root exudates of a specific age involved growing the hairy roots in B5 medium for the required time. Once the required age was attained, roots were separated from medium, and the medium was filtered through a 0.2 μm filter membrane [Millipore, Bedford, MA] to remove microorganisms, root cells or cell debris from the root exudates. After filtration the root exudates (filtrate) were refrigerated until use in the experiment.

Experimental conditions and setup

The OTC-plant interactions described in this study were carried out in aqueous medium. Stock solution of OTC [Sigma-Aldrich, St. Louis, MO] was prepared by dissolution in RO water. For *in vivo* experiments (ones with the live root cultures), the stock solution was added to the root cultures on the 15^{th} after the cultures were transferred. Before addition, the OTC stock solution was filter-sterilized through 0.2 μm syringe filters [Millipore, Medford, MA]. A test concentration of 5 mg/L of OTC was used for all the OTC-plant interaction studies. Control experiments were conducted to determine the natural degradation of OTC in the growth medium (Gamborg B5 medium supplemented with 3% sucrose and vitamins), in the absence of the plant catalysts (live roots or the root exudates). The experiment involving the study of *in vitro* interactions between root exudates from *H. annuus* hairy root cultures and OTC, in the presence of 0.5 mM SA, was carried under conditions similar to that with the *in vivo* experiments involving the root cultures. For the elicitation experiments, stock solutions of SA [Aldrich, Milwaukee, WI] and MeJA [Sigma-Aldrich, St. Louis, MO] were prepared in de-ionized (DI) water and 95% ethanol, respectively. The stock solutions of MeJA and SA were added to the root cultures, of ages 15 and 16 days, respectively, by passing them through 0.2 μm syringe filters. The treatment concentrations of MeJA and SA studied were 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 mM; each treatment had OTC at 5 mg/L concentrations.

Sampling and analysis

After addition of OTC, the flasks were transferred to the shaker table for about 10 minutes to distribute OTC uniformly in the root exudates preparations and hairy root

cultures. The flasks were sampled in a laminar flow hood. The 0^{th} day samples represented the initial concentrations of OTC for the remediation studies. Samples were then taken at time intervals indicated in the graphs and immediately frozen for later analysis. High performance liquid chromatography (HPLC) was used for analyzing OTC concentrations in the growth media. After thawing, the samples were centrifuged in a micro-centrifuge [Brinkman Instruments Inc., Westbury, NY] for 10 minutes. The supernatant from the centrifuged samples was then filtered through a 0.45 μm centrifuge tube filter [Millipore, Medford, MA], transferred to a 250 μL silanized inert insert [Agilent Technologies, Wilmington, DE] and placed in a HPLC auto-sampler sample vial [Agilent Technologies, Wilmington, DE].

The HPLC system with photodiode array detector [Hewlett Packard 1050 series] was used with a 150 x 4.6 mm C-18 column [Phenomenex, Torrance, CA] for analyzing OTC concentrations in the samples. The mobile phase consisted of 0.1% (by volume) formic acid [Aldrich, Milwaukee, WI] in HPLC grade water [Fisher-Scientific, Fairlawn, NJ] (solvent A) and 2-propanol containing 0.1% (by volume) formic acid [Fisher-Scientific, Fairlawn, NJ] (solvent B). Before each sample injection, the column was preconditioned with solvent A to ensure optimum hydrophobic interaction between OTC and the column packing. An 82/18 ratio of solvent A to B was used to elute OTC from the column at combined flow rates of 1 mL/minute. The concentrations of OTC in the samples were determined by measuring their UV absorbance at 355 nm. The retention time for OTC was found to be around 5.0 minutes. Periodically, slight changes in the retention times

were encountered, possibly due to an increase in the pressure drop across the column. The detection limit of the method was 0.2 mg/L of OTC.

Enzyme assays

At the end of the respective experiments, the roots that were to be used for the enzyme assays were dried on a paper towel, weighed and frozen until their use in the assay. The enzyme extract for studying APX activity was prepared by homogenizing 0.7 g of root biomass in 8 mL of 25 mM sodium phosphate [Phytotechnology Laboratories, Shawnee Mission, KS] buffer (pH 7.8) containing 0.2 mM EDTA [Fisher-Scientific, Fairlawn, NJ] and 10 g/L of polyvinylpyrrolidone [Sigma, St. Louis, MO]. The homogenate was then filtered through four layers of cheesecloth and centrifuged at 8000 rpm at 4 C for 20 minutes. The supernatant was collected and used for enzyme assays as the enzyme extract. The enzyme extract for studying CAT and GPX activity was prepared by homogenizing 0.5 g of root biomass in 6 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 0.2 mM EDTA and 10 g/L of polyvinylpyrrolidone. The homogenate was then filtered through four layers of cheesecloth and centrifuged at 8000 rpm at 4 C for 15 minutes. The supernatant was collected and used for enzyme assays as the enzyme extract.

The procedures for preparing the enzyme extracts and for carrying out the assays were adapted from Zhang and Kirkham (1996a and 1996b). Changes made in the assay method were necessary because the assays reported in the above reference were carried out either on *H. annuus* leaves or on roots obtained from the seedlings of *H. annuus*. The enzyme

extract obtained from our hairy root cultures, when used at the prescribed amounts in the assay, gave a constantly fluctuating base absorbance in the spectrophotometer cell. This observation might, probably, be due to the high levels of phenolic compounds in the homogenate. So a lesser volume of the homogenate (20 μL) was used for all the enzyme assays reported here.

APX assay

The assay mixture was made in a 25 mM sodium phosphate buffer (pH 7.0) that contained L-ascorbic acid [Fisher-Scientific, Fairlawn, NJ] at 0.25 mM concentration and 0.05 mM EDTA. 20 μL of the enzyme extract were added to 1 mL of the assay mixture. The reaction was started by adding 40 μL of 5 mM H_2O_2 [Aldrich, Milwaukee, WI]. The reaction mixture had a total volume of 1.06 mL. APX activity was assayed by following the decrease in absorbance at 290 nm for 10 minutes, resulting due to the disappearance of L-ascorbic acid. In the time frame of the assay, no significant oxidation of L-ascorbic acid was observed in the absence of either the enzyme extracts or hydrogen peroxide.

CAT assay

A 25 mM sodium phosphate buffer (pH 7.0) was used as the assay mixture. 40 μL of 62.5 mM H_2O_2 were added to 1 mL of the assay mixture. The reaction was started by adding 40 μL of the enzyme extract. The reaction mixture had a total volume of 1.06 mL. CAT activity was assayed by following the decrease in absorbance at 240 nm for 10 minutes, resulting due to the decomposition of H_2O_2. In the time frame of the assay, no significant decomposition of H_2O_2 was observed in the absence of the enzyme extracts.

GPX assay

The assay mixture was made in a 10 mM sodium phosphate buffer (pH 7.0) that contained pyrocatechol [Sigma, St. Louis, MO] at 0.25 mM concentration. 20 μL of the enzyme extract were added to 1 mL of the assay mixture. The reaction was started by adding 40 μL of 5 mM H_2O_2. The reaction mixture had a total volume of 1.06 mL. GPX activity was assayed by following the increase in absorbance at 470 nm for 10 minutes, resulting due to the formation of the oxidation product of pyrocatechol. In the time frame of the assay, no significant oxidation of pyrocatechol was observed in the absence of either the enzyme extracts or hydrogen peroxide.

Bioassay for determining the anti-microbial potency of the phyto-oxidized OTC

The bioassay, which was adapted from Rodriguez et al. (2004), was a test to determine if the ROS-mediated oxidation product(s) of OTC still retained its anti-microbial potency. *Enterococcus hirae* (formerly *E. faecalis*) ATCC 10541 was used as the test microorganism for the bioassay; the culture was maintained on tryptone soy agar [Difco, Detroit, MI] slopes at 4^0 C. Before use in the bioassay, *E. hirae* was cultured on the tryptone soy agar slopes at 37^0 C for 24 hours. A loop of *E. hirae* was then transferred to 20 mL of an assay broth containing: trypticase (9 g/L) [Becton Dickinson, Cockeysville, MD, USA], glucose (20 g/L) [Mallinckrodt, Paris, KY], yeast extract (5 g/L) [Fisher-Scientific, Fairlawn, NJ], sodium citrate (10 g/L) [Fisher-Scientific, Fairlawn, NJ], K_2HPO_4 (1 g/L) [Fisher-Scientific, Fairlawn, NJ], and KH_2PO_4 (1 g/L) [Fisher-Scientific, Fairlawn, NJ]. The assay broth was autoclaved and then cooled to approximately room temperature before inoculation with the bacterial loop. The assay broth, inoculated with

E. hirae, was then incubated at 37^0 C for 18 hours. At the end of the incubation period, 13 mL of the culture were transferred to a liter of fresh assay broth. This cell suspension containing broth was then used as the inoculum for the assay experiments. For the bioassay four parts of the inoculum were added to one part test solution, in sterile 15-mL centrifuge tubes [Fisher-Scientific, Fairlawn, NJ]. The tubes containing the *E. hirae*-inoculated controls and test solutions were incubated in a water bath at 37^0 C for the required amount of time (up to 24 hours). For the cell density measurements, samples were drawn from the incubation tubes and the cell densities of the test solutions were determined as the visible light absorbance at 600 nm. As described in Rodriguez et al. (2004), the absorbance at 600 nm was used as a surrogate for the cell density of *E. hirae* suspension cultures.

The bioassay experiment was conducted in two parts. The purpose for the first part of the experiment was to compare the growth of *E. hirae* in four different test solutions, which were: de-ionized water, 5 mg/L OTC solution in DI water, root exudates from *H. annuus* hairy roots cultures exposed to 5 mg/L OTC, and root exudates from *H. annuus* hairy roots cultures exposed to 5 mg/L OTC and 0.6 mM MeJA (X). The cell densities were measured at incubation periods of 0, 4, 8, 12 and 24 hours. The second part of the experiment was conducted to check whether the elicitation of ROS activity of the *H. annuus* hairy roots cultures on MeJA-treatment leads to a further reduction in the toxicity of the dissolved OTC. As control experiments, the inoculum was added in the same proportions, to: a) de-ionized (DI) water, b) 5 mg/L solution of OTC in DI water, and c) root exudates (RE) from *H. annuus* grown for 5 weeks that had not been exposed to

either OTC or MeJA. The RE collected at the end of the MeJA-treatment experiments were frozen and then used later as the test solutions for this bioassay. The cell densities were measured at incubation periods of 4 hours and 24 hours.

Results and Discussion

Equation [I] is used for quantitation of the observed OTC oxidation only as a fit to the data.

$$C_t = C_0.e^{-k.t} \qquad [I]$$

Where,

C_0 and C_t are the concentrations of OTC at time 0 and t, respectively, and k is the first order rate coefficient for the process. Exponential decay does not necessarily model the actual reactions kinetics for the reported OTC-hairy roots/root exudates interactions (See Gujarathi et al. (b)). The first order rate coefficient value, k, is used to compare the OTC oxidations rates between treatments to gain an understanding of the effect of SA or MeJA elicitation on the hairy roots.

Effect of varying concentrations of MeJA on *H. annuus* roots and their OTC oxidation activities

Figure 1 shows the image of the root cultures of *H. annuus*, three hours after MeJA treatment. Starting, from the left, with the root culture not treated with either MeJA or OTC to the successive ones with increasing concentrations of MeJA treatment (and OTC at 5 mg/L), the effect of oxidative stress on the roots is clearly visible in the form of dark coloration. The shade of the roots gets darker with an increase in the treatment MeJA

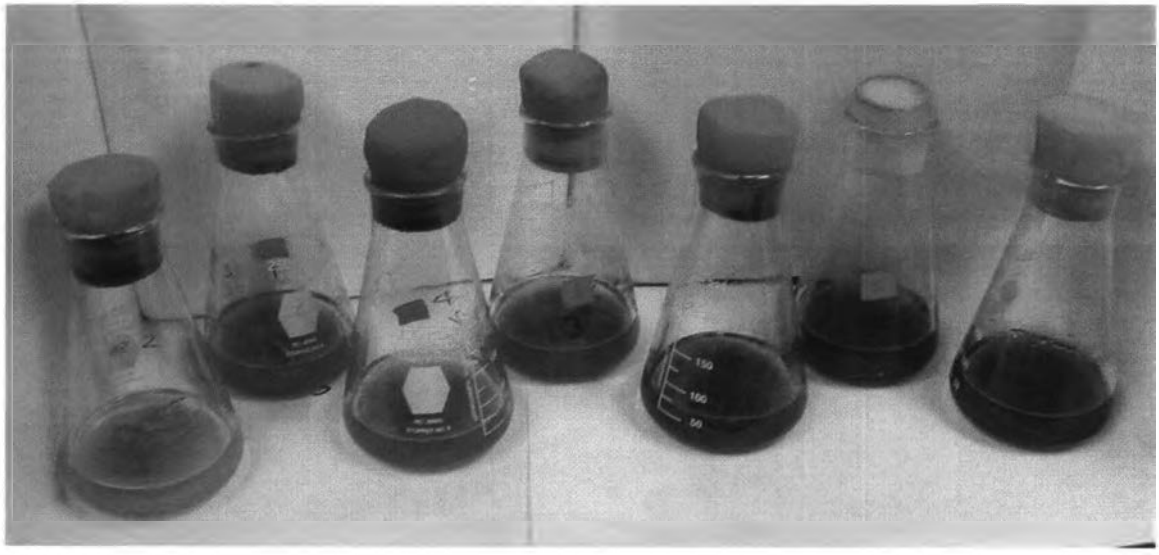


Figure 1. Oxidative burst in *H. annuus* root cultures in response to treatment with OTC and MeJA. The flasks shown in the figure are (starting from the left): control roots (no OTC or MeJA treatment); roots treated with OTC; roots treated with OTC and 0.2 mM MeJA; roots treated with OTC and 0.4 mM MeJA; roots treated with OTC and 0.6 mM MeJA; roots treated with OTC and 0.8 mM MeJA; and roots treated with OTC and 1.0 mM MeJA.

concentration. Such manifestations of oxidative stress on roots of *H. annuus*, caused due to MeJA, has been discussed elsewhere [Garrido et al., 2003]. Apparently, the ROS excreted by the plant roots are consumed by extracellular peroxidases in cross-linking and phenolics oxidation in the cell walls, resulting in the darkening of the roots. Thus, the greater the concentration of the extracellular ROS the darker would be the roots. Interestingly, the roots treated only with 5 mg/L of OTC too show a darker shade as compared to the non-treated roots. Therefore, OTC by itself also appears to elicit ROS formation in the roots, thus supporting our hypothesis that OTC oxidation by ROS is the primary mechanism of phytoremediation by the roots. It is important to note that manifestation of oxidative stress (darkening of roots) may not necessarily mean oxidative damage. As shown later, oxidative damage is usually seen in the form of reduction in growth and/ or reduction in the levels of soluble proteins. When subjected to oxidative stress, plant antioxidant systems usually prevent (or at least reduce) the oxidative damage caused by the stress.

As described earlier, in the materials and methods section, two days after the elicitation with MeJA, the roots were harvested, dried on paper towels, weighed and frozen for later enzyme assays. And at the end of the enzyme assays the total soluble protein concentration was determined by the method of Bradford [Bradford, 1976]. The fresh weights (g) for the roots from the different MeJA treatments and the corresponding soluble proteins (g/L), extracted from the roots during the preparation of the enzyme extracts, are graphed in Figure 2. Both, the fresh weights and the total soluble protein concentrations, appear to follow a similar trend with the values decreasing at higher

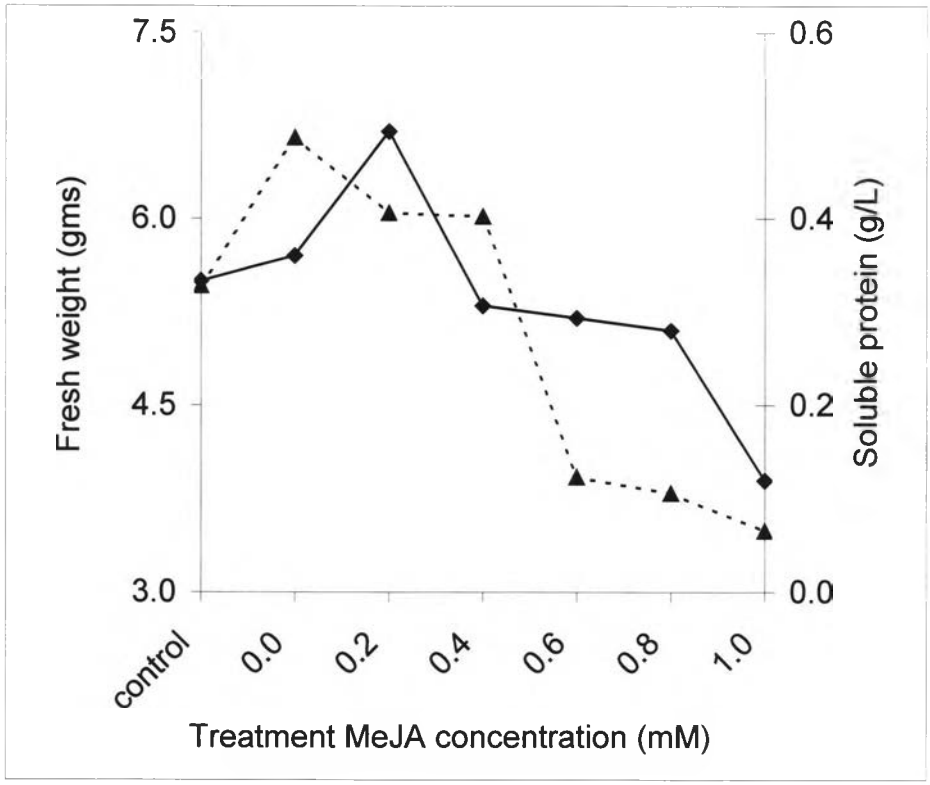


Figure 2. Effect of increasing concentrations of MeJA on the fresh weights (♦) and the total soluble protein content of the roots (▲).

MeJA concentrations. The total soluble protein content of the roots decreases appreciably on increasing the treatment concentration of MeJA from 0.4 to 0.6 mM. Thus, at the treatment concentration of 1.0 mM MeJA, the effects of oxidative damage on the roots are evident in the form of notable reduction in the fresh weight and total soluble protein content of the roots.

The dose-dependant ROS elicitation by MeJA results in an expected dose-dependant enhancement of the OTC oxidation activity of the root cultures (see Figure 3). A treatment concentration of 0.6 mM of MeJA appears to induce the maximum OTC oxidation activity. As compared to the root culture not treated with MeJA, a three-fold increase in the first-order rate coefficient for OTC oxidation is observed in the 0.6 mM treatment. The decrease in the OTC oxidation activity at concentrations higher than 0.6 mM might be due to the inhibitory effects of such concentrations of MeJA on growth and protein activities of the root cultures (Figure 2). For optimal ROS activity there should be an optimal level of the ROS producing enzymes such as the NAPH oxidases and other extracellular peroxidases (see the introduction section for background information on these enzymes). Although the specific activities of these enzymes do not decrease, a decrease in the total soluble protein content reduces the net ROS production.

The specific activity of an enzyme is defined as the amount of substrate consumed or product formed (normally expressed in molar units) per unit time and per unit mass of protein present in the enzyme extract of a certain volume. The net enzyme activity of an enzyme can then be defined as the amount of substrate consumed or product formed per

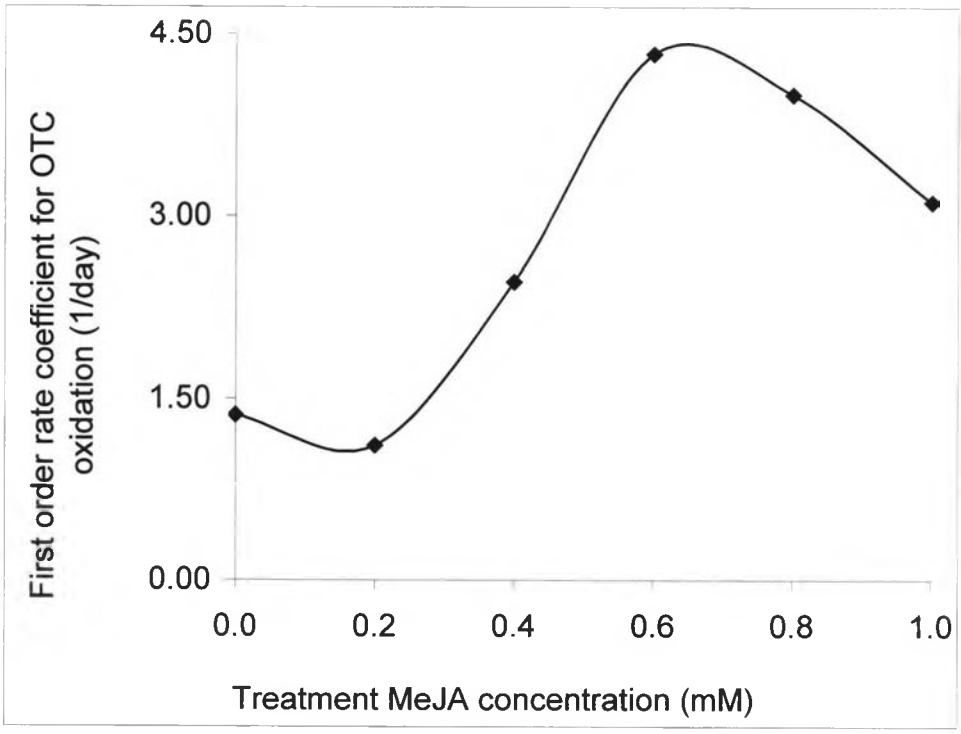


Figure 3. Effect of increasing concentrations of MeJA on OTC oxidation by *H. annuus* root cultures.

unit time by the same volume of the enzyme extract. Usually, for monitoring an enzymatic activity, the specific activity of the enzyme is relevant. But for the interaction between *H. annuus* roots and OTC, which takes place in the extracellular nutrient medium, the net enzyme activity might be of greater relevance. We say this because, as illustrated in the earlier paragraph, the total protein content of the enzyme extracts reduces drastically at higher MeJA concentrations. The results, in the form of graphs, from the enzyme assays that are presented in this study give the specific enzyme activities on the primary Y-axis and the net enzyme activities on the secondary Y-axis (Figures 4 and 8). Units are the same on both the axes in Figures 4 and 8.

Figure 4 shows the enzyme activities observed in the extracts obtained from the MeJA-treated roots. As compared to the control roots (no OTC and no MeJA treatment), the net activities of the two peroxidases, APX and GPX, increase for the roots treated solely with 5 mg/L of OTC. Since peroxidases are usually associated with hydrogen peroxide scavenging, a higher peroxidase activity can be linked with the presence of higher concentration of the peroxide in response to which the net activity of the peroxidases increases. The APX and GPX activities decrease on treatment with increasing concentrations of MeJA, especially at and above 0.4 mM. This, to an extent, might explain why we observe greater oxidative stress (in the form of darkened roots), in the four treatments between 0.4 and 1.0 mM of MeJA. Also, since the net antioxidant activity is low, the ROS-mediated OTC oxidation rates can be expected to be high, as evident from Figure 4. Even in the case of CAT, the net activity is almost absent for the 0.6, 0.8 and 1.0 mM treatments. For CAT, the maximum activity is observed in the non-OTC

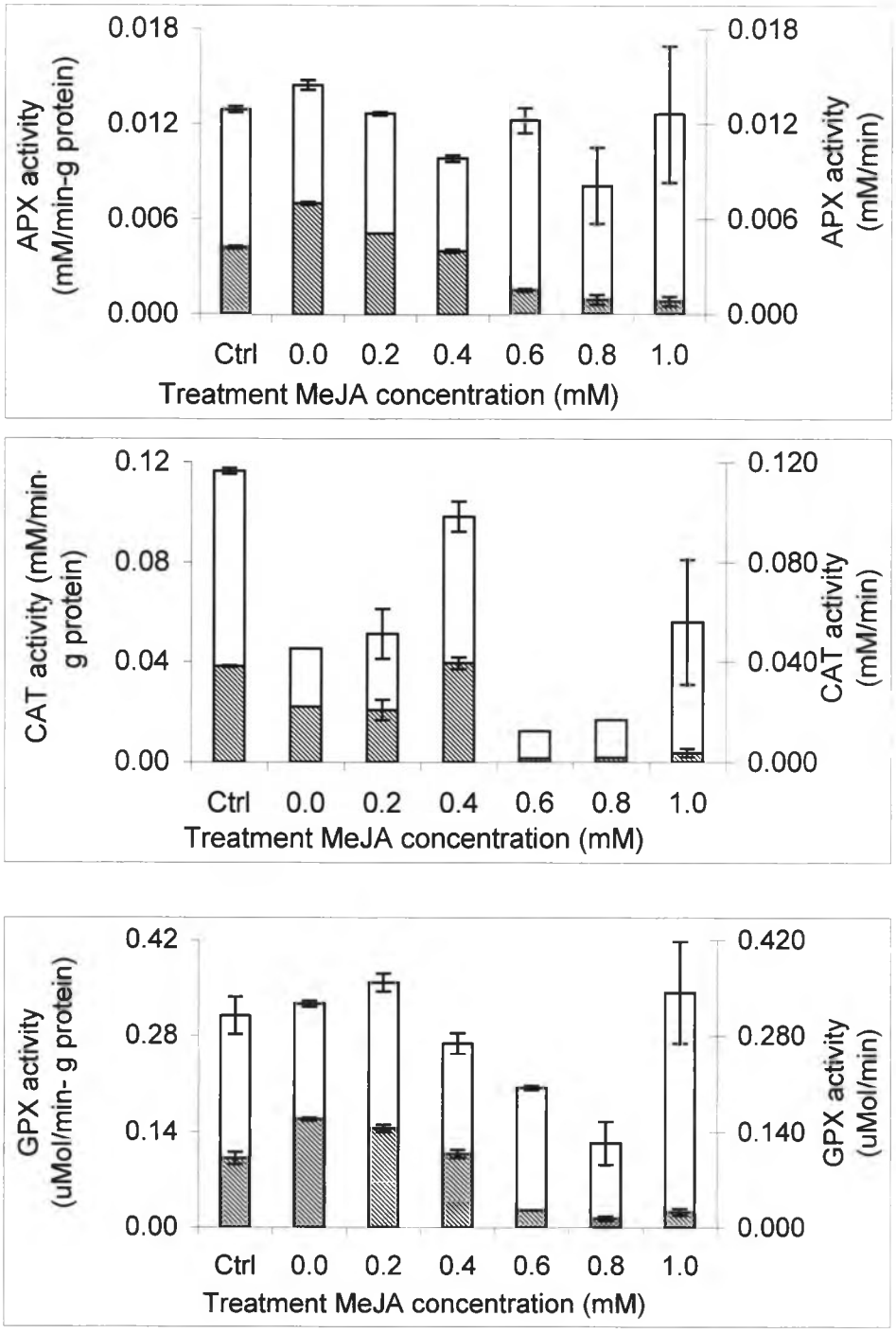


Figure 4. Enzyme activities in the roots treated with MeJA. The shaded columns give the values for the non- specific enzyme [units: mM/min], while the non-shaded columns give the values for the specific enzyme activities [units: mM/min-g of protein].

non-MeJA control and in the 0.4 mM treatment. Overall, the net activities of these three antioxidant enzymes are markedly lower in the roots treated with MeJA at concentrations higher than 0.4, thus enabling greater ROS production for those treatments. But, owing to the reduced antioxidant activities at these treatment MeJA concentrations, the roots undergo greater oxidative damage.

Effect of varying concentrations of SA on *H. annuus* roots and their OTC oxidation activities

Since the oxidative stress caused by 5 mg/L OTC alone in comparison with the non-treated control is compared in the earlier section, the control roots are not included in the following involving SA. Instead, comparison is made between the roots treated solely with OTC and the ones treated with OTC and steadily increasing concentrations of SA as an ROS elicitor.

As in the case with MeJA, increasing concentrations of SA induce greater oxidative stress in *H. annuus* (Figure 5). The fresh weights of the roots remain relatively constant up to a treatment concentration of 0.8 mM SA (Figure 6). The total soluble protein content of the roots appears to increase on SA treatment. According to the preliminary experiments reported by Minibaeva and Gordon (2003), exogenous SA increases the total content of extracellular proteins. Both, the fresh weight and the total protein content, drop drastically for the 1.0 mM treatment concentration. While for the 1.0 mM MeJA treatment the fresh weight and the soluble content drop to about half of the non-elicited control, in the 1.0 mM SA treatment they drop to less than one third of the non-elicited

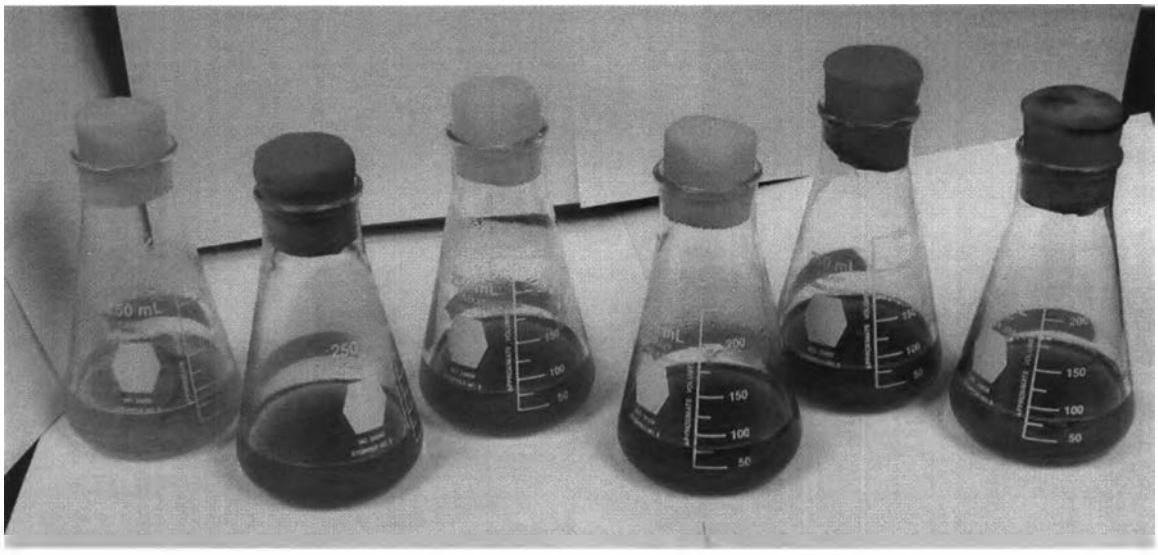


Figure 5. Oxidative burst in *H. annuus* root cultures in response to treatment with OTC and SA. The flasks shown in the figure are (starting from the left): roots treated with OTC; roots treated with OTC and 0.2 mM SA; roots treated with OTC and 0.4 mM SA; roots treated with OTC and 0.6 mM SA; roots treated with OTC and 0.8 mM SA; and roots treated with OTC and 1.0 mM SA.

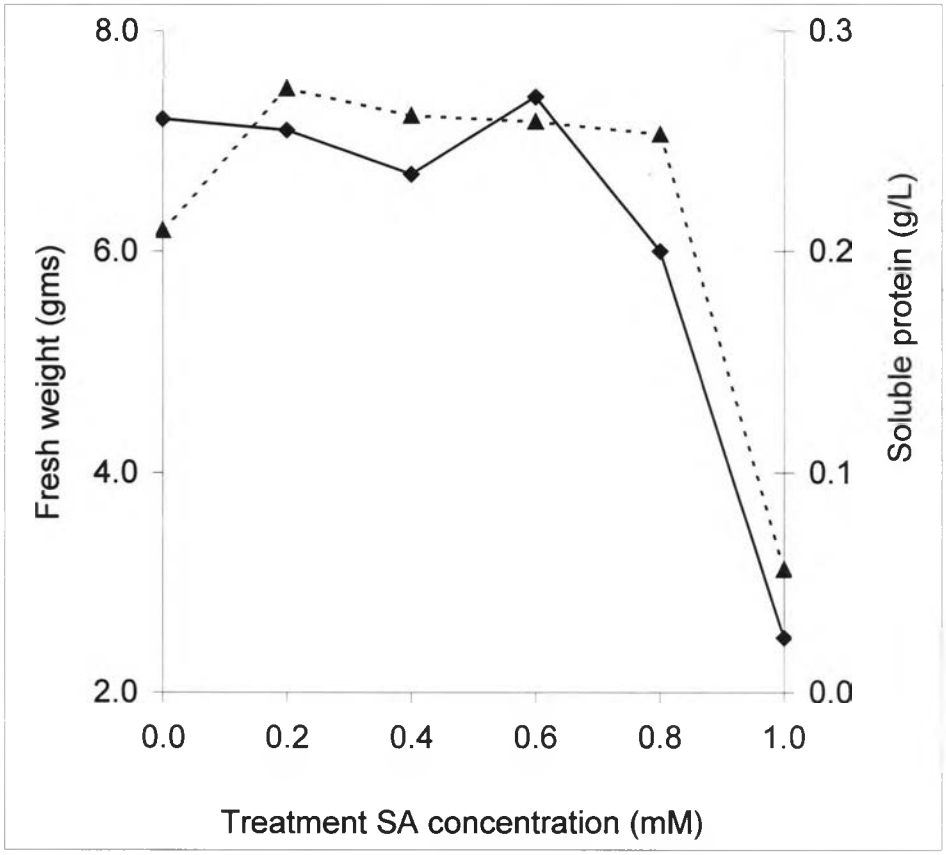


Figure 6. Effect of increasing concentrations of SA on the fresh weights (♦) and the total soluble protein content of the roots (▲).

control. We suspect that by the time we froze the roots, the root culture treated with 1.0 mM SA was already dead. At the same time it is important to note that the inhibitory effects of MeJA on the *H. annuus* root cultures appear to occur at lower treatment concentrations than those due to SA (compare Figure 2 and Figure 6).

The OTC oxidation activity of the *H.* annuus root cultures increases with an increase in the treatment SA concentration (See Figure 7), with concentrations of 0.6 and 0.8 mM yielding maximum OTC oxidation rates. As in the case with MeJA, a maximum of three-fold increase in the first-order OTC oxidation rate coefficient, with respect to the non-treated control, is observed. The OTC oxidation rate for the 1.0 mM treatment is almost equal to that obtained for control, which might be because, as mentioned earlier, the roots might have died and the ROS production, consequently the OTC oxidation, might have ceased.

The enzyme activities for the SA treatment are shown in Figure 8. The net APX activity appears to stay almost constant until the treatment SA concentration reaches 0.8 mM, after which the net activity reduces markedly. For CAT, as compared to the non-SA control, the net activity increases for 0.2 mM SA treatment; the net activity is approximately same as that in the control for the higher treatment concentrations of SA. In the case of GPX, the net activity steadily increases with increasing treatment concentration of SA, but only up to the 0.8 mM treatment. As in the case with APX, the net GPX activity is almost absent for the 1.0 mM treatment. Thus, sustained activities of the three enzymes in the concentration range of 0.0 to 0.8 mM appears to avoid the

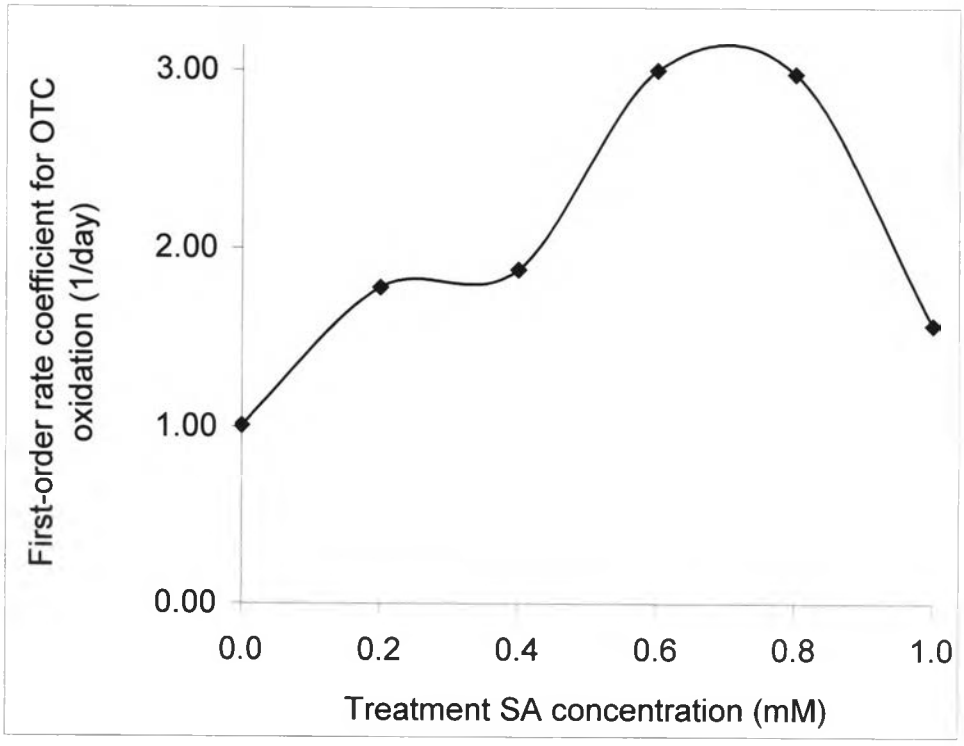
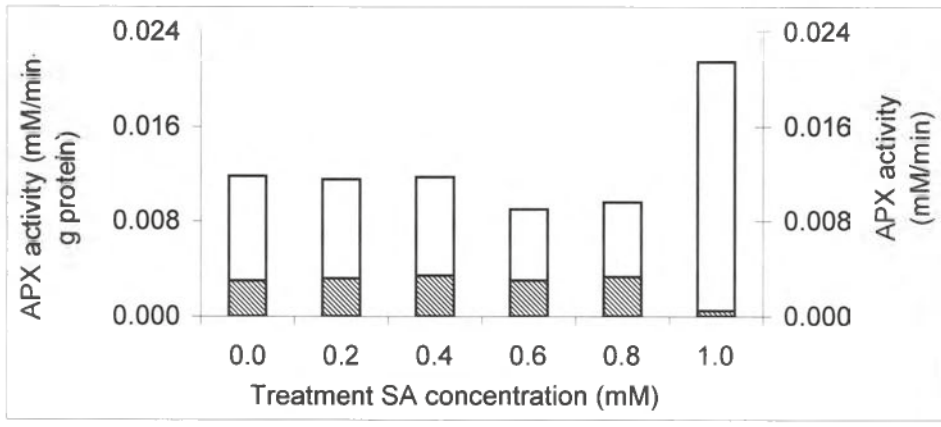
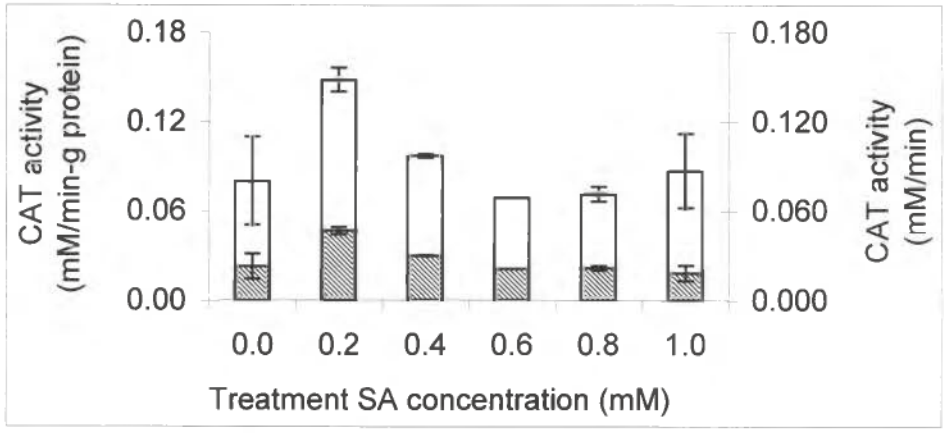


Figure 7. Effect of increasing concentrations of SA on OTC oxidation by *H. annuus* root cultures.





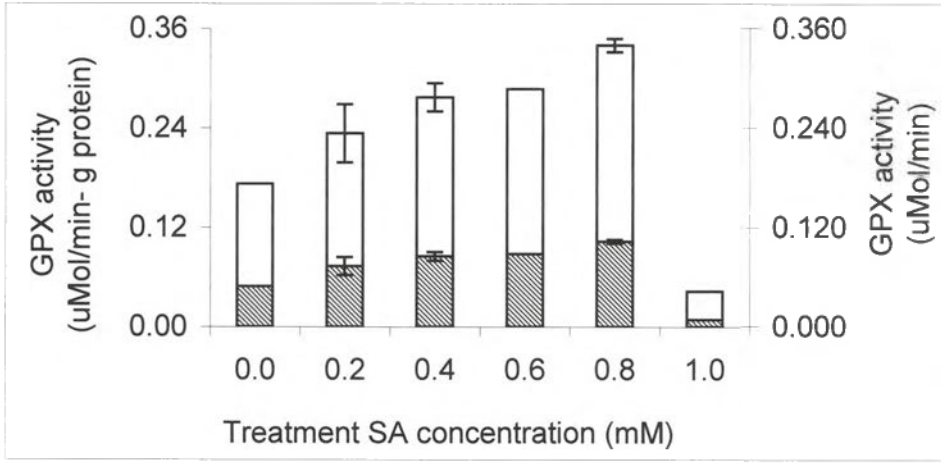


Figure 8. Enzyme activities in the roots treated with SA. The shaded columns give the values for the non- specific enzyme [units: mM/min], while the non-shaded columns give the values for the specific enzyme activities [units: mM/min-g of protein].

inhibitory effects of oxidative stress in the SA treatments. Therefore, SA elicits ROS formation in *H. annuus*, but it does not appear to affect the antioxidant activity. It is also interesting to note that since the net activities of the antioxidant enzymes appears to remain constant, for most part i.e. up to 0.8 mM SA treatment, the ROS formation, and consequently the OTC oxidation rates increase in a dose-dependant fashion without any significant oxidative damage to the roots.

SA as an antioxidant and as an elicitor of ROS

In the live root cultures (see R-OTC in Figure 9), SA appears to elicit ROS activity and thus increases the rate of OTC modification. However, in the root exudates (see RE-OTC in Figure 9), SA appears to scavenge the ROS and thus decreases the rate of OTC modification. The OTC control, with the same concentration of OTC in the nutrient medium, shows no appreciable OTC modification. Thus, primarily SA is an antioxidant that, as discussed earlier, reacts with the hydroxyl radical to produce the subsequent products of oxidation. But in the presence of live roots, the SA is inducing the secretion of cell wall peroxidases in to the growth medium that in combination with SA is producing superoxide ions [Minibaeva and Gordon, 2003; and Minibayeva et al., 2003]. These superoxide ions then breakdown to give hydrogen peroxide, which in turn interact with transition metals (or transition metal based enzymes) to give the highly reactive hydroxyl radical. There is also the possibility of SA being involved in signal transduction in the roots that would lead to induction of defense responses of the root cultures, including the secretion of ROS. Thus SA acts as an antioxidant in the absence of the ROS

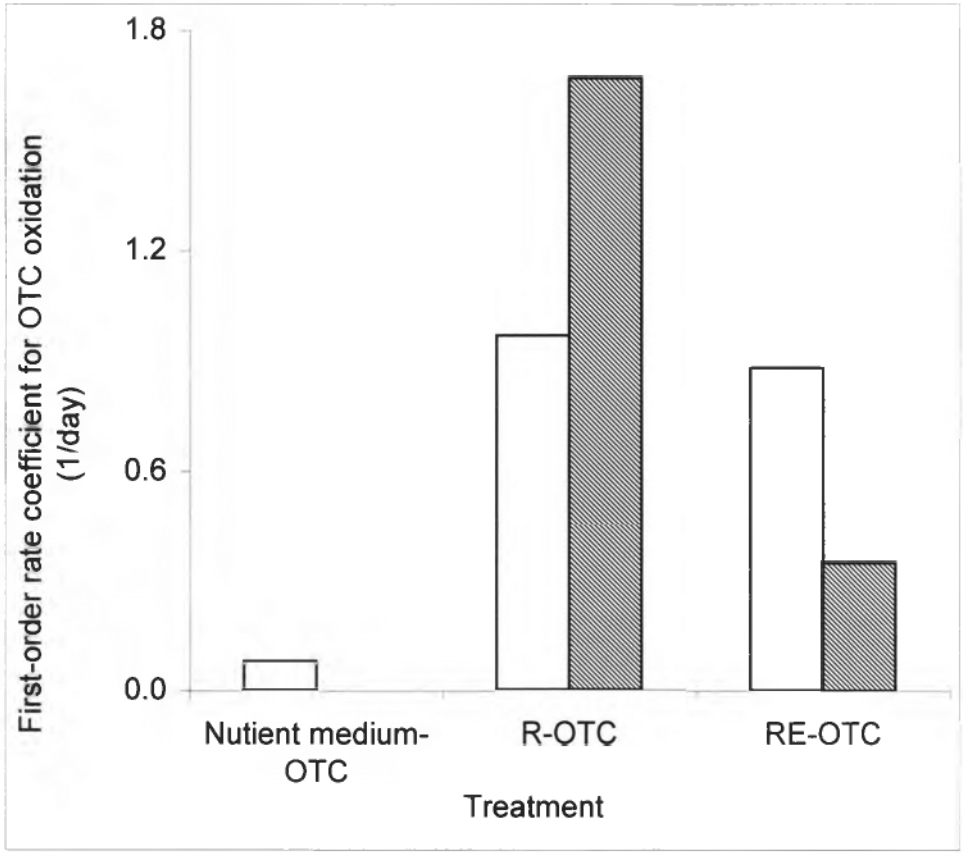


Figure 9. Effect of 0.5 mM SA treatment on OTC oxidation by *H. annuus*. The non-shaded columns represent the results for the roots and root exudates not treated with SA, while the shaded columns represent the results for the SA-treated roots and root exudates. (Symbols: R- roots and RE- root exudates from *H. annuus*).

producing *H. annuus* roots, while in the presence of the live roots it elicits ROS production by the roots.

Bioassay for determining the anti-microbial potency of the phyto-oxidized OTC

The primary purpose for conducting the bioassay was to determine whether the oxidation product(s) of OTC, presumably obtained after interaction with the ROS from *H. annuus*, had retained the anti-microbial potency of the parent OTC molecule. It was carried out in two parts. The first part of the assay experiment compared the growth curves for *E. hirae* in the treated-OTC medium with that in the controls, while the second part compared the growths (cell densities) in medium obtained from various OTC treatments.

The growth curves for *E. hirae* shown in Figure 10 represent the four test solutions: de-ionized water, 5 mg/L OTC solution in DI water, root exudates from *H. annuus* hairy roots cultures exposed to 5 mg/L OTC, and root exudates from *H. annuus* hairy roots cultures exposed to 5 mg/L OTC and 0.6 mM MeJA. Even after an incubation period of 24 hours, the growth in the OTC control is negligible. As is evident from the results, the oxidation product does not appear to be toxic to *E. hirae*. In the initial 8-hour period, maximum growth is observed in the DI control; slower growth is allowed by the 0.6 mM MeJA-treated root exudates and the root exudates-OTC test solution. Since the 0.6 mM MeJA treatment gives maximum OTC oxidation (See Figure 3), greater OTC detoxification can be expected, compared to that in the purely OTC treated roots. After 24 hours of incubation, the cell densities for *E. hirae*, in the three solutions are similar. Observation of slow growth in the initial 12 hours, followed by that in DI-controls later

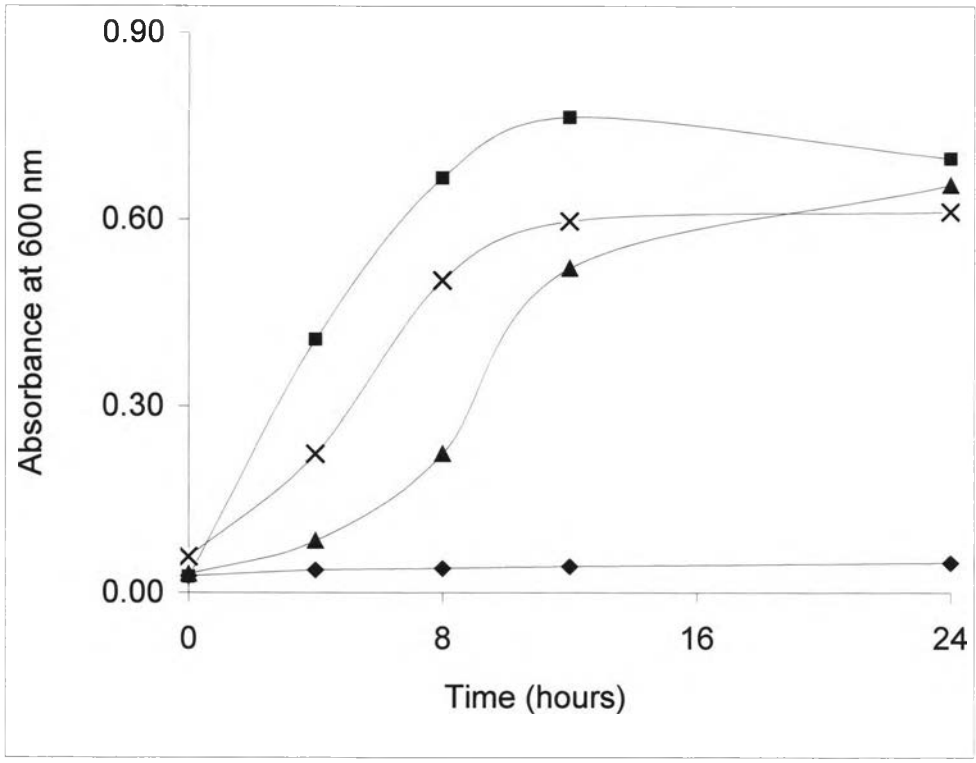


Figure 10. Growth curves for *E. hirae* in DI water (■), 5 mg/L OTC solution (♦), root exudates from *H. annuus* hairy roots cultures exposed to 5 mg/L OTC (▲), and root exudates from *H. annuus* hairy roots cultures exposed to 5 mg/L OTC and 0.6 mM MeJA (X).

on, might be due to acclimatization time required by the microorganisms to adapt to the root exudates containing the oxidation product(s) of OTC.

The OTC concentrations in the test/ treatment samples after 24 hours are given in Figure 11 [A]. The DI and RE (root exudates) controls had no OTC added to them. The OTC control has OTC at 5 mg/L. While the six treatment samples from the MeJA-elicitation experiments were each treated with 5 mg/L of OTC, the root exudates were recovered and frozen for the bioassay after an incubation period of two days for OTC in the respective MeJA-elicited cultures. OTC concentrations in the cultures after 48 hours are not available since the OTC sampling from MeJA-elicitation experiment was terminated after the first 24 hours of MeJA treatment. The OTC concentrations in the MeJA treated samples, as expected, follow a trend consistent with the trend observed for OTC oxidation by the MeJA-treated roots in Figure 3.

As compared to the DI control, the DI-OTC control gives very low cell densities for both incubation periods (See Figure 11[B]). Unlike the case of the other test solutions and controls, the cell densities did not change appreciably over the 19 hour time period (Figure 11 [B]). The RE-control, with no OTC or MeJA treatment, gives the highest cell density, which can be expected considering the fact that plant rhizosphere provides rich nutrients for high microbial activity. The trend for the cell densities of *E. hirae* in the RE from the MeJA treatment is consistent with the trend for the OTC concentrations in those treatments (compare parts [A] and [B] of Figure 11). Excluding the OTC control, the 0.6 mM treatment concentration MeJA gives the highest cell density while the 0.2 mM

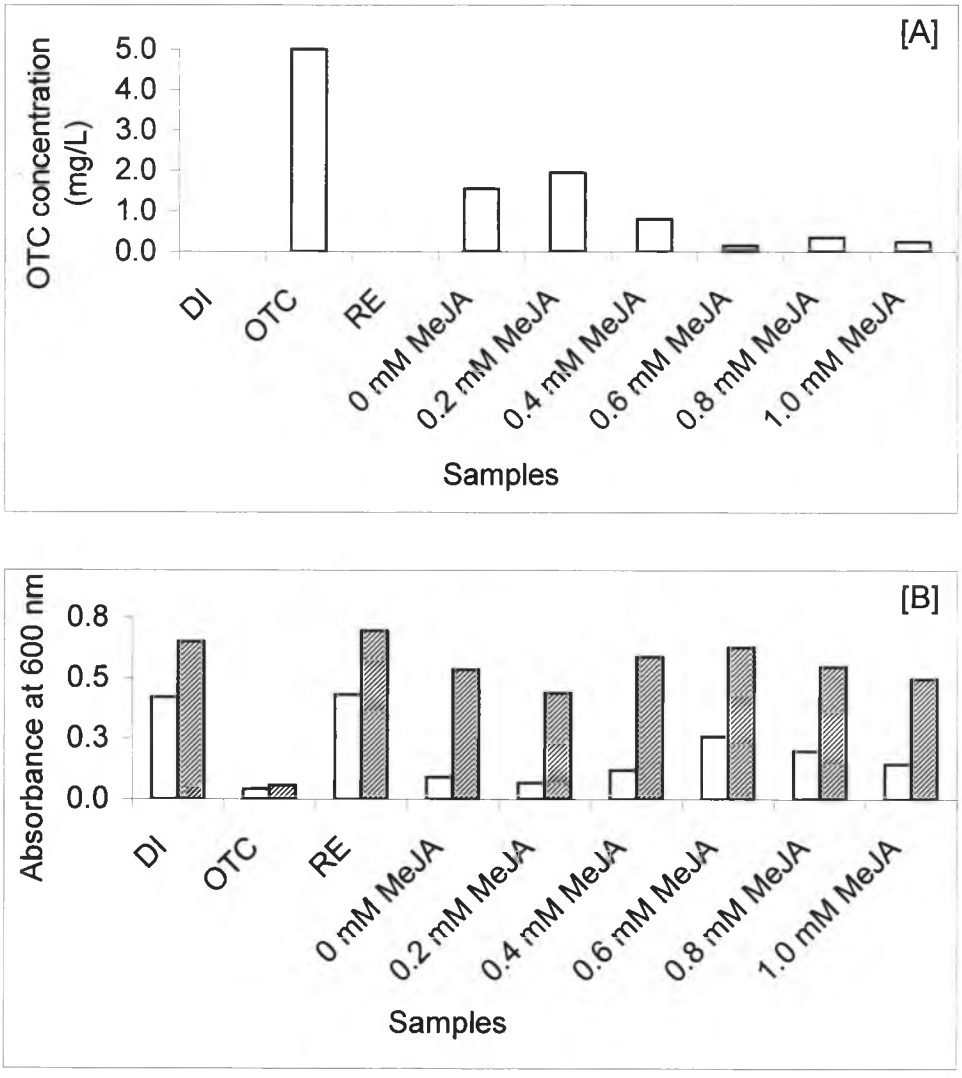


Figure 11. [A] OTC concentrations in the treatment samples, 24 hours prior to freezing. [B] Absorbance at 600 nm for the *E. hirae* suspension cultures exposed to the treatment samples. (DI- de-ionized water, OTC solution in DI water; RE- root exudates from *H. annuus*). The non-shaded columns represent the absorbance values for the cultures after 4 hours, while the shaded columns represent the values after 23 hours.

treatment concentration gives the lowest cell density. This consistency in the trends in Figures 11 [A] and 11 [B], though expected, further confirms our hypothesis that MeJA, which is known to elicit ROS activity in plants, results in enhanced levels of oxidation of OTC to give oxidation product(s) devoid of antibiotic activity. An important implication of the above findings is that MeJA, and presumably SA too, can be used for optimizing the phytoremediation of OTC when the technology is used in field operations.

Conclusions

Hairy root cultures of *H. annuus* oxidize OTC through the action of ROS that are secreted into the nutrient medium. MeJA and SA, which are known elicitors of ROS in plants, elicit ROS production by the hairy root cultures when added exogenously, and result in enhanced rates of OTC oxidation. OTC, by itself, also elicits ROS formation in the roots. Treatment concentrations of approximately 0.6 mM of MeJA or SA in the nutrient medium both resulted in maximum OTC removal. The oxidative burst caused due to elicitation of the ROS activity in the hairy root cultures is demonstrated by darkening of the roots. The oxidative burst is followed by an oxidative damage, which is evident from the reduced biomass and lowered total soluble protein content of the roots. The hairy root cultures show greater tolerance towards SA, compared to that towards MeJA. Oxidative damage caused by treatment concentration up to 0.8 mM MeJA exceeds that caused by similar concentrations of SA. At the treatment concentrations of 1.0 mM of MeJA and SA, the hairy roots are killed. In the case of SA elicitation, sustained net activities of the antioxidant enzymes (APX, CAT and GPX) appear to protect the root cultures against oxidative damage. With MeJA, the net activities of the three enzymes decrease with

increasing treatment concentrations, which result in oxidative damage. When SA is added to the root exudates obtained from the hairy root cultures in the absence of the live roots, SA acts as an oxidant and reduces the OTC oxidation activity of the root exudates. Thus, when added to the root exudates-containing nutrient medium, SA acts as an antioxidant and an elicitor of ROS in the absence and presence of the live roots, respectively.

The major concern regarding the release of antibiotics in to the environment is the evolution, and the subsequent proliferation, of antibiotic resistance among microbial populations in the exposed environment. Therefore, it is important to ensure that the method adopted to treat the antibiotics should result in biologically inactive product(s). The ROS-mediated oxidation of OTC by the hairy root cultures of *H. annuus* yields oxidation products(s) that have lost the antimicrobial potency of the parent OTC molecule. *E. hirae*, a microorganism that is known to be susceptible to tetracyclines, shows good growth in the root exudates that contain the oxidation product(s) of OTC; no growth is observed in an OTC solution in water. The extent of detoxification of the OTC molecule by the hairy root cultures exhibits a trend similar to that observed in OTC removal on treatment with increasing concentration of MeJA; 0.6 mM treatment concentration of MeJA gives maximum detoxification of OTC.

Acknowledgements

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Chapter V. Effect of Physiological Conditions on Oxytetracycline Remediation by the Plant Root Exudates

Introduction and Background

Most biological reactions show maximum activity in some specific pH range. As described in Chapters III and IV, plants produce ROS through the reductive action of enzymes such as cell wall peroxidases, NADPH oxidase and xanthine oxidase that usually reduce oxygen to superoxide radical. The ROS producing activity of the cell wall peroxidases is pH dependant [Custers et al., 2004; and Raeymaekers et al., 2003]. In bean cells, the production of hydrogen peroxide using horseradish peroxidase and two cell wall peroxidases, FBP1 and FBP2, have pH optima of 8.5, 7.5 and 9.0, respectively [Bolwell et al., 1995]. Peroxidase-dependant hydrogen peroxide production is inhibited in the presence of biological buffers. ROS-scavenging activity of the buffers is suggested as a possible explanation.

Since oxygen is required for the formation of superoxide radicals, unlimited oxygen supply may play a role in the reported ROS-mediated OTC oxidation. In aquatic plants, the reactions occurring in the rhizosphere can be expected to be aerobic as root aeration and rhizosphere oxidation are intimately involved in growth and survival [Sorrell 2004]. Similarly, hairy root cultures are known to require high levels of dissolved oxygen in the

growth medium to maintain optimal growth and physiological activity [Williams and Doran, 1995].

Thus the pH and aeration of root exudates may have a significant bearing on OTC oxidation activity. An important aspect of studying the remediation of an organic pollutant is to recognize and differentiate between the pathway for natural degradation of the compound and that for the mechanism of the adopted remediation, under the given physiological conditions.

OTC is documented to degrade naturally under conditions of high pH and temperature, as well as light intensity [Doi and Stoskopf, 2000; and Halling-Sorensen et al., 2003]. The half-lives of OTC degradation under the influence of different environmental conditions are given in Table 1. The starting concentration for all the treatments was 10 mg/L of OTC for the data presented in Doi and Stoskopf (2000). At 43^0 C, the half-life of OTC is more than 50-fold less than that at 25^0 C; while at 4^0 C OTC appears to be stable. Similarly, as compared to the neutral pH, OTC appears to be stable at low pH values with a 3-fold higher half-life at pH of 3.0, but is relatively less stable at a pH of 10.0 with a half-life approximately 35% less than that at the neutral pH. Light exposure reduces the half-life of OTC 3-fold. Thus, high temperature, high pH and light exposure appear to enhance OTC degradation in aqueous systems. Since OTC is a moderately hydrophilic molecule, with a log K_{ow} (octanol-water partition coefficient) value of –1.22, it is more likely to migrate to water than to be adsorbed on the soil.

Table 1. Half-life of OTC under varying conditions of pH, temperature and light [Doi and Stoskopf, 2000].

Solution characteristics in the treatment	**Half-life (days)**
Control (pH 7.0, temperature 25^0 C, and dark)	14.04 ± 5.41
Treatment 1 (pH 7.0, temperature 4^0 C, and dark)	No degradation
Treatment 2 (pH 7.0, temperature 43^0 C, and dark)	0.26 ± 0.11
Treatment 3 (pH 3.0, temperature 25^0 C, and dark)	46.36 ± 4.92
Treatment 4 (pH 10.0, temperature 25^0 C, and dark)	9.08 ± 4.22
Treatment 5 (pH 7.0, temperature 25^0 C, and light exposure at 4500 lux)	3.94 ± 0.66

Materials and Methods

It is important to note that the reported experiments were carried out before we determined that ROS produced by the roots are involved in the antibiotic removal mechanism. In fact, these experiments provided a hint in that direction. All the experiments reported in this section were carried out using the root exudates, either from *P. stratiotes* or from *H. annuus* hairy root cultures. The effect of pH, temperature and gas sparging on OTC removal by the root exudates was studied. The results for effect of pH and temperature on OTC removal are reported only for the *P. stratiotes* root exudates. The pH and temperature dependency experiments involving the *H. annuus* root exudates gave inconsistent and non-repeatable results. The experiment to study the effect of gas purging was performed only for the *H. annuus* root exudates.

P. stratiotes root exudates of age 7 days, obtained from mason jars containing approximately 250 g/L of live biomass, were used at pH values of 5.5, 6.5, 7.5, and 8.5. The original pH of the *P. stratiotes* root exudates was ~7.5. The pH was adjusted to the required value by adding either sodium hydroxide [Fisher-Scientific, Fairlawn, NJ] or sulfuric acid [Mallinckrodt, Paris, KY]. The pH measurements were carried out using a pH meter from Fisher-Scientific, Fairlawn, NJ. OTC was added at 5 mg/L final concentrations. As a control, OTC [Sigma-Aldrich, St. Louis, MO] was added at similar concentrations to 3 flasks containing reverse osmosis water at a pH of 5.5 (we later realized that due to auto-degradation of OTC at higher pH values we should have had controls at each test pH). All the flasks were maintained at room temperature (25^0 C).

The temperature dependence experiments were carried out using the *P. stratiotes* root exudates from the same source, and of the same age. The temperatures tested were 25, 37 and 50^0 C. For this experiment, controls were included for all the three temperatures. For attaining and maintaining temperatures of 37 and 50^0C, flasks were transferred to water baths at those temperatures. Again, OTC was added at a final concentration of 5 mg/L. For both sets of experiments, samples were taken at regular time intervals for the first four days after the addition of OTC. The pH of the root exudates was left unaltered at ~ 7.5.

The pH and temperature dependency experiments were repeated in the summer, with assistance from the REU student, Heidi Park. They were repeated using a biological buffer, PIPES (piperazine-N, N`-bis [2-ethanesulfonic acid]) [Sigma, St. Louis, MO], to control the pH of the root exudates of *H. annuus* at 6. We later found that addition of such pH buffers adversely affect the ROS production/ activity of the root exudates. As compared to the non-buffered experiments at the same pH values, very low levels of OTC oxidation are observed in the buffered treatments (compare Figure 1-A with Figure 1-B). This observation is consistent with observations cited in the literature (See the Introductory section of this chapter).

The effect of gas sparging was studied only for the *H. annuus* root exudates. These experiments were carried out in autoclaved 25-mL test tubes with rubber stoppers. Before autoclaving, two holes were drilled through the stoppers to fit sparging tubes (made of rubber). Two tubes were fitted per cap, one for the inflow of the gas and the other for the

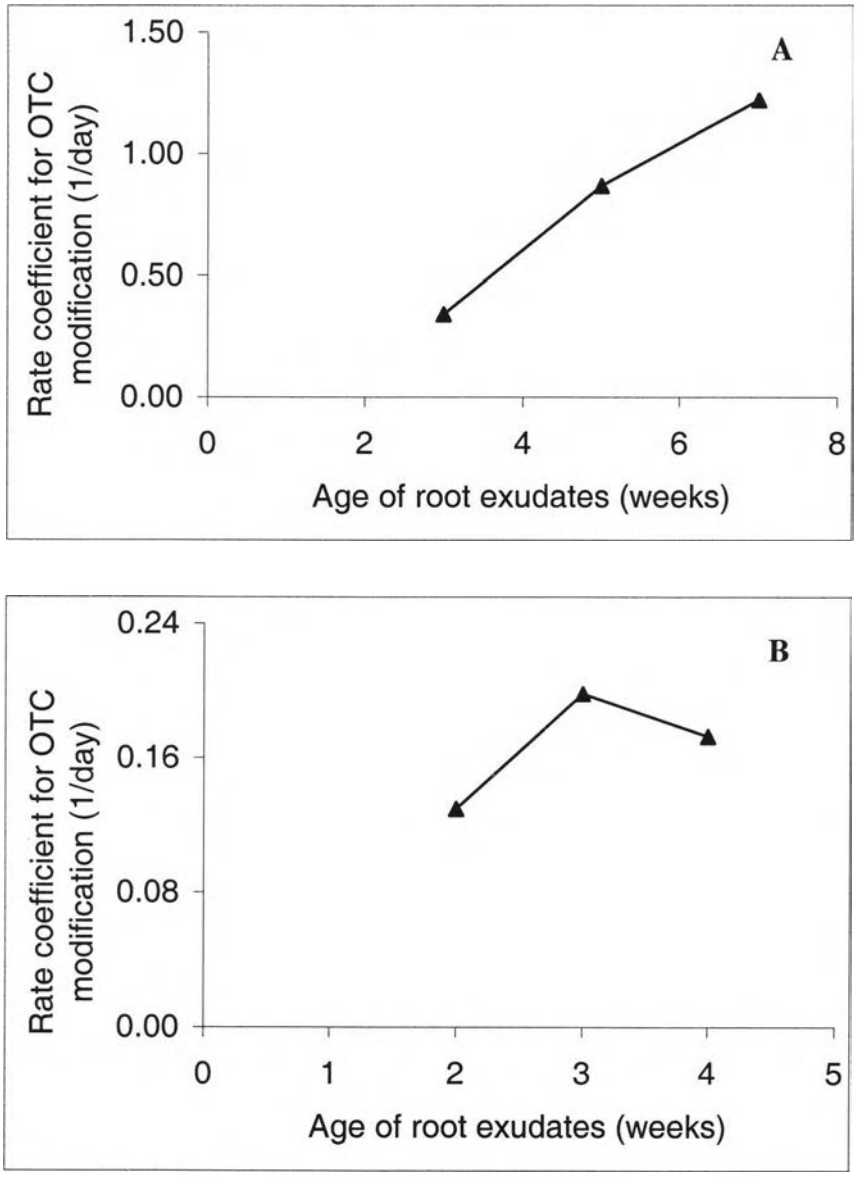

Figure 1. OTC modification rate, at pH 6, as a function of the age of the *H. annuus* root exudates. A- non-buffered root exudates; B- buffered root exudates.

outflow. Once the test tubes were autoclaved, 0.2 μm syringe filters were fitted to the outer ends of the tubes to avoid contamination of the root exudates during the course of the experiment. Since the rate of sparging was too low for the lowest mark on the available rotameter, the sparging rate was adjusted to approximately one bubble per 5 seconds in each of the test tubes. The tube carrying the gas in to the test tube was lowered to the bottom of the test tube to ensure maximum gas contact with the root exudates. OTC was added to the root exudates at a final concentration of 5 mg/L. Samples were drawn after 0, 0.5, 1 and 2 days of addition of OTC to the root exudates.

Results and Discussion

For the root exudates of *P. stratiotes*, the first-order rate coefficient for OTC removal increases appreciably with an increase in the initial root exudate pH (See Figure 2). At pH 5.5 the difference between the RO water control and the root exudate-treatment is negligible. The rate coefficient for OTC removal increases more rapidly with increasing pH from 5.5 to 6.5 than above 6.5. Except for the treatment with pH of 5.5, final pH values for the all the other unbuffered treatments change to approximately 8.0. The original pH of the root exudates, when obtained from the plant-source, is approximately 7.5.

The results for the temperature dependency of OTC removal by *P. stratiotes* root exudates are shown in Figure 3. Since the controls were performed for all three temperatures in RO water, the normalized rate coefficients for OTC removal were obtained by subtracting the removal rate coefficients for OTC removal in RO water from

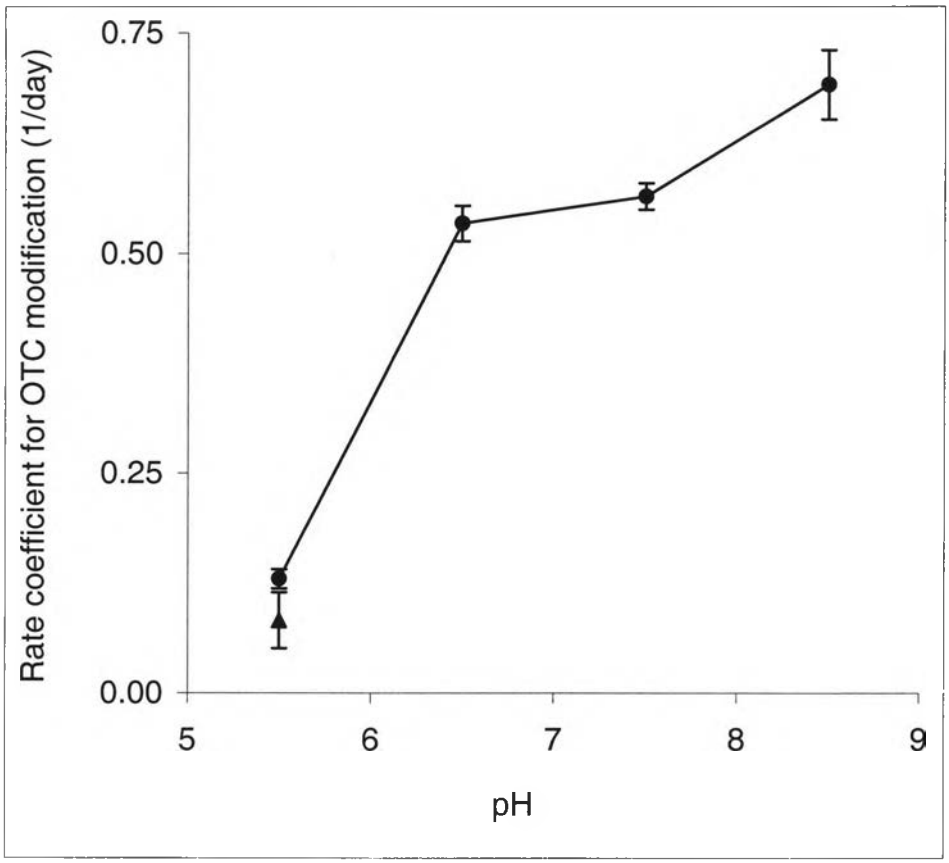

Figure 2. Effect of pH on OTC removal by the root exudates of *P. stratiotes*. The (♦) represent the rate coefficients for OTC removal in the root exudates, while the (▲) represents the rate coefficient for OTC removal in RO water at pH 5.5.

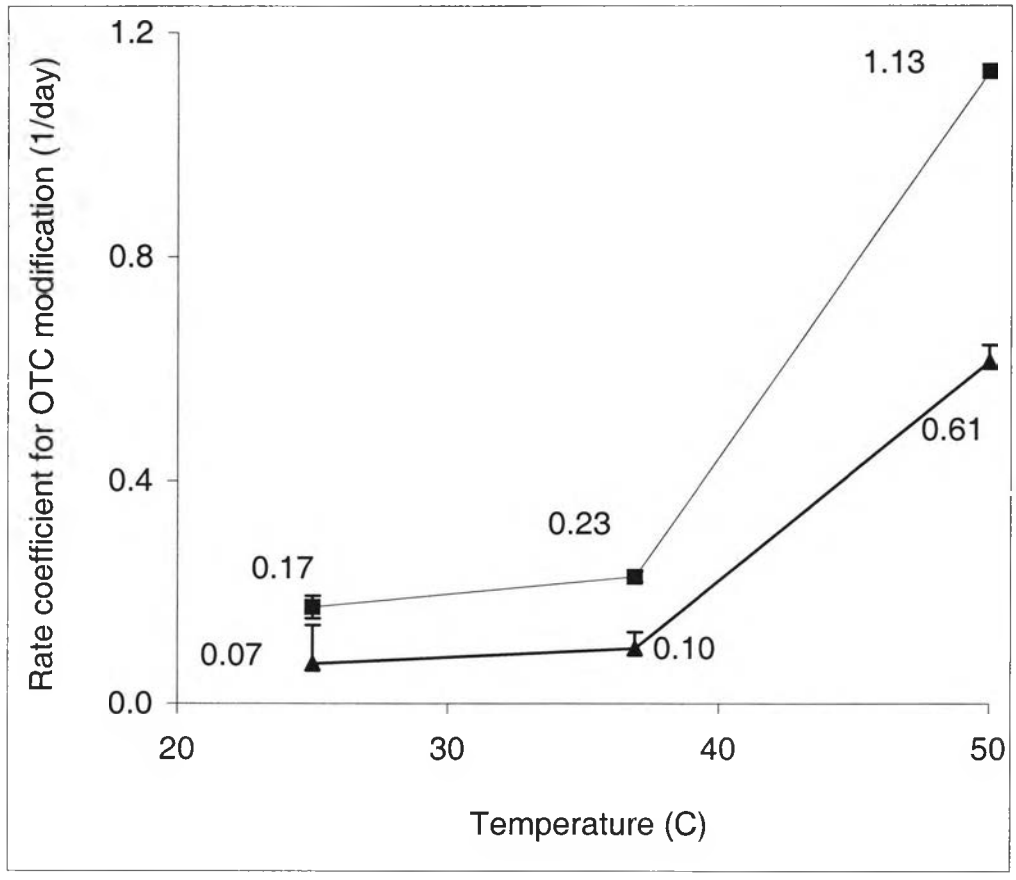

Figure 3. Effect of temperature on OTC removal by the root exudates of *P. stratiotes*. The (■) represent the rate coefficients for OTC removal in the root exudates, while the (▲) represents the normalized rate coefficient for OTC removal in the root exudates, obtained by subtracting the OTC removal rate coefficients in RO water.

the rate coefficients obtained in the root exudates at the same temperatures. This method of data treatment helps to distinguish between the effects of temperature increase on the actual OTC removal by the root exudates (represented by the normalized rate coefficients) and the combined effects of removal due to thermolysis and root exudate action. Although the normalized removal rate coefficient does not change considerably for an increase in the temperature from 25 to 37^0 C, it increases nearly 6-fold for the increase from 37 to 50^0 C. Therefore the OTC removal reaction by the root exudates of *P. stratiotes* appears to be temperature dependant with higher activity at 50^0 C.

Interestingly, gas sparging has a negative effect on OTC removal by the root exudates. The rate coefficient for OTC removal is significantly higher in the root exudates not purged with any gas compared to the ones purged with either oxygen or nitrogen (Figure 4). The basis for differences may be the displacement of ROS caused by sparging. Nevertheless, the rate coefficient for the oxygen-sparged root exudates is greater than that for the nitrogen-sparged root exudates. Therefore, sparging appears to affect the ROS interaction with OTC, but in the case of oxygen the effect is less drastic probably due to utilization of molecular oxygen in ROS formation in the root exudates. The concentration profiles of OTC in the root exudates of *H. annuus* sparged with air, oxygen, and nitrogen are shown in Figure 5. The profiles are similar up to the 0.5-day mark after which the OTC removal in the nitrogen-sparged root exudates ceases. The concentration profiles remain similar for the air and oxygen sparged root exudates. Oxygen does not appear to be the limiting component in the OTC removal by the root exudates of *H. annuus*. But the OTC removal process appears to be more efficient under aerobic conditions than under

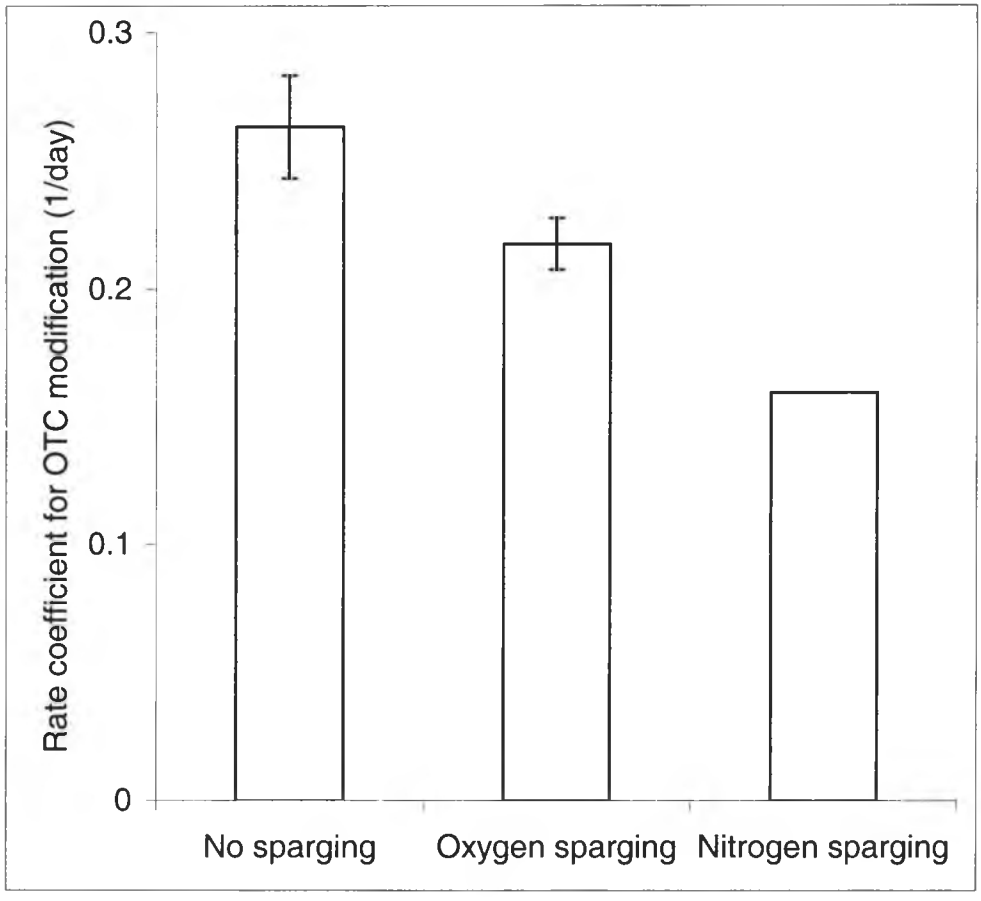

Figure 4. Reduction in OTC modification due to gas sparging.

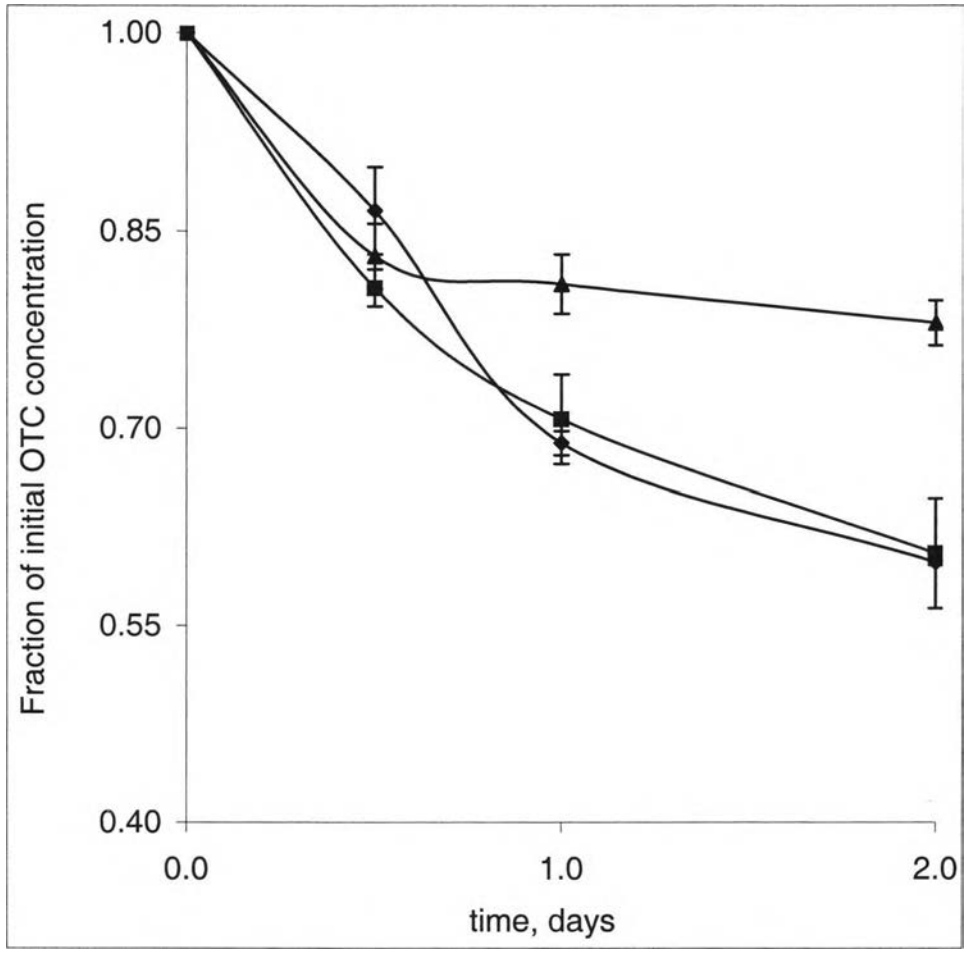

Figure 5. Concentration profiles of OTC in the root exudates of *H. annuus* sparged with air (■), oxygen (♦) and nitrogen (▲).

anaerobic conditions. Oxygen may be involved as a non-rate-limiting component either in ROS-mediated oxidation of OTC, or in the ROS formation reactions. The rate coefficient for OTC removal in the nitrogen-sparged root exudates is significantly lower than that in the air or oxygen-sparged root exudates (Figure 6).

Conclusions

The removal of OTC by the root exudates of *P. stratiotes* is pH and temperature dependant with greater removal at higher values of both the pH and the temperature. Since the production of ROS by the roots of plants involves a pH dependent, physiological response of the plant enzymes, it is important to maintain the temperature and pH conditions of the growth medium at values optimal for plant growth. But when using the root exudates for antibiotic removal, under *in vitro* conditions, the pH and temperature conditions can be manipulated for maximum antibiotic oxidation. The experiments reported in this section should be repeated under field conditions. Since ROS are involved in OTC removal by the plant root exudates, OTC removal rate is greater under aerobic conditions than that under anaerobic conditions. But some removal is observed in the anaerobic (nitrogen-sparged) system. Since the removal process almost ceases after the first 12 hours, the observed removal might be due to the residual ROS left in the root exudates that are eventually used up by the end of the 12-hour period. Note that the perceived use up of the ROS might be happening in a time period much less than the 12-hour period, but since no samples were taken between 0 and 12 hours it is difficult to make comments on this aspect. It is also clear that molecular oxygen by itself does not oxidize the OTC molecule.

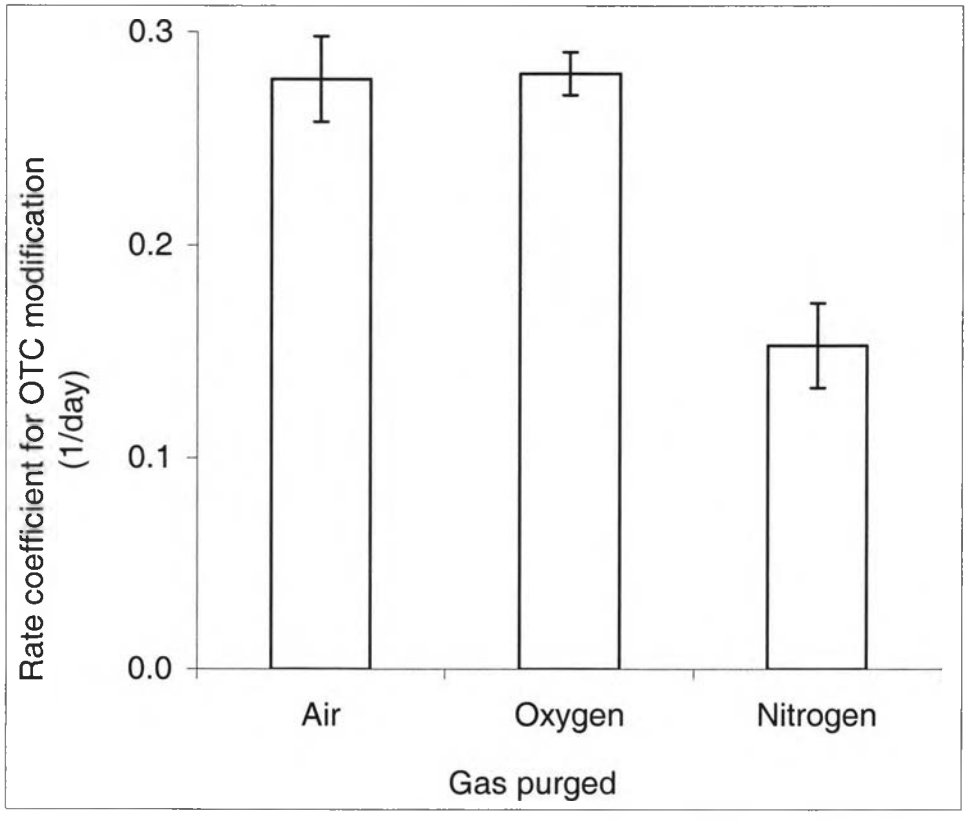

Figure 6. The effect of gas sparging on the OTC removal by the root exudates of *H. annuus*.

From a practical point of view, for field OTC remediation systems utilizing the root exudates of plants, the manipulation of the pH is more feasible compared to providing external heating. The heating operations would be, both, expensive and difficult to achieve considering the total volume and high solids content of the wastewater streams emerging out of the animal feeding operations. On the other hand, pH can be increased through the addition of chemicals such as calcium carbonate.

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Chapter VI. An Integrated Bioreactor System Designed for Removal of Oxytetracycline from Water

Abstract

Plant produced reactive oxygen species (ROS) in root exudates inactivate oxytetracycline, rendering it non-toxic to microorganisms. The OTC oxidation activity occurs completely in the root exudates, suggesting a possibility of using the plants only as a production bed for the ROS-containing root exudates. An integrated bioreactor system, with a miniature microcosm-pond coupled to a bioreactor, is studied for its efficacy in removing oxytetracycline (OTC) from water. The use of two bioreactor configurations, a plug flow reactor (PFR) and a continuous stirred tank reactor (CSTR), in the preliminary experiments is reported.

Introduction

The use of antibiotics in concentrated animal feeding operations (CAFO) as growth promoters and feed efficiency promoters has led to widespread exposure of antibiotics to the environment. Antibiotics added to animal feeds are eventually released to the environment through run-off from CAFO or leakage from storage structures. Use of animal manure as fertilizer also results in a substantial exposure of the antibiotics to the environment. Tetracyclines are found in pig manure in concentrations up to 4 mg/L [Thiele-Bruhn, 2003]. Meyer et al. (2000) report tetracyclines at concentrations up to 0.8

mg/L in hog pen wastewater lagoons. Kolpin et al. (2002) report tetracycline concentrations as high as 0.7 μg/L in water samples taken from rivers across the United States. Antibiotics are also used in aquaculture for fish production, where they are directly added to fishponds and are commonly found in sediments at concentrations in the range of 0.5 to 4 mg/L [Thiele-Bruhn, 2003]. Thus antibiotics are found in water, soil and sediments, making their exposure to microorganisms inevitable. Induction and the consequent propagation of antibiotic resistance among bacteria is a cause of great environmental concern and is discussed in literature [EPA 2001; Smalla and Sobecky, 2002; and Thiele-Bruhn, 2003], as well as in Chapter I. Effective treatment strategies for antibiotic remediation need to be determined for countering this problem.

The research focus of our laboratory is to study the phytoremediation of tetracycline antibiotics (tetracycline and oxytetracycline) from water, and to develop strategies for effective field applications of the technology. In our previous research communications, we have reported the phytoremediation potential of two aquatic plant species [Gujarathi et al., 2005a] and the hairy root cultures of *Helianthus annuus* [Gujarathi et al., 2005b] in remediating the two antibiotics. Reactive oxygen species (ROS) produced in the medium of *H. annuus* inactivate OTC to form oxidation product(s) non-toxic to microorganisms [Gujarathi and Linden, 2005].

In all three research communications we show that root exudates (secretions of the roots in the nutrient medium) of the plants/hairy root cultures are involved in OTC modification. This observation led to design of a pond-microcosm. The layout of the

pond-microcosm bioreactor system is shown in Figure 1. *P. stratiotes* is chosen over *M. aquaticum* due to its greater effectiveness, per unit propagule, in modifying OTC from the nutrient medium [Gujarathi et al. (a)]. Also *P. stratiotes* propagates much faster than *M. aquaticum* (growth data not shown). The bioreactor system (Figure 1) consists of:

1. A pond-microcosm, which is constructed of wood and plastic, to grow *P. stratiotes*
2. A bioreactor for the modification of OTC by the root exudates of *P. stratiotes*

The ultimate motive behind the unique layout of the bioreactor system is to collect the root exudates from the pond-microcosm and react them with the antibiotic-contaminated wastewater in an isolated bioreactor. Note that the reduction in the OTC concentration in the outlet from the bioreactor would be due to both dilution with and modification by the root exudates stream. This layout, if proven effective, would have several advantages over the commonly used designs where the plants come in direct contact with the pollutant, as the polluted medium is sometimes phytotoxic. The advantages are:

1. The plants will be protected from the toxic effects of the wastewater.
2. Bioaccumulation of the antibiotics in the plants would be avoided.
3. The plants can be efficiently elicited for production of the phytoremediation factors (ROS).
4. The scale-up of the pond-microcosm to a field-pond would be straightforward.
5. The physiological conditions and the chemical composition of the reaction system can be optimized without affecting the plants.

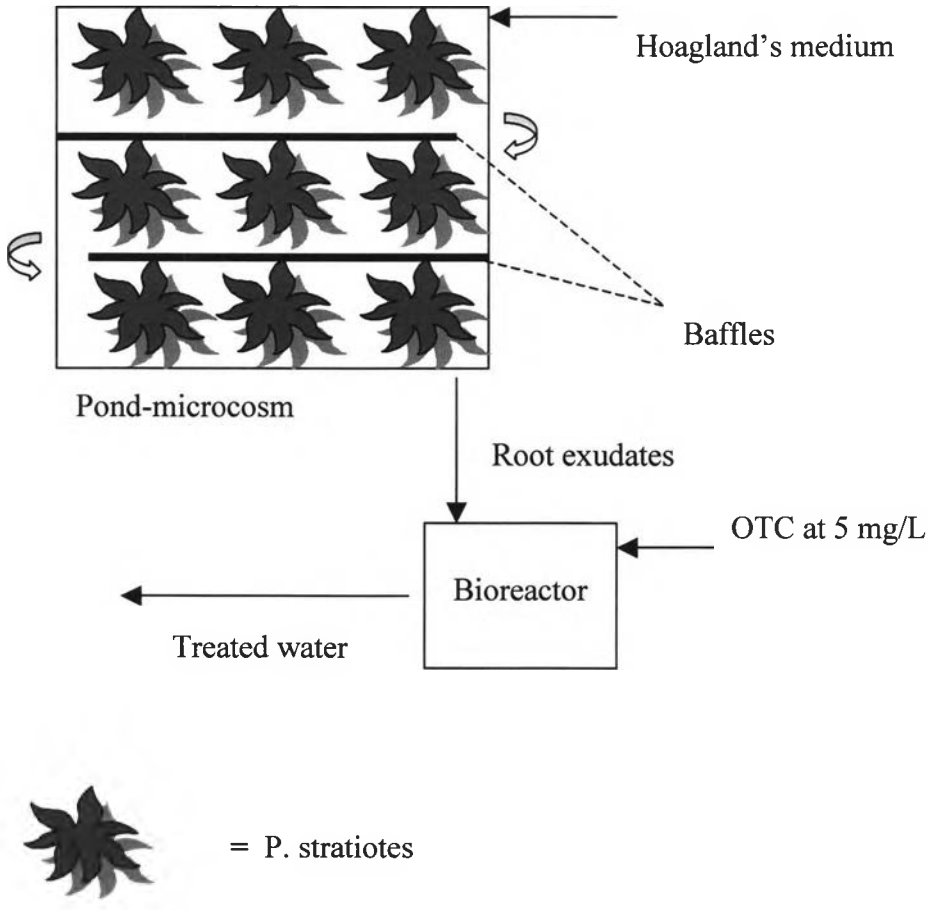


Figure 1. Layout of the integrated bioreactor system.

As shown in Figure 1, baffles are provided as channel separators between successive rows of plants. This is done to maximize the retention time of the nutrient medium in the pond-microcosm by eliminating short-circuiting of the nutrient flow path through the pond-microcosm.

Pond-microcosm design

The pond-microcosm design is based on the results obtained in the laboratory for *P. stratiotes* interactions with OTC [Gujarathi et al., 2005a]. A biomass concentration of 250 kg fresh weight/m^3 of Hoagland's medium was used as a design parameter, but in the operation of the bioreactor system the total plant biomass increases continuously, until the pond-microcosm is totally occupied by the *P. stratiotes* propagules. Therefore it is difficult to maintain the biomass constant at a given value. It is also important to realize that the concentration of the phytoremediation factors (ROS) in the root exudates is more relevant than is the biomass concentration of the propagules.

While setting up the pond-microcosm it was observed that the maximum possible biomass (Bm) of *P. stratiotes* that can be placed per m^2 of the microcosm is 8 kg. Therefore equation [I] can be considered as a design constraint for the pond microcosm.

$$\frac{Bm}{W \times L} \leq 8 \text{ kg/m}^2 \qquad [I]$$

Where,

W is the width of the pond-microcosm (m)

L is the length of the pond-microcosm (m)

From the preliminary experiments described earlier, the required biomass concentration per unit volume of the microcosm should be at least 250 kg/m^3. Therefore,

$$\frac{Bm}{W \times L \times D} = 250 \text{ kg/m}^3 \qquad [II]$$

Where,

D is the depth of the pond-microcosm (m)

And as in the case of the preliminary experiments, the residence time, t, of the nutrient medium in the microcosm should be 7 days. Therefore,

$$t = \frac{W \times L \times D}{Q} = 7 \text{ days} \qquad [III]$$

Solving equations from constraints I and II, we get,

$$D = D_{optimum} = 0.032 \text{ m} \qquad [IV]$$

It is important to note that production of the ROS by the plant roots is not directly growth associated. Factors such as nutrient limitation, environmental stress and elicitation have a greater bearing on ROS production by the plant roots.

The pond-microcosm was set on a table in the Greenhouse. The maximum possible width of the pond-microcosm due to size restriction of the table was 1.2 m. Substituting the values for W (1.2 m) and D from [IV] in equation [III], we get,

$$\frac{L}{Q} = 182.29 \text{ day/m}^2 \qquad [V]$$

Therefore to obtain the desired concentration of the phytoremediation factors in the root exudates, the L/Q ratio should be greater than 182 day/m^2.

For the *P. stratiotes*-OTC interactions reported in Gujarathi et al. (a), the plants were grown in the nutrient medium for 7 days before their exposure to OTC. This was done in order to attain a sufficiently high concentration of the phytoremediation factors in the root exudates.

The parameter values for the pond-microcosm that satisfy the constraint equations [I]-[V] are given below:

- Volume of the pond-microcosm = 0.092 m^3
- Flow rate of the root exudates from the pond-microcosm into the second stage bioreactor = 0.0132 m^3/day

Pond-microcosm bioreactor systems

Since the ROS are the rate-limiting components in the ROS-OTC interaction the design of the bioreactors should use rate equations based on ROS concentrations. But the half-lives of such ROS are very short; the hydroxyl radical, which appears to be involved in the OTC modification process, has a half-life shorter than 10^{-3} seconds [Pryor, 1986]. Therefore the approach adopted is to determine the flow configuration that gives best results and then determine the optimum conditions for the operation of the chosen configuration. The image of the pond-microcosm is shown in Figure 2.

Figure 2. The pond-microcosm with *P. stratiotes* growing in it.

The performance of two second-stage bioreactor configurations, plug-flow and continuous-stirred, are evaluated for removal of OTC from water. For both the configurations the root exudates flow in to the bioreactor at 0.0132 m^3/day and the 5 mg/L OTC stream flows in at 0.01 m^3/day.

The sizing parameters chosen for the PFR system are,

- Volume of the cylindrical PFR = 0.045 m^3
- Residence time in the PFR = $\frac{0.045}{0.01+0.0132}$ = 1.94 days.

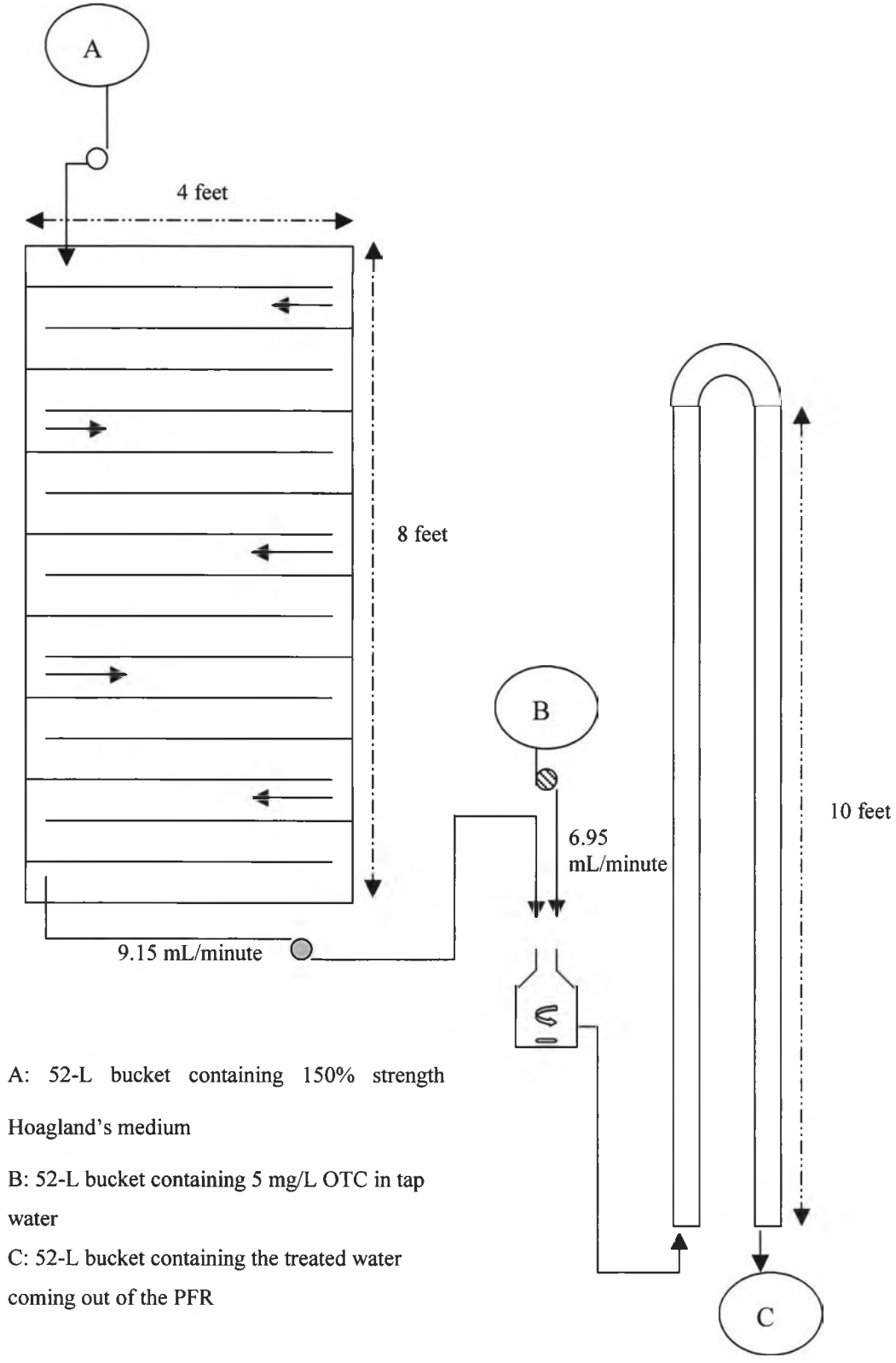
The sizing parameters chosen for the CSTR system are,

- Total volume of the CSTR = 0.014 m^3
- Maximum possible residence time in the CSTR = $\frac{0.014}{0.01+0.0132}$ = 0.60 days.

The mechanical drawing for the pond-microcosm PFR system is shown in Figure 3. For the pond-microcosm CSTR system (the integrated bioreactor system) the flash mixer and the PFR are replaced by the 0.014 m^3 CSTR.

Materials and Methods

The pond-microcosm was built using wooden plywood and boards purchased from a local lumberyard. In accordance with the design values, the dimensions chosen for the pond-microcosm were 8 feet X 4 feet (i.e. 2.4 m X 1.2 m). A rim constructed of 2 X 4 inch boards was screwed to the ½-inch plywood sheet base. The rectangular, box-shaped



A: 52-L bucket containing 150% strength Hoagland's medium

B: 52-L bucket containing 5 mg/L OTC in tap water

C: 52-L bucket containing the treated water coming out of the PFR

Figure 3. Mechanical drawing of the pond-microcosm PFR system

set-up of about 3-inch depth was then lined from the inside with a thick pond-liner (made of rubber). The pond-microcosm was then filled with Hoagland's medium [Hoagland, 1938] to the design depth of 0.032 m. Approximately 75 *P. stratiotes* propagules were introduced to microcosm. Baffles, made of 1/8-inch thick, 4-inch wide rubber baseboard material [local lumberyard], were placed in between consecutive rows of plants, as shown in Figure 3. Placing baffles approximately 6 inches apart created 16 rows of plants. The baffles were designed with notches on alternating ends such that the nutrient medium would follow a zigzag pattern across the microcosm, thereby maximizing the residence time of the nutrient medium inside the microcosm. The lightweight flexible baffles were held vertical with notched lumber (2 × 2 inches) on each end and supported in the center with string through holes in the baffles around a ½-inch plastic pipe, which spanned the length of the microcosm. A single layer of cheesecloth was laid over the entire microcosm to shade the *P. stratiotes* propagules to protect the plants from direct sunlight in the University greenhouse.

The PFR was built out of two 4-inch diameter plastic sewer pipes, each of length 10 feet, obtained from a local lumberyard. The two pipes were connected by two 90^0 elbow joints with a 4-inch diameter connector, which further increased the length of the PFR by approximately 1 foot. The ends of the PFR were sealed with 4-inch caps that fit tightly after cementing to the pipes using PVC cement. After placing the PFR under the greenhouse table on which the microcosm was placed (See Figure 4), holes (1/4 inch) were drilled at the upper end of the caps for the inlet and outlet of the reactants and at 2.5-foot intervals along the length of the pipes for sampling through serum caps cemented

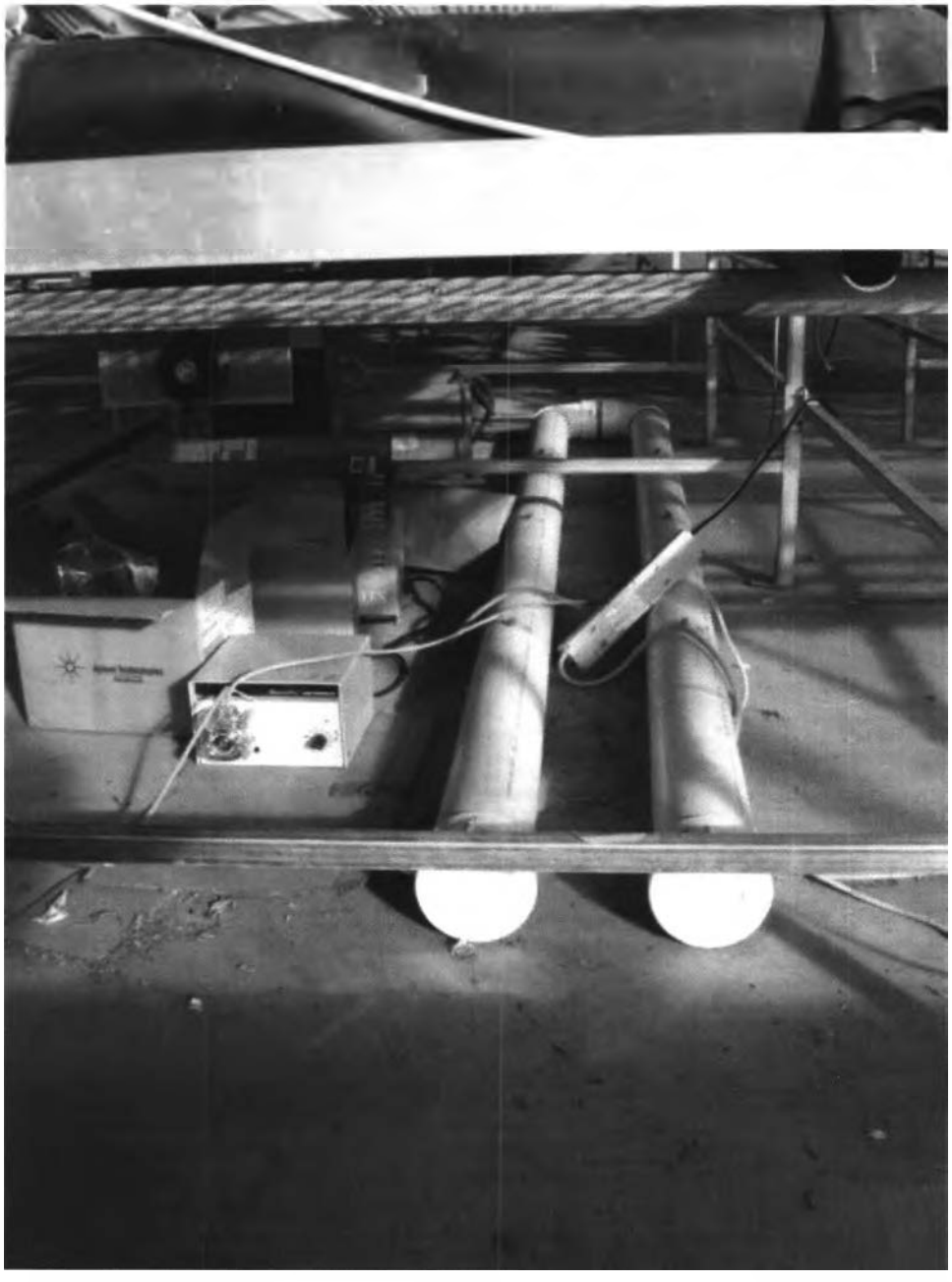


Figure 4. The plug flow reactor (PFR) built from two 4-inch diameter pipes.

in the holes. A 2-L flask (flash mixer) was placed upstream of the PFR to facilitate mixing of the reactants before their introduction into the PFR. The mixer was placed on a magnetic stirrer, which rotated at approximately 400 rpm. The residence time in the mixer was approximately 30 minutes. Three peristaltic pumps were placed in the layout as depicted in the mechanical drawing (Figure 3).

The CSTR consisted of laboratory fermentor [Model No. MF 114; New Brunswick Scientific, New Brunswick, NJ] with a variable speed impeller system, heating/cooling pump, provision for aeration control with a rotameter. The 14-L reaction vessel, which was made of glass, was covered with aluminum foil to protect the reaction chamber from sunlight. Since the metal body of the CSTR warms up under direct sunlight, a green-colored shade (kindly provided by the University Greenhouse manager, Jennifer Matsuura) was placed over the CSTR. A 3-gallon air compressor [Model # 15210; Craftsman, Sears Roebuck, & Company] was connected to the CSTR for providing aeration. An image of the CSTR set –up is shown in Figure 5. The root exudates from the microcosm and OTC solution were added directly to the CSTR. The residence time of the reactants was controlled by adjusting the depth to which the tubing for withdrawal of the product stream was placed. Pump 4, associated with the withdrawal, was operated at a rate greater than the sum of the CSTR input streams.

Figure 5. The 14-L continuous stirred tank reactor (CSTR) covered with a shade.

The following pumps were used:

1. Masterflex (model # 7565): For pumping the Hoagland's medium into the pond-microcosm- ~ 14 mL/minute (variable, depending upon the climatic conditions). Norprene tubing from Masterflex (# 6485-14) was used.
2. Watson-Marlow (model # 502-S): For pumping the root exudates from the pond-microcosm to the bioreactor, in case of the PFR, first to the flash mixer, - ~ 9.15 mL/minute (design value). Norprene tubing from Masterflex (# 6402-16) was used.
3. Masterflex (model # 7553-60): For pumping the 5 mg/L OTC solution into the bioreactor, in case of the PFR, first to the flash mixer, - ~ 6.95 mL/minute (design value). Norprene tubing from Masterflex (# 6402-16) was used.
4. Masterflex (model # 7520-25): For pumping the product stream out of the CSTR, ~ 16 mL/minute. Norprene tubing from Masterflex (# 6402-16) was used.

Note that the flow-rate of the Hoagland's medium in to the pond-microcosm was set at a higher value than the outlet from the pond-microcosm to account for both evaporation and plant transpiration processes. The Hoagland's medium and OTC solutions were diluted using tap water into 52-L plastic tubs, covered with aluminum foil and pumped from each tub accordingly.

For the microcosm-PFR system, the concentration of OTC in the sample drawn from the inlet OTC-stream to the flash mixer (sample # 1) was treated as the inlet concentration of OTC to the PFR bioreactor system (~ 5 mg/L). A sample was also drawn from the outlet

stream emerging out of the flash mixer (sample # 2), which was treated as the inlet concentration of OTC in the PFR. Samples were then drawn from six equally spaced points along the length of the PFR (samples #'s 3 → 8). The last sample, representing the outlet concentration of OTC from the PFR was drawn from the outlet stream emerging from the PFR (sample # 9). The samples from #3 to #8 were drawn using a syringe through rubber-septa cemented in the holes drilled on the top surface of the pipe (See Figure 4). Each of the samples, from #3 to #8, constituted a mixture of identical volumes of sample liquid drawn from the top, center and the bottom of the flow current in the pipe. The sampling regime accounted for effects due to the non-ideality in the plug-flow-pattern (axial dispersion), although only to an extent.

For the microcosm-CSTR system, samples were drawn from the inlet OTC stream to the CSTR (inlet OTC concentration for the bioreactor system) and from the outlet stream from the CSTR (outlet OTC concentration for the bioreactor system). A dissolved oxygen probe [Model # 5100; YSI, Yellow Springs, OH] was placed in-line with the exit stream from the CSTR.

In the case of the microcosm-CSTR system, experiments were conducted to study the effects of varying mixing speed, aeration rate and residence time on the OTC removal by the system. As a control experiment, 6.95 mL/minute of 5 mg/L OTC was passed through the CSTR at a mixing speed of 350 rpm, retention time of 6 hours and a temperature of 30^0 C. This control was performed to determine the OTC degradation due to the high shear rates encountered in the CSTR. The pH of the root exudates entering the CSTR and

that of the product stream from the CSTR was consistently observed to be in the range 7.2-7.6.

Results

A first-order equation of the form shown in equation [VI] is used as a curve-fit to represent the concentration vs. time data.

$$\frac{C}{C0} = \text{Constant} \times e^{-k.t} \qquad [VI]$$

Where,

C is the instantaneous OTC concentration at time t

C0 is the initial OTC concentration

k is the rate coefficient, day-1

Plug flow reactor

As described in Materials and Methods, sample #2 represents the OTC concentration in the water emerging from the flash mixer and entering the PFR. It is evident from the results (Figure 6) that the flash mixer with a retention time of ~30 minutes gives greater OTC removal than did the PFR with a retention time of 1.94 days. The error bars in Figure 6 represent normalized standard deviations from 9 samples taken daily from each sampling point. The concentration of OTC in the PFR decreases from 33% of initial concentration to 27% of the initial concentration (5 mg/L). This observation of greater removal in the flash mixer compared to that in the PFR might be due to:

1. Limited concentration of the ROS in the root exudates.

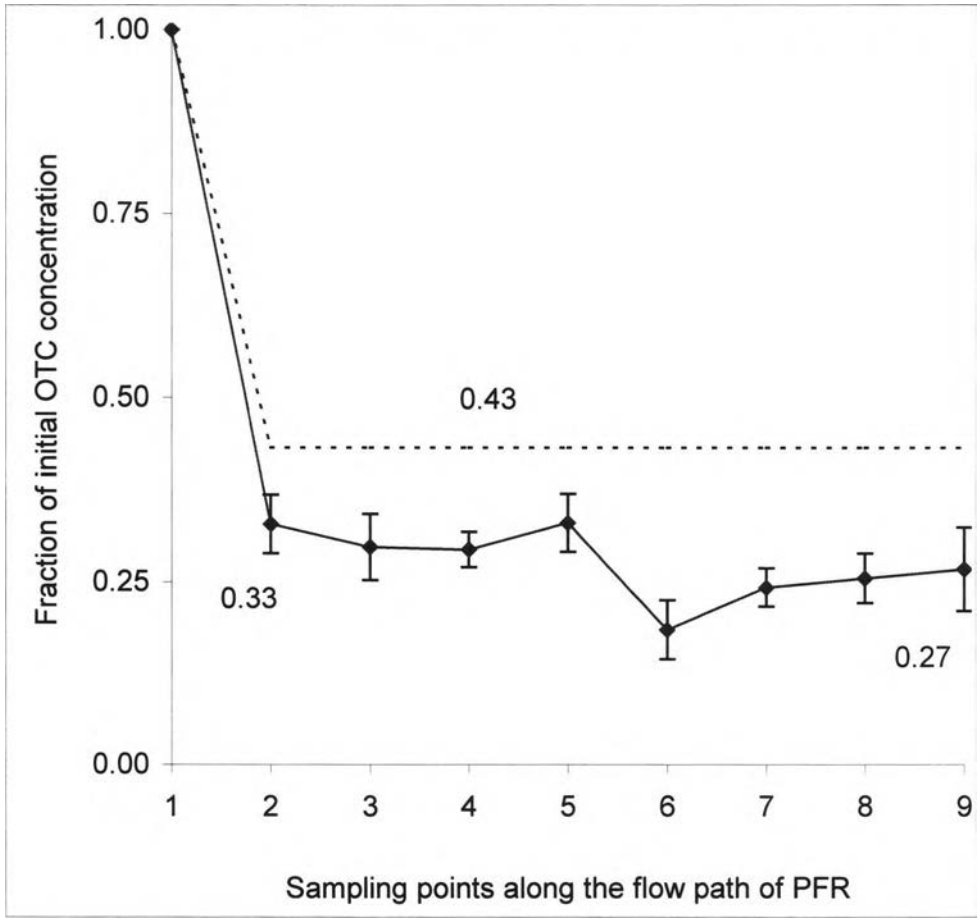


Figure 6. OTC modification in the microcosm-PFR system. The dotted lines give the expected OTC concentrations that would result purely due to dilution with the root exudates.

2. Diffusion limitation being the rate-controlling factor for the reaction between OTC and the ROS.
3. Oxygen limitation (or anoxic conditions) in the PFR limiting the extent of OTC oxidation in the PFR.
4. A combination of the above possibilities.

Continuous stirred tank reactor

In all the results discussed from the CSTR experiments, the error bars represent normalized standard deviation from triplicate samples taken on a given day. The control experiment with no input from the microcosm or aeration gives no OTC removal at 350-rpm agitation rate and 6 hours retention time (results not shown).

The integrated microcosm-CSTR system with an aeration rate of 6 L/min and a retention time of 7 hours yielded rate coefficients for OTC modification, which increased as the mixing speed of the impeller system was increased (Figure 7). Therefore, the reaction between the ROS and OTC molecules appears to be diffusion limited. Hydroxyl radicals, being highly reactive and unselective, react with rate constants that are nearly diffusion controlled and they usually react within 1 to 5 molecular diameters of their site of formation [Pryor, 1986]. Therefore, ROS interaction with OTC can be expected to be diffusion limited, if hydroxyl radical is involved in the process. The dissolved oxygen level increased from 45% (mixing speed of 50 rpm) to 60% (mixing speed of 350 rpm) with the aeration rate set at 6 L/minute.

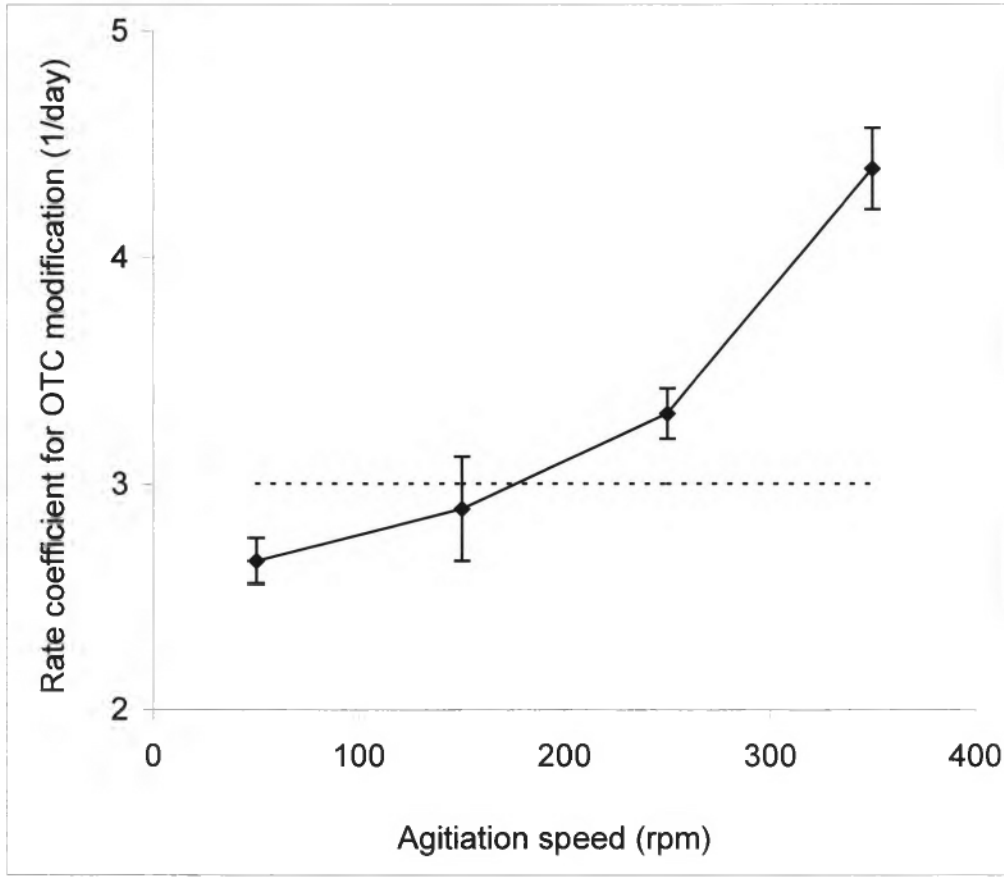


Figure 7. Effect of mixing speed on OTC removal by the microcosm-CSTR system. The dotted line gives the expected OTC removal that would result purely due to dilution with the root exudates. Aeration at 6 L/min and retention time of 7 hours was used for this set of experiments.

The effect of aeration on OTC removal by the microcosm-CSTR system is reported for a constant mixing speed of 350 rpm and a retention time of 7 hours (Figure 8). At an aeration rate of 2 L/min, significantly lower OTC modification is observed compared to that with no external aeration provided. As the aeration rate is increased beyond 2 L/minute, the rate coefficient for OTC modification also increases. For the aeration rate from 6 to 10 L/minute, there is no significant increase in the OTC modification rate and appears to level off to at a value similar to that obtained with no external aeration. Note that at a mixing speed of 350 rpm the large head space in the CSTR (about 9 L) should itself provide significant aeration. This observation of decreased OTC oxidation rates on aeration is consistent with the laboratory experiments with the *H. annuus* root exudates, where sparging root exudates with air, oxygen or nitrogen results in reduced OTC removal compared to non-sparged controls (see chapter V). Displacement of ROS by sparging is believed to be the cause of these observations. For the mixing speed of 350 rpm, the dissolved oxygen level in the CSTR did not change much with the aeration rates. It was constant in the range of 55%- 60% of the saturation DO value.

Lastly, changes in the retention time of the reactants in the CSTR do not affect the rate of OTC removal (Figure 9). The temperature was set at 30^0 C, agitation at 350 rpm and aeration at 6 L/min. Therefore, the reaction between OTC and the ROS appears to be rate limiting with respect to the ROS content of the system. It appears that once the ROS are used up in the OTC modification, OTC modification terminates.

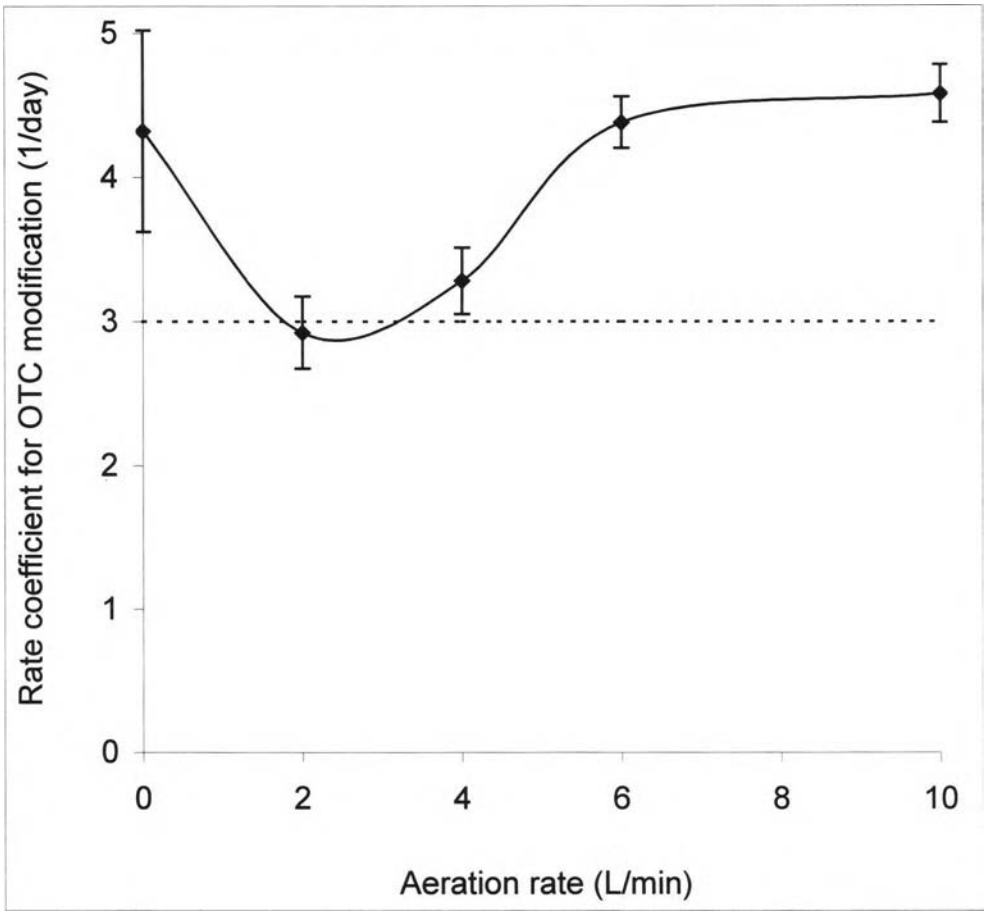


Figure 8. Effect of aeration rate on OTC removal by the microcosm-CSTR system. The dotted line gives the expected OTC removal rate that would result purely due to dilution with the root exudates. Agitation rate of 350 rpm and retention time of 7 hours were used for this set of experiments.

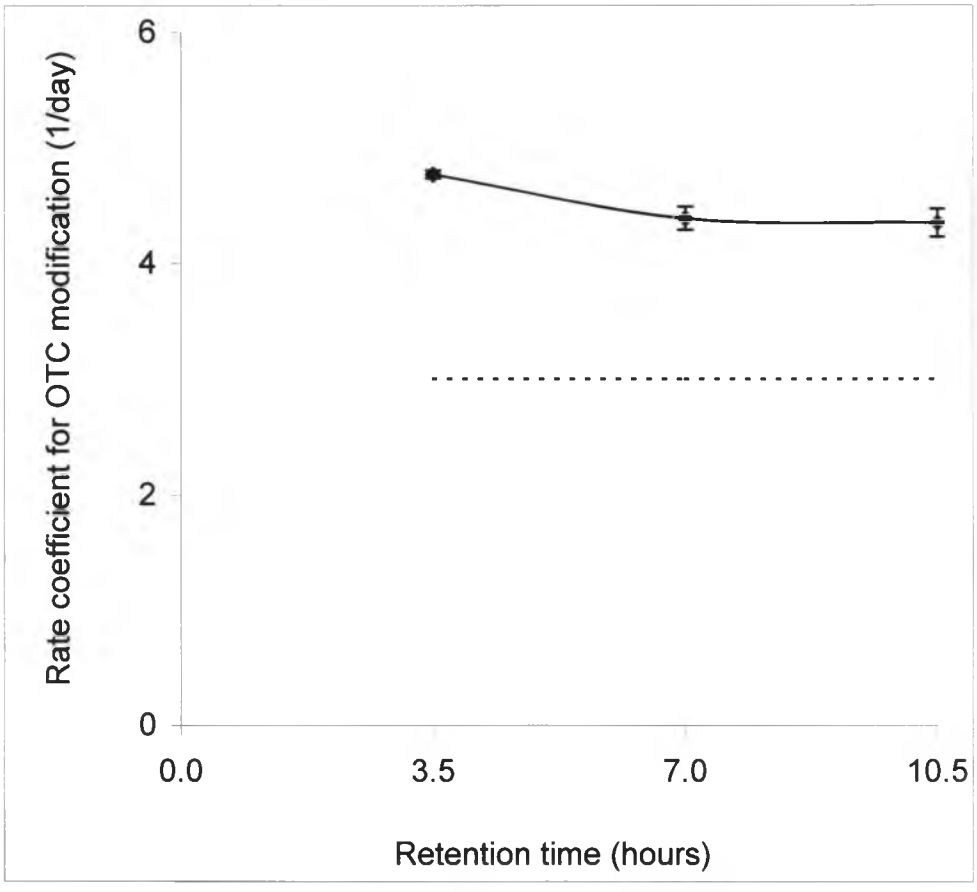


Figure 9. Effect of retention time on OTC removal by the microcosm-CSTR system. The dotted line gives the expected OTC removal rate that would result purely due to dilution with the root exudates. Agitation rate of 350 rpm and aeration at of 6 L/min were used for this set of experiments.

For all the reported results from the CSTR system, the length of time to establish steady state was estimated by determining the residence time (RT) of the reactants in the CSTR. To keep the sampling process consistent, the sampling times for determining the inlet concentration of OTC to the CSTR and the outlet concentration of OTC in the effluent stream from the CSTR were spaced at time intervals equal to the RT of the reactants in the CSTR.

Discussion

For the microcosm-CSTR system a maximum of ~75% OTC removal is observed for at aeration rate of 6 L/min, mixing speed of 350 rpm and a retention time of 3.5 hours. Although this extent of removal is approximately equal to that observed with the PFR system (~73%), the retention time in the PFR was about 13 times longer than that in the CSTR. Most of the removal is attained in the 30-minute flash mixer that is placed upstream of the PFR, whereas the PFR itself results in very little OTC removal. It appears that the ROS are quickly used up in the OTC oxidation reaction, after which further contact of the root exudates with OTC proves ineffective. So a CSTR with a much smaller volume than the PFR, but with better mixing conditions, gives greater OTC removal.

In the results presented from the CSTR system (see Figures 7 and 8), the concentration of OTC in the bioreactor effluent appears to increase with respect to the value expected from dilution with the root exudates, which in turn results in rate coefficient values for OTC removal lower than that expected from pure dilution. This observation might be due

to the incomplete oxidation of the OTC molecule. Since the ROS-OTC interaction is diffusion limited, conditions of low mixing speed, lower contact time, lower ROS concentration, or a combination of one or more of these, can be expected to result in incomplete oxidation of OTC. Incomplete oxidation of OTC would result in an increase in the unsaturated carbonyl bonds (C=O) owing to the formation of reaction intermediates. Electrons in the unsaturated bonds need lesser energy for excitation, specifically the $\pi \rightarrow \pi^*$ transitions, and therefore absorb energy at longer wavelengths (the near UV-range ~ 360 nm) [Feinstein, 1995]. The rule is 'higher the wavelength of light, the lower is the energy content'. The HPLC-based detection of OTC is carried out at 355 nm where the BCD chromophore of the OTC molecule absorbs light [Gujarathi et al., 2005a, b]. The BCD chromophore consists of the phenolic group in the D-ring and the enol group in the B-ring. With an increase in the unsaturated character of the chromophore, with the above-mentioned incomplete oxidation, the absorbance of the OTC molecule at this wavelength can be expected to increase.

Preliminary experiments were performed to test the effectiveness of salicylic acid (SA) and methyl jasmonate (MeJA) in eliciting ROS production by *P. stratiotes*. These experiments were performed under batch conditions with one *P. stratiotes* propagule per jar as where the experiments described in Chapter II, except that various concentrations of SA and MeJA were added to the Hoagland's medium, between 0 to 1 mM. Both SA and MeJA prove effective in enhancing the OTC removal efficiency of *P. stratiotes* (Figure 10). At dosage levels greater than 0.4 mM SA, and 0.2 mM MeJA, the propagules died after 3 days. This observed plant necrosis might be due to limiting concentration of

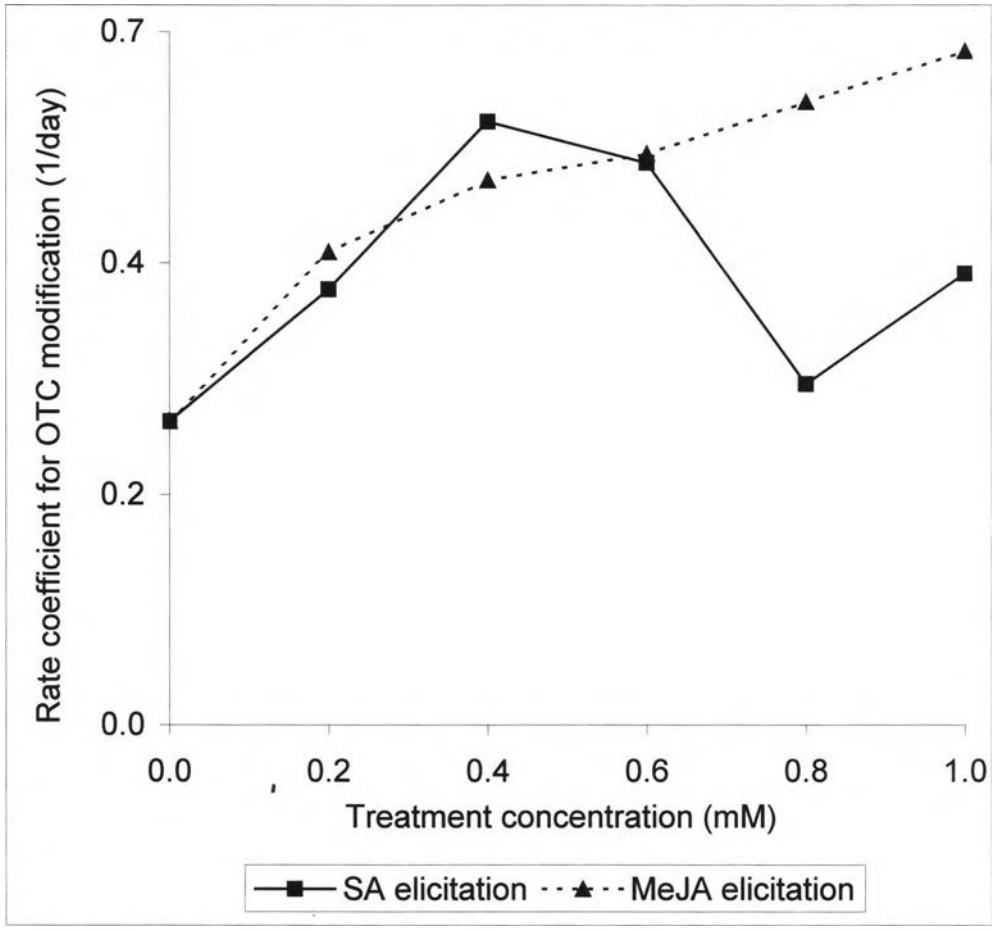


Figure 10. Effect of SA and MeJA elicitation on OTC removal by *P. stratiotes*. The ■'s represent the results obtained with SA, while the ▲'s represent the results obtained with MeJA.

nutrients that, in turn, would limit the plants ability to produce or express excess antioxidant compounds or enzymes. If this argument holds true, then elicitation of the pond-microcosm with SA, in which 150% strength Hoagland's medium is continuously pumped through it, should not result in plant necrosis.

The pond-microcosm, when elicited with 0.6 mM SA, indeed, shows greater adaptability to elicitor treatment. Interestingly, on treatment with 0.6 mM SA, several manifestations of ROS activity are observed in the microcosm. First, extensive bubble formation is observed that may be due to oxidation of the organic matter (OM) present in the microcosm (See the bottom picture in Figure 11). No such activity is observed in the non-elicited microcosm (compare the top and bottom pictures in Figure 11). Second, large debris of floating organic matter is observed along with the bubbles. Actually, the bubbles are observed within the debris. This, again, might be due to the oxidation of the OM (soluble or insoluble) by the ROS. It was observed that the bubble formation, and the subsequent appearance of floating debris, was predominant in the first half of the microcosm. The manifestations of the apparent ROS activity decreased along the length of the microcosm, with no such manifestations in the final few rows. This can be attributed to the non-availability of SA in the nutrient medium by the time it reaches those rows. Therefore, the residence time and the configuration of the microcosm appear to be unsuitable for SA elicitation. Ideally, all the propagules should be exposed to 0.6 mM of SA and the residence time of the nutrient medium in the microcosm should be low so as to avoid consumption of the ROS in OM oxidation.

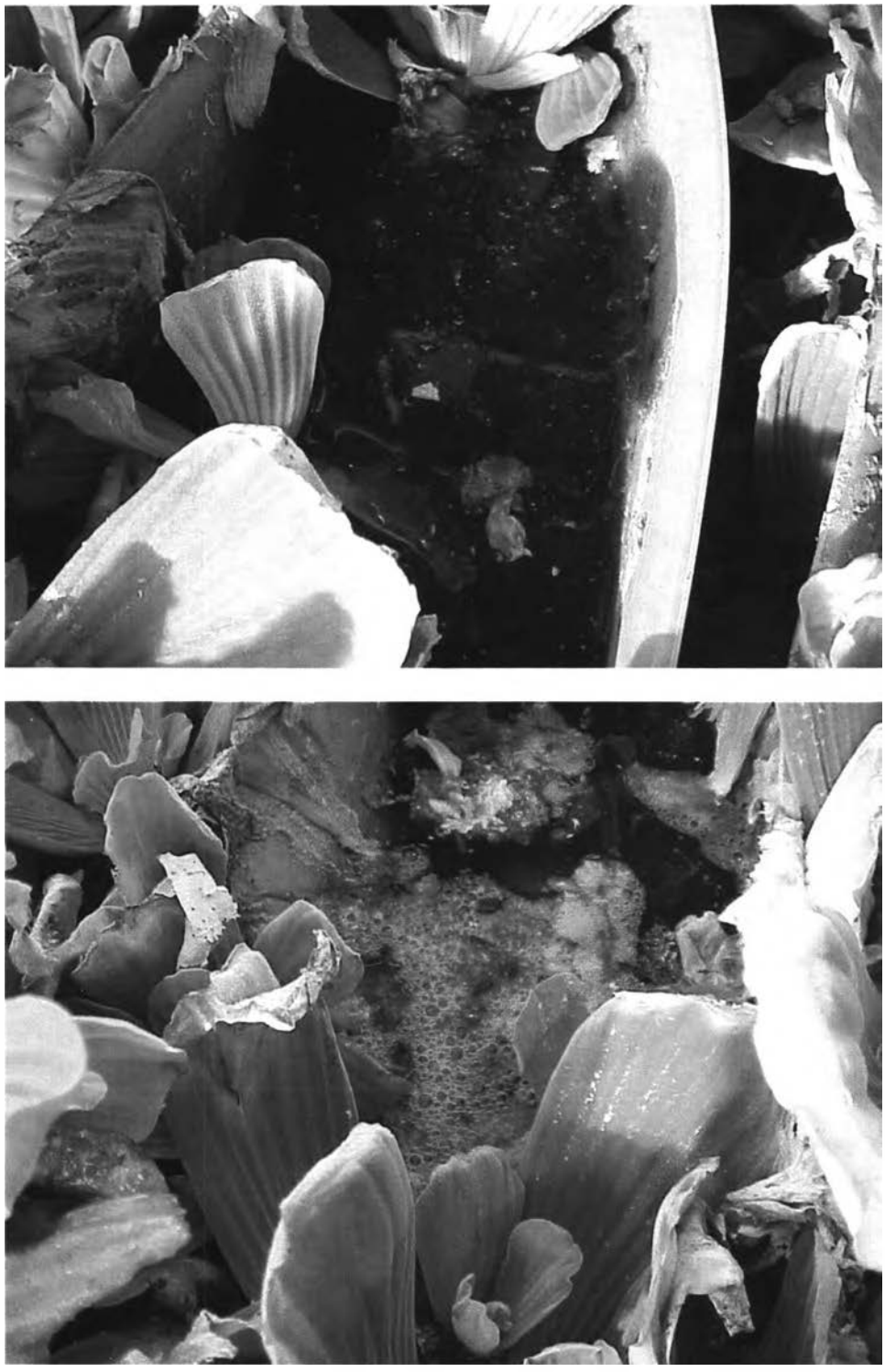


Figure 11. Non-elicited (top) vs. elicited (bottom) microcosm. Bubble formation is observed in the elicited microcosm, probably due to ROS activity on organic matter.

The root exudates from the microcosm elicited with 0.6 mM SA were then used for treating OTC in the CSTR system. The experimental conditions were similar to those in the experiments described earlier. The effect of increasing mixing speeds and aeration rates on the OTC removal by the CSTR were studied. Figures 12 and 13 show the results for the effect of mixing speed and aeration rate, respectively. Although the trends observed in Figures 12 and 13 are different to those observed in Figures 7 and 8, the rates of OTC oxidation are not enhanced to any significant extent, on SA treatment. These findings are consistent with the hypothesis presented in the paragraph above that the residence time and the configuration of the microcosm are unsuitable for elicitation. Excess ROS that is produced in the elicited microcosm apparently is utilized in oxidation of OM.

An improved design for the integrated pond-microcosm CSTR system is suggested in Figure 14, where two changes have been made to the original design (See Figure 3). First, the residence time of the nutrient medium in the bioreactor is reduced from 7 days to approximately 4.9 days. Second, the pond microcosm is divided into four equally distributed sections. Therefore, the inlet from the nutrient bucket is divided into four streams such that each section of the pond-microcosm would receive fresh nutrient medium, and an optimum concentration of the elicitor (SA). The exit streams from each section would be collected together and pumped into the CSTR.

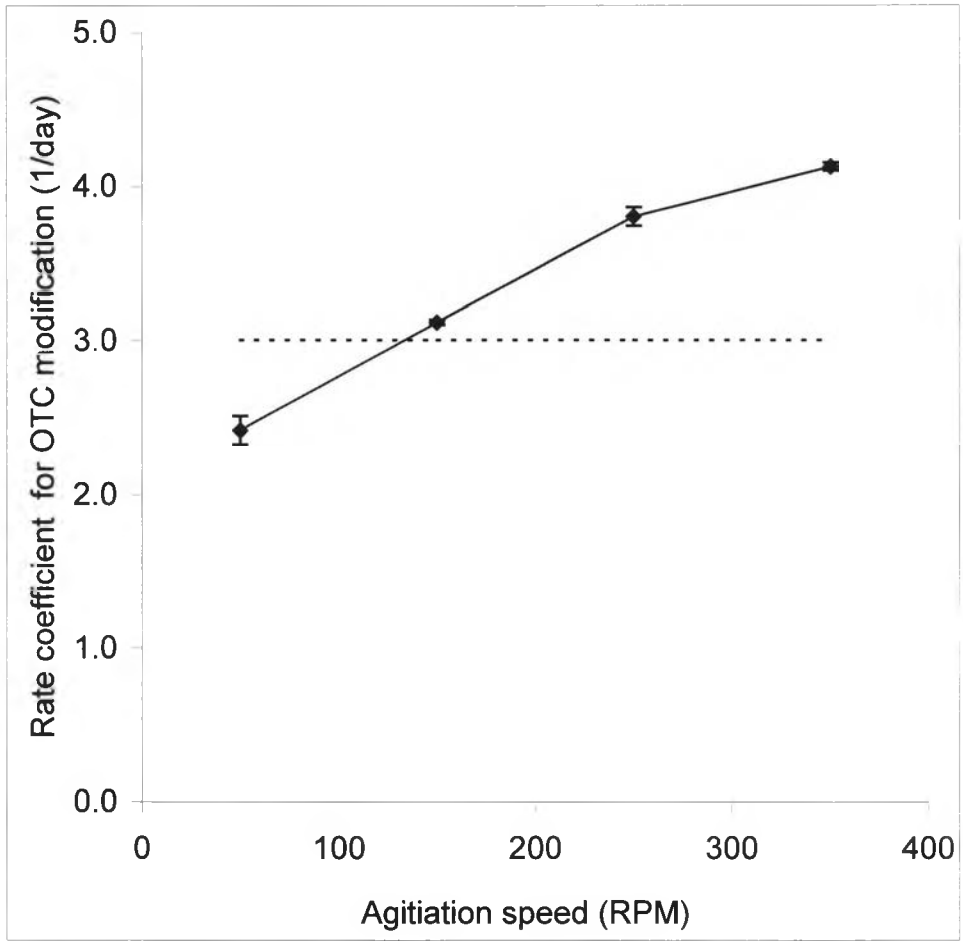


Figure 12. Effect of mixing speed on OTC removal by the SA-treated microcosm-CSTR system. The dotted line gives the expected OTC removal coefficient that would result purely due to dilution with the root exudates. Aeration at 6 L/min and retention time of 7 hours was used for this set of experiments.

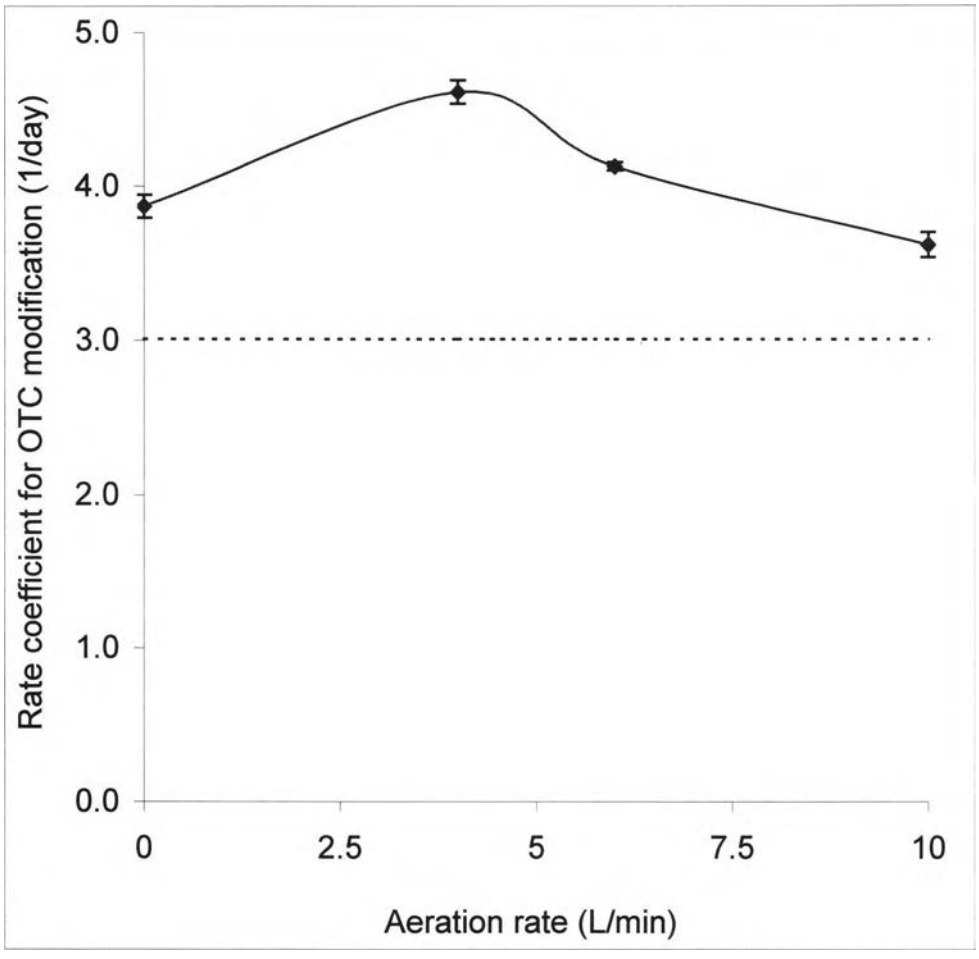
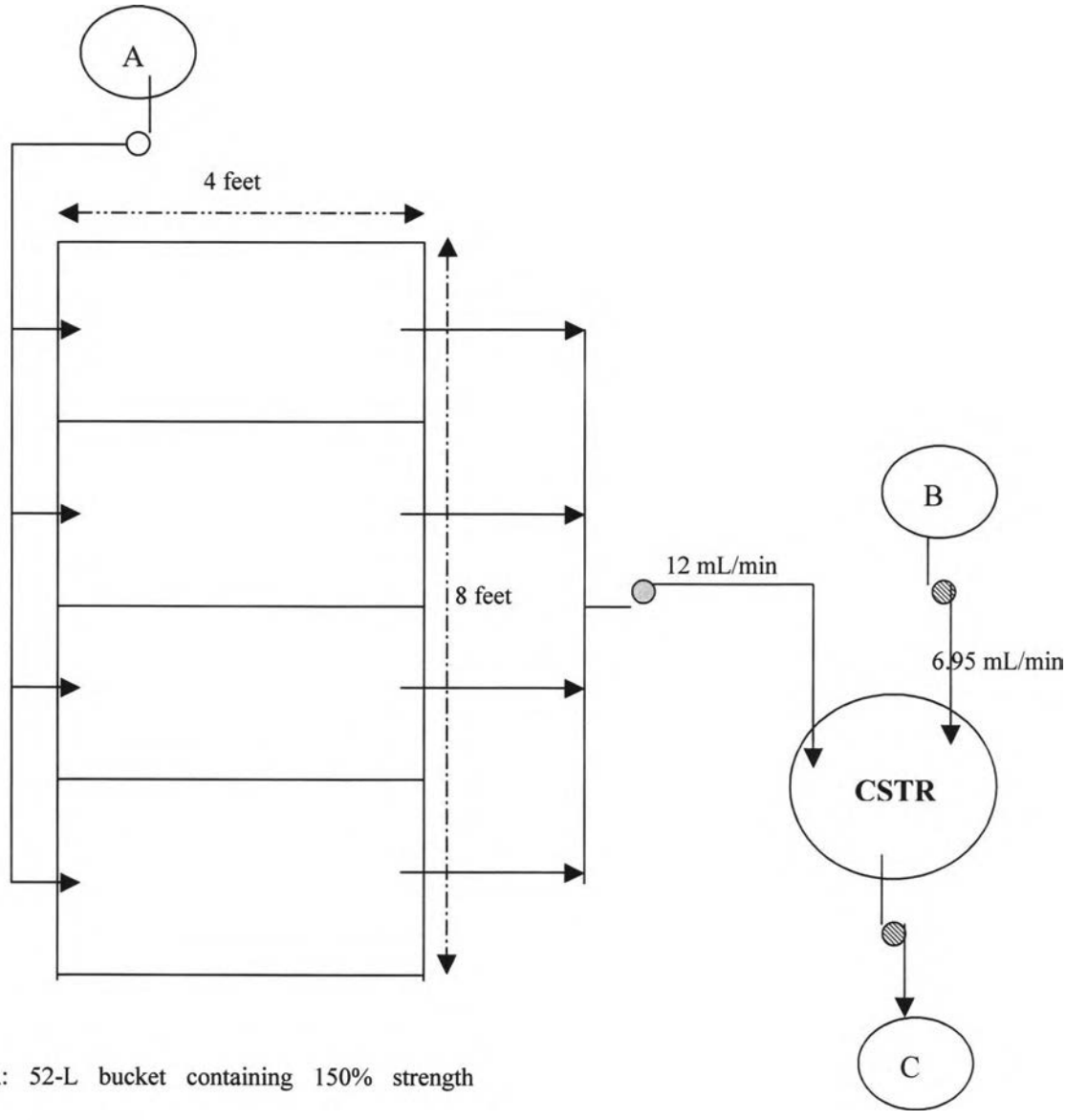


Figure 13. Effect of aeration rate on OTC removal by the SA-treated microcosm-CSTR system. The dotted line gives the expected OTC removal coefficient that would result purely due to dilution with the root exudates. Agitation rate of 350 rpm and retention time of 7 hours were used for this set of experiments.



A: 52-L bucket containing 150% strength Hoagland's medium

B: 52-L bucket containing 5 mg/L OTC in tap water

C: 52-L bucket containing the treated water coming out of the CSTR

Figure 14. Mechanical drawing of the integrated pond-microcosm CSTR system with modifications for effecting better utilization of the ROS.

Conclusions

An integrated bioreactor system with a pond-microcosm connected to a continuous-stirred tank reactor (CSTR) gives more efficient OTC removal from water compared to the microcosm-plug flow reactor (PFR) configuration. Based on the experiments reported in this study using OTC dissolved in tap water and microcosm-generated ROS, a high mixing speed with a sufficient retention time is required for significant ROS-OTC interaction and is recommended for the operation of the CSTR. Based on the batch experiments, elicitation of the plants in the pond-microcosm for higher ROS secretion in the root exudates may significantly improve the performance of the integrated bioreactor system. Optimum levels of elicitor treatment need to be determined to ensure plants are not killed by the treatment. In the continuous flow system, the plants adapt better to SA treatment than in batch culture, probably due to non-limiting conditions of nutrients. Modifications in the flow pattern of the nutrient medium in the pond-microcosm are required in order to obtain, and sustain, optimum ROS concentration in the root exudates that are subsequently contacted with OTC, in the CSTR.

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Chapter VII. Effectiveness of Root Exudates in Oxidizing Oxytetracycline from Lagoon Water

Introduction

The goal of this doctoral study is to assess the suitability of phytoremediation in treated antibiotic-contaminated water from confined animal feeding operations (CAFO). The earlier chapters show that the three plant systems used in this project can successfully degrade OTC and TC through ROS-mediated oxidation. The oxidation product(s) of the antibiotic are non-toxic to microorganisms susceptible to the antibiotic. The next step would be to adapt the ROS-producing phytoremediation systems in removing the antibiotics from wastewater samples taken from storage lagoons around CAFO. Note that the experiments described in the earlier chapters dealt with either TC or OTC dissolved in water. The wastewater samples (hereafter referred to as the lagoon water samples) can be expected to contain high levels of nutrients and other metabolized products introduced through animal faeces and urine. There is a possibility that the ROS-mediated antibiotic degradation process might be affected due to interferences from compounds present in the lagoon water samples.

Materials and Methods

The lagoon water samples were obtained from an animal operation in northern Colorado, on the promise of anonymity. Mrs. Kathy Doesken-Corwin, a research associate for

Professor Jessica Davis in the Department of Soil and Crop Sciences at CSU, provided valuable help in communicating with the animal operation owner, as well as in taking the samples. Approximately, 10 liters of lagoon water were collected from four locations around the lagoon. The pooled samples were mixed and stored in the University Greenhouse. Approximately 100 mL of supernatant were prepared after two days of storage. The lagoon water was first pre-filtered over regular coffee-filter paper and then through a 0.2 μm mini filter capsule [Gelman Sciences, Ann Arbor, MI] that was driven by vacuum.

A 5 mL aliquot of the filtered lagoon water supernatant was added to 5 mL of 0.2 μm-filtered root exudates (age 7 weeks), in sterile 15 mL centrifuge tubes [Fisher-Scientific, Fair Lawns, NJ]. An aliquot of stock solution of 100 mg/L OTC was added in to the tube to give a final concentration of 5 mg/L of OTC in the lagoon water-root exudate solution. As a control experiment, similar volumes of the root exudates and the OTC stock solution were added to 5 mL of de-ionized (DI) water. Another control was performed with 5 mg/L in DI water. After the addition of all the reactants, the tubes were mixed well and the 0 day samples were drawn from each. Samples were then taken after two days. For both the sampling times, 5 mL of sample were drawn from each tube. Since the lagoon water can be expected to contain high concentrations of nutrients, the samples were extracted through solid phase extraction (SPE) cartridges [Alltech, Deerfield, IL] before analysis. The SPE cartridges were preconditioned with 5 mL of 2-propanol (0.1 % Formic acid) [Aldrich, Milwaukee, WI] followed by 5 mL of HPLC grade water containing 0.1 % Formic acid. HPLC grade water and 2-propanol were purchased from

Fisher-Scientific, Fair Lawns, NJ. A given fixed volume of the sample was then passed through the SPE cartridge followed by elution with the same volume of 30:70 2-propanol:HPLC grade water mixture containing 0.1% formic acid. The eluted sample was then analyzed by HPLC for OTC concentration, as described earlier.

In a separate set of experiments, root exudates from *P. stratiotes* were tested for their efficiency in removing the biological oxygen demand (BOD) of the lagoon water. The procedure for the BOD_5 determination of the samples was adapted from Metcalf and Eddy (1995). The BOD of the lagoon water samples can be expected to be in the range 1200-25000 mg/L [EPA, 2001]. Four similar-sized *P. stratiotes* propagules were grown in four 1-L mason jars, each containing 300 mL of Hoagland's medium. The propagules were grown for a period of one week. One propagule was kept as a control, with no OTC or elicitor treatment. The other three propagules were each treated with 5 mg/L of OTC. Prior to the OTC treatment, one propagule was elicited with SA at a treatment concentration of 0.2 mM, while another was elicited with the same concentration of MeJA. After two days of incubation, the propagules were removed from all the four jars and 300 mL of the lagoon water was added to each jar, containing the root exudates. The lagoon water was then incubated in the root exudates for two days in the greenhouse. As a control experiment for the lagoon water, 300 mL of lagoon water was incubated with 300 mL of tap water in a 1-L mason jar. At the end of the 2 day incubation period, the five jars containing: lagoon water and tap water (L); lagoon water and root exudates (LR); lagoon water and root exudates treated with OTC (LRO); lagoon water and root exudates elicited with 0.2 mM SA and treated with OTC (LROS); and lagoon water and

root exudates elicited with 0.2 mM MeJA and treated with OTC (LROM); were sampled for the BOD_5 determination.

A dilution series from each treatment was established in BOD bottles using air-saturated water. The BOD was determined from the dilution with 0.4 mL of sample in 299.6 mL of the air-saturated water. The dissolved oxygen content of each of the bottles was determined [YSI, Yellow Springs, OH]. The BOD-bottles were then incubated at 22^0 C for 5 days. At the end of the 5-day incubation period the dissolved oxygen content of each bottle was determined. To determine the background oxygen consumption, a control bottle was maintained with 300 mL of the air-saturated tap water. The BOD_5 value was determined as,

$BOD_5 = (DO_{i0} - DO_{i5})-(DO_{c0} - DO_{c5})/d$

Where,

DO_{i0} and DO_{i5} are the dissolved oxygen contents of the samples at 0 and 5 days, respectively,

DO_{c0} and DO_{c5} are the dissolved oxygen contents of the air-saturated tap water control at 0 and 5 days, respectively, and

d is the overall dilution factor for the lagoon water in the BOD-bottle

Results and Discussion

The lagoon water treated with the root exudates from the hairy root cultures shows a higher level of precipitation, and consequently greater clarification, compared to non-treated filtered lagoon water. An image of the lagoon water samples, along with the

controls, is shown in Figure 1. Starting from the left, the first tube contains OTC dissolved in DI water at 5 mg/L initial concentration. The second tube contains 2.5 mL of, each, DI water and 0.2 μm-filtered root exudates from *H. annuus*, with OTC added at 5 mg/L. The third tube contains 2.5 mL of, each, DI water and lagoon water. The fourth tube contains 2.5 mL of, each, DI water and 0.2 μm-filtered lagoon water. The fifth tube contains 2.5 mL of, each, 0.2 μm-filtered root exudates from *H. annuus* and 0.2 μm-filtered lagoon water, with OTC added at 5 mg/L. The precipitation observed in the root exudates-treated lagoon water might be due to the oxidation of dissolved organic matter from the lagoon water. Samples from the DI-OTC control were ruined, probably due to a bad SPE cartridge, so the results for that control are not included in Figure 2. The control tube with the root exudates and DI water shows 56% OTC oxidation, while the root exudates oxidize approximately 42% of OTC from the lagoon water.

The BOD_5 for the lagoon water (L) is 4526 mg/L. The $\%BOD_5$ removal in each treatment (LR, LRO, LROS and LROM) is calculated by comparing the BOD_5 values of each treatment to that of the lagoon water (L). The values for the BOD_5 removal for all the treatments are represented in Figure 3. Plant treatment with OTC increase the BOD_5 removing capacity of the root exudates, probably due to the increased production of ROS that in turn remove the BOD, along with OTC. Expectedly, elicitation with ROS-elicitors, SA and MeJA, further increases the BOD removing capacity of the root exudates. The root exudates treated with neither OTC nor the elicitors, give the least BOD removal (approximately 25%).

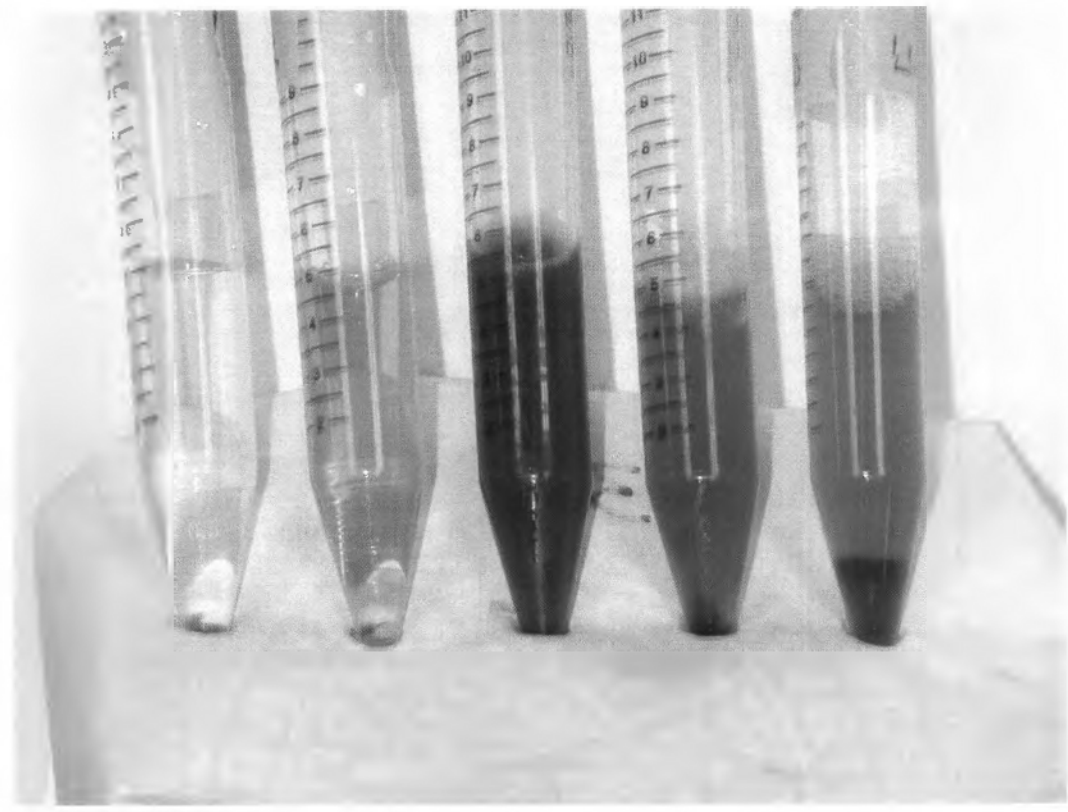

Figure 1. Observation of precipitation in the root exudates-treated lagoon water. Starting from the left, a) 5 mg/L OTC in DI water after two days of incubation, b) 5 mg/L OTC in 50-50 DI water and *H. annuus* root exudates after two days of incubation, c) 50-50 unfiltered lagoon water sample and DI water, d) 50-50 filtered lagoon water sample and DI water, e) 5 mg/L OTC in 50-50 filtered lagoon water and *H. annuus* root exudates after two days of incubation.

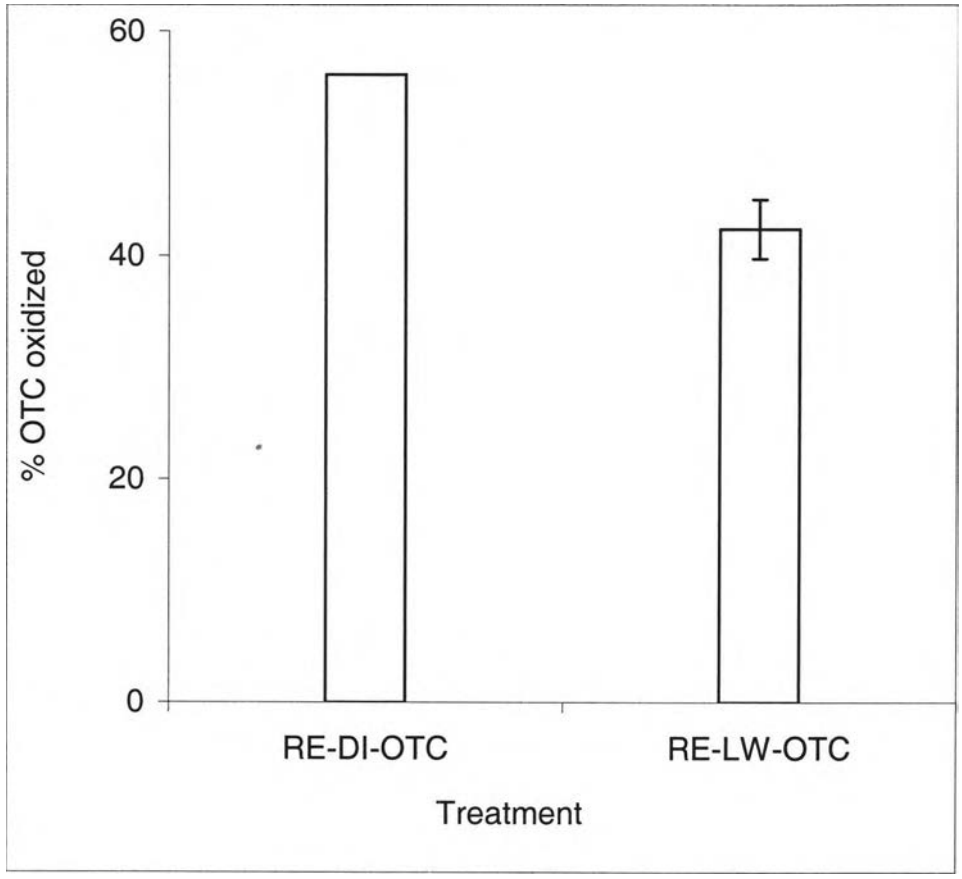

Figure 2. OTC oxidation in the root exudate treated lagoon water (RE-LW-OTC) compared to the root exudates- DI water control (RE-DI-OTC).

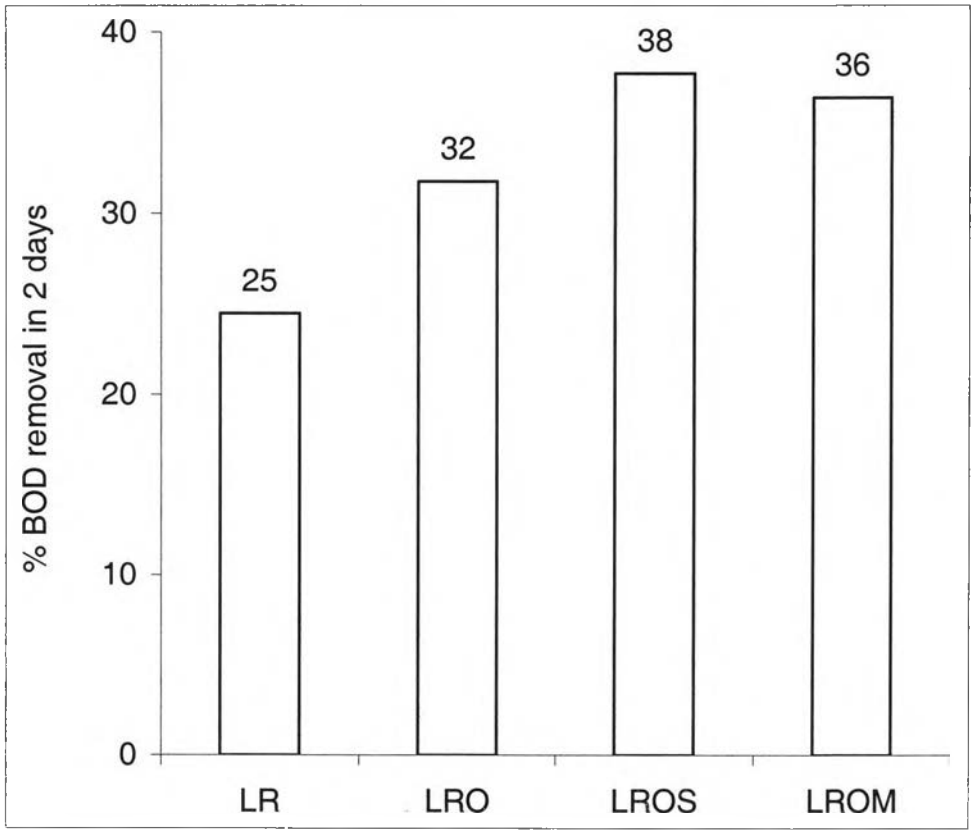

Figure 3. BOD removal achieved by the root exudates from *P. stratiotes*. (LR- Lagoon water and root exudates from *P. stratiotes*; LRO- Lagoon water and root exudates from *P. stratiotes*, treated with 5 mg/L OTC; LROS- lagoon water and root exudates elicited with 0.2 mM SA and treated with 5 mg/L OTC; and LROM- lagoon water and root exudates elicited with 0.2 mM MeJA and treated with 5 mg/L OTC.

Conclusions

The effectiveness of *H. annuus* root exudates in oxidizing OTC decreases by approximately 20% in the lagoon water compared to that in DI water. The observed decrease in OTC oxidation might be due to the simultaneous oxidation of other dissolved organic matter that precipitates out in the lagoon water treated with the root exudates. Therefore the constituents of the lagoon water appear to hinder the ROS-mediated oxidation of OTC. On the other hand, treatment with root exudates might be helping in reducing the nutrient (or organic) load of the lagoon water. Thereby, making the lagoon water more suitable for environmental release. Root exudates from *P. stratiotes* remove as high as 38% of the BOD in the lagoon water, with the SA elicited root exudates giving the maximum BOD removal and the untreated root exudates giving the least BOD removal. Therefore, the root exudates not only remove the OTC, but they also remove the BOD from the lagoon water.

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Conclusions and Recommendations

Conclusions

The antibiotics, tetracycline (TC) and oxytetracycline (OTC), pose serious health hazards due to their persistence in the environment. Long-term exposure of these antibiotics results in induction, and the subsequent propagation, of antibiotic resistance genes among the microbial populations of the environment. Conventional wastewater treatment processes prove ineffective in removing the two tetracyclines from water. Phytoremediation of the two antibiotics by the aquatic species *Pistia stratiotes* and *Myriophyllum aquaticum* and the hairy root cultures of *Helianthus annuus* can potentially lead to an effective and inexpensive method for antibiotic remediation. All three plant systems give high antibiotic removal rates from water, with *P. stratiotes* and the hairy root cultures of *H. annuus* giving better results due to the associated higher volumetric root biomass levels. Sterile 0.2 μm-filtered plant cell-and microbe-free root exudates from the three plant species yield significant antibiotic removal. Hairy root cultures of *H. annuus* provide an excellent model system for determining the mechanism of antibiotic remediation.

The observed antibiotic removal by the plant systems is through oxidative modification at the three possible sites of oxidation on the tetracycline molecule; two on the BCD chromophore and one on the A chromophore. ROS secreted by the plant root exudates

oxidize the antibiotics. ROS are stress related compounds produced by plants, intracellularly and extracellularly, under stress conditions such as nutrition deficiency, pathogen attack, heat, cold, elicitation and exposure to toxic compounds. ROS are also involved in the signal transduction system of plants.

The involvement of ROS in the antibiotic modification process is supported by,

1. Modification of the antibiotic (OTC) molecule at the functional groups that are known to be possible sites of oxidation
2. Reduction in the OTC modification rates by the root exudates of *H. annuus*, on addition of the antioxidants ascorbic acid and salicylic acid.
3. Increase in the OTC modification rates by the hairy root cultures of *H. annuus*, on addition of the ROS elicitors salicylic acid and methyl jasmonate.
4. Observance of higher oxidative damage in elicited root cultures that show reduced activities of the antioxidant enzymes ascorbate peroxidase, catalase and guaiacol peroxidase; significantly lower damage is observed in the elicited root cultures that show high activities of the above antioxidant enzymes.

The hydroxyl radical (OH) appears to be involved in the observed antibiotic modification by the plants because,

1. OTC treatment with hydrogen peroxide does not result in significant OTC removal, but when treated with a combination of hydrogen peroxide and horseradish peroxidase (HRP), OTC is completely removed from the water [See Appendix A]. HRP is basically an iron-based enzyme that transitions between the

'2^+' and '3^+' oxidation states to reduce substrates like hydrogen peroxide. HRP and hydrogen peroxide interact through Fenton's reaction to give the highly reactive hydroxyl radical, as illustrated below.

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^{-} + Fe^{3+}$$

2. Salicylic acid, a ROS scavenger that is fairly specific to the hydroxyl radical, when added to the root exudates from *H. annuus* hairy root cultures results in lower OTC modification rates by the root exudates.

Although oxygen/air sparging reduces the OTC modification capability of the root exudates, the OTC modification rate is significantly higher than that when nitrogen is sparged through the root exudates (see Chapter VII). It is also observed that autoclaved root exudates retain some OTC modification activity (see Appendix A). Therefore there appears to be a non-enzymatic mechanism that is involved in ROS (hydroxyl radical) production in the root exudates. As described in the above paragraph, transition metals in their lower oxidation states can reduce hydrogen peroxide, and probably oxygen too, to initiate the reactions for hydroxyl radical formation. This mechanism may not be the primary source of ROS in the root exudates; it is more likely to be one of the contributing mechanisms. Most of the ROS present in the root exudates appear to be directly released by the roots.

The oxidative modification of the antibiotic (OTC) by the ROS results in oxidation product(s), which become non-toxic to *Enterococcus hirae*. *E. hirae* is a microorganism known to be susceptible to tetracyclines. The root exudates, when used to treat lagoon

water samples spiked with 5 mg/L OTC, result in significant OTC removal. The OTC removal from the lagoon water is compromised by the presence of other organic/ inorganic constituents in the lagoon water. The root exudates from *P. stratiotes* reduce the BOD of the lagoon water by approximately 35% in two days. Therefore the proposed phytoremediation application, if used on field at a CAFO operation, can potentially serve two purposes, antibiotic removal as well as BOD removal from the wastewater.

An integrated bioreactor system, consisting of a pond-microcosm coupled to a bioreactor that utilizes the root exudates from *P. stratiotes* in removing OTC from water, suggests a possibility of scaling up the antibiotic-phytoremediation process to an actual field-operation. The integrated bioreactor system using a second stage CSTR gives more efficient results compared to that using a PFR. The OTC remediation reaction ceases on depletion of the ROS from the reaction mixture. Since the ROS are present in rate-limiting concentration and are known to have short half-lives, a CSTR with a short retention time gives high OTC removal from water. The root exudate-OTC reaction is diffusion limited; higher mixing speeds give greater OTC removal rates. The above reaction, though not oxygen-limited, performs better under aerobic conditions.

In summary, phytoremediation provides an innovative and inexpensive option for controlling the release of antibiotics to the environment.

Recommendations

Identification of the oxidation product(s) of TC/OTC on modification by the ROS

It is difficult to identify the oxidation(s) products of TC/OTC from the root exudate-OTC reaction mixture because plant root exudates contain several hundred organic and inorganic compounds. Spectroscopic methods such as mass spectrometry (MS) and Fourier transform infrared spectroscopy (FTIR) were used for the identification purpose, but without much success. As suggested earlier, the hydroxyl radical is responsible for the reported OTC/TC phytoremediation and it can be formed through Fenton's reaction by adding hydrogen peroxide and HRP in water. Therefore, reaction mixtures containing OTC, hydrogen peroxide and HRP in HPLC grade water can be analyzed using either a MS or FTIR over a period of time to follow the OTC oxidation process under a variety of conditions. With just three known compounds in the system, the new compounds appearing in the analysis can be identified as the oxidation products of OTC. Characterization of these products will enable understanding of the organic mechanism of the transformation.

Nutritional manipulation to maximize the ROS production by plants/ plant cell cultures

Nutritional manipulation can be considered to maximize ROS production by the plant cell cultures. It is reported that salinity stress induces cellular accumulation of ROS in plants [Shalata and Neumann, 2001]. Accordingly, hydroponically grown tomato seedlings (*Lycopersicon esculentum*) treated with 300 mM NaCl in the nutrient medium demonstrated maximum oxidative damage in the roots. Although 300 mM NaCl causes

plant death, lower levels of treatment could be investigated for ROS production using hairy root cultures of *H. annuus*. Heavy metals act as abiotic elicitors. Experiments reported by Zhang et al. (2004) show that 15 to 30 μM Ag^{+} induce ROS production in hairy root cultures of *Salvia miltiorrhiza*, an herbal plant. Zinc is directly linked to superoxide dismutase (SOD) and other antioxidant enzymes, whereby bean leaves demonstrate increased production of ROS under Zn deficiency [Cakmak and Marschner, 1993]. Copper acts directly on the production of ROS through the Fenton or the Haber-Weiss reactions, as demonstrated in *Nicotiana tabacum* cell suspension cultures, whereby treatment with 10 μM $CuSO_4$ gives approximately 8-fold increase in hydrogen peroxide production within 200 minutes of treatment [Raeymaekers et al., 2003].

As suggested earlier, nutrition deficiency triggers ROS production in plants. Some evidence of this was observed in our experiments with the pond-microcosm bioreactor system. Experiments can be performed to identify the nutrients that lead to ROS production in the root exudates, but do not lead to plant death under deficient conditions.

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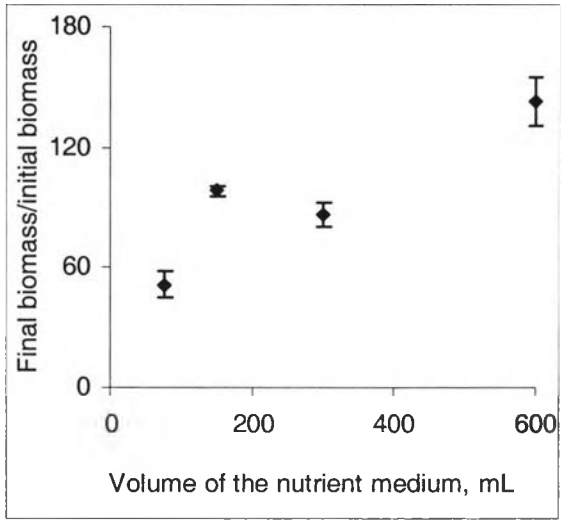
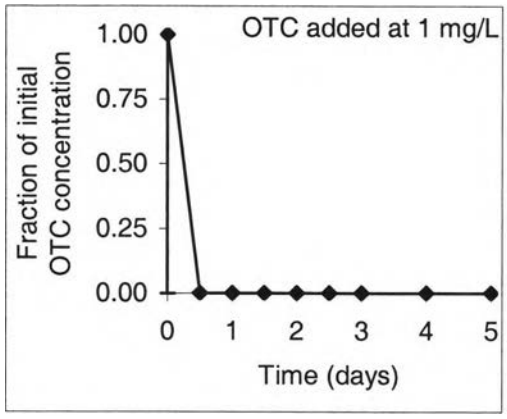
Appendix A. Some interesting results not represented in the primary text of the dissertation

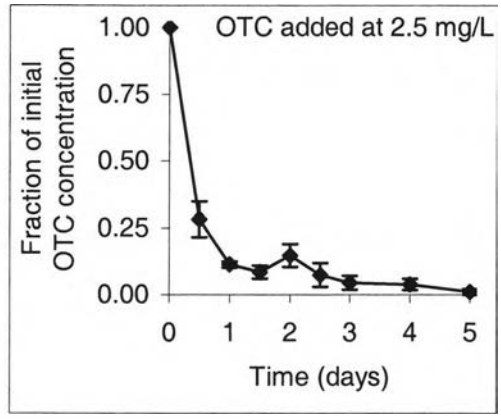
Experiment conducted to study the effect of container/ media volume on the growth of *H. annuus* hairy root cultures

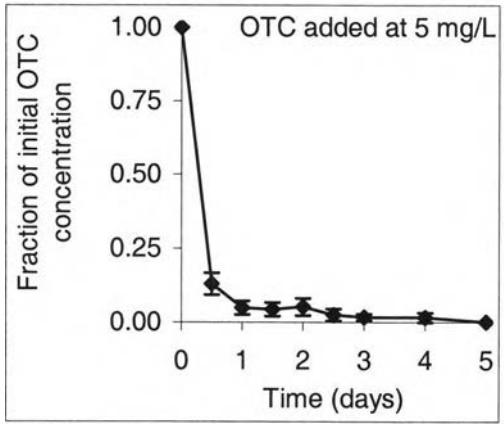


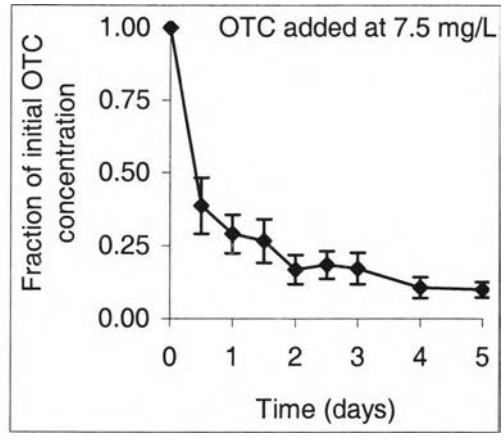
Note: The volumes of the respective Erlenmeyer flasks were proportional to the volume of the medium contained in them. Each of the flasks had the same inoculum volume of 0.2 g of growing root tips. All the flasks were inoculated and harvested at the same time. Since the biomass of the root cultures increases almost linearly with the container (media) volume, scaled-up designs for growing the hairy root cultures can be effectively used for achieving greater OTC removal from water.

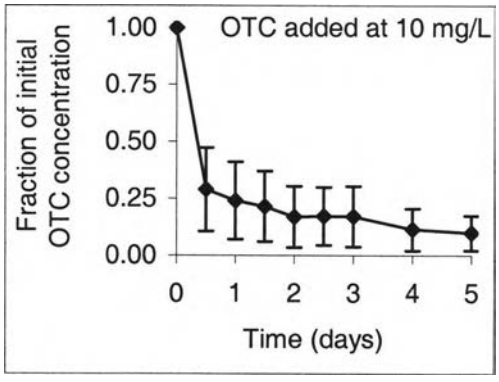
Phytoremediation of Oxytetracycline by *Eichhornia crassipes* (water hyacinth)





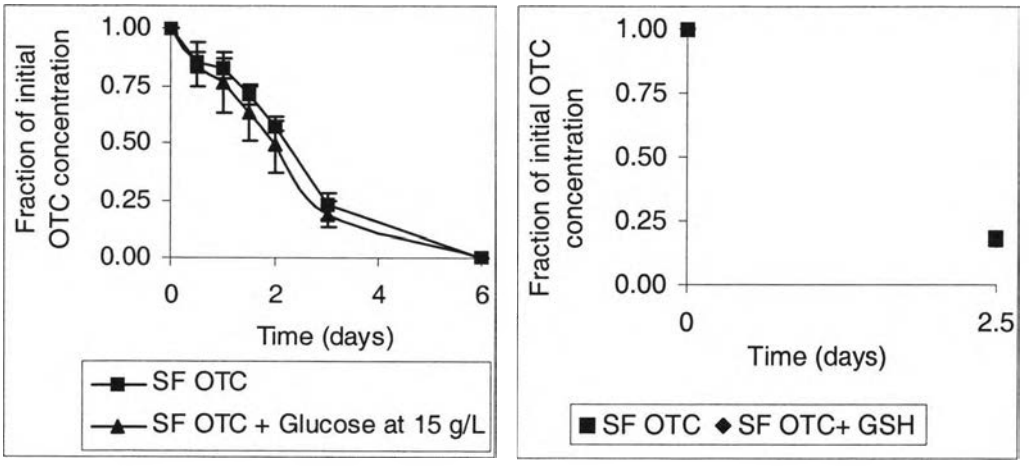






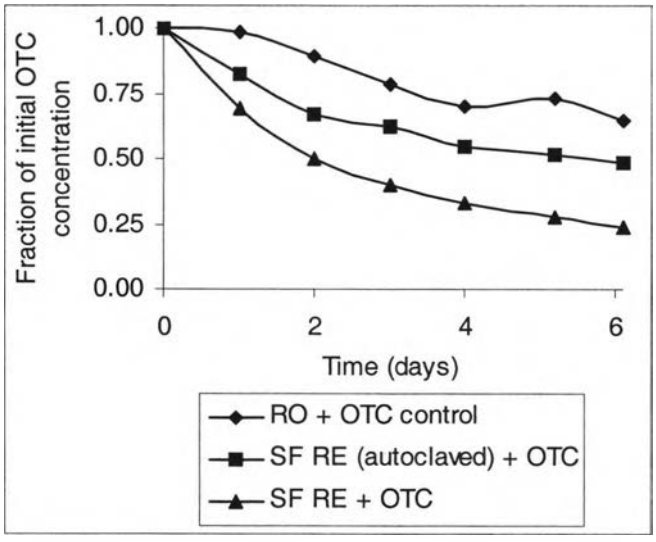
Note: *E. crassipes* gives good OTC removal from the nutrient medium (Hoagland's medium).

Experiments to investigate the possibility of OTC conjugation with glucose and glutathione



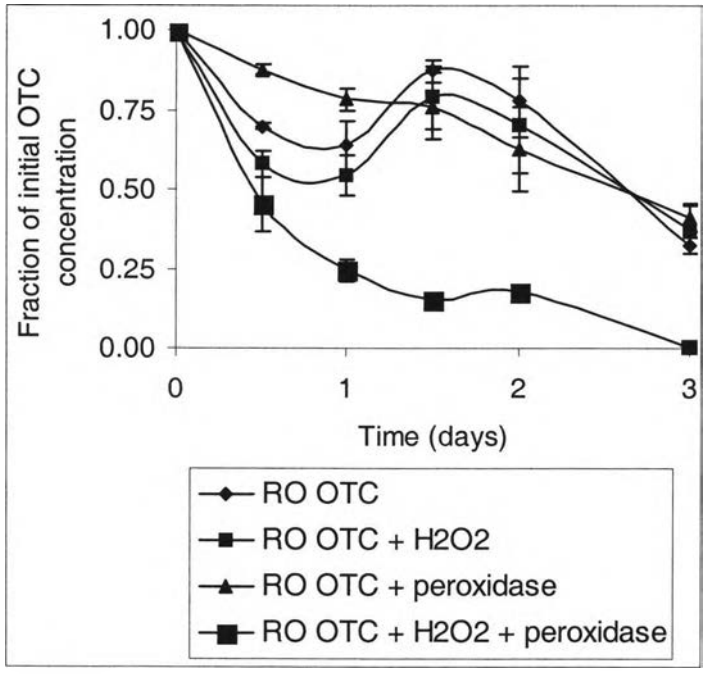
Note: Glucose and glutathione (GSH) when added to *H. annuus*, i.e. sunflower (SF), hairy root cultures, at 15 g/L and 40 μM/L, respectively, does not result in any significant increase in the OTC removal by the root cultures. Thus OTC uptake in to the roots via conjugation with glucose or glutathione is not supported.

Experiment conducted to determine the effect of autoclaving on the OTC remediation capacity of the root exudates of *H. annuus*



Note: Although autoclaving reduces the OTC remediation capacity of the root exudates, activity still persists. Thus, the results suggest that OTC modification by the root exudates is not directly enzyme-catalyzed. (RO is reverse osmosis water).

Experiment conducted to check to if hydrogen peroxide, horseradish peroxidase, or a combination of the two can oxidize OTC from water.

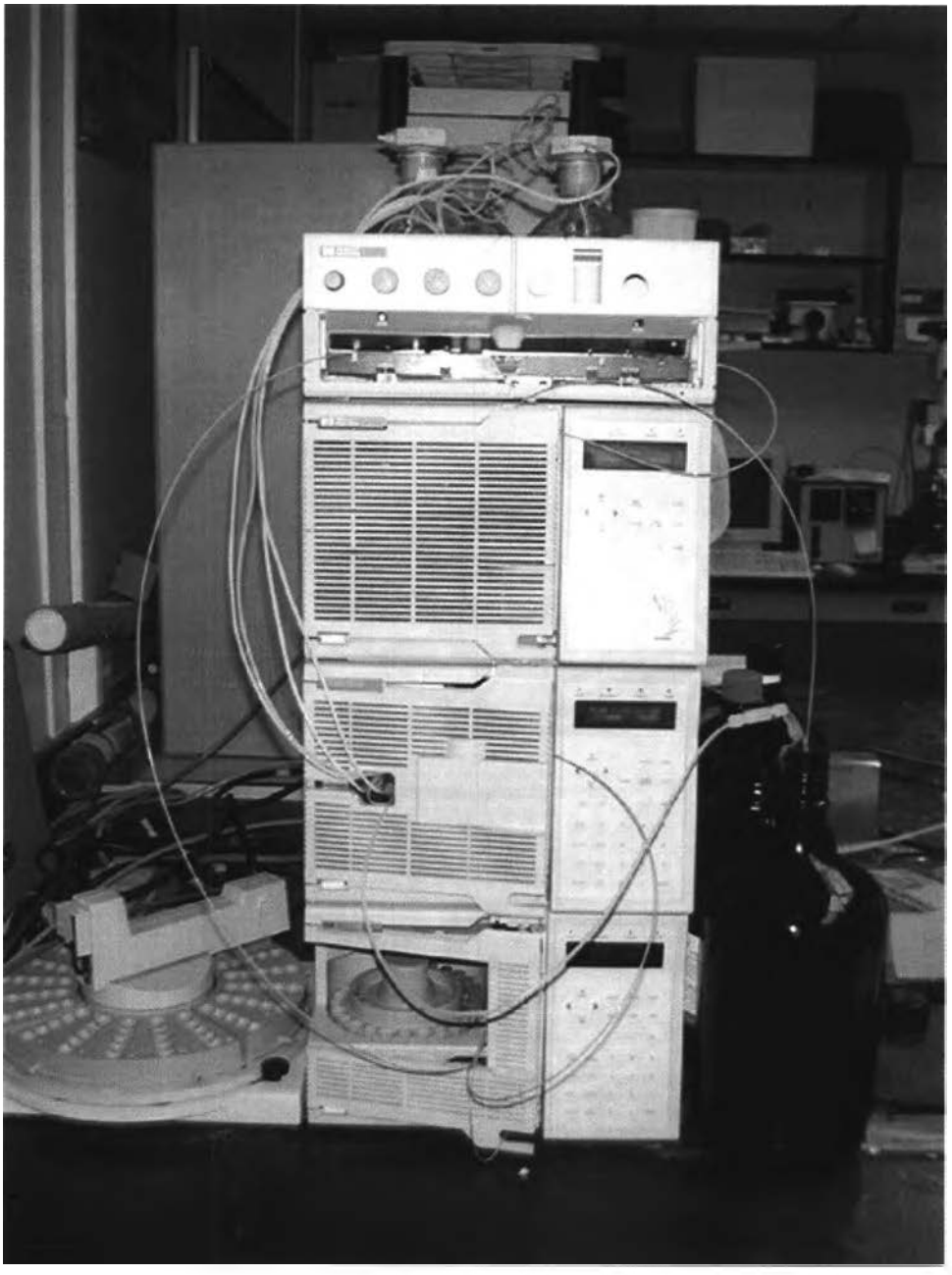


Note: Hydrogen peroxide does not oxidize OTC to any significant extent, on its own. But when added with horseradish peroxidase, hydrogen peroxide probably combines with the peroxidase and produces the highly reactive hydroxyl ion through Fenton's reaction. Hydroxyl ion efficiently oxidizes all the OTC from the reverse osmosis (RO) water. Thus the hydroxyl radical is the likely ROS that actually oxidizes OTC when added to the roots or root exudates of *H. annuus* and other plant systems investigated in this project.

Appendix B. Equipment used during the study but not illustrated in the primary text of the dissertation

Appendix C. Recipe for the preparation of Hoagland's medium

High performance liquid chromatograph (HPLC)



HP 1050 series- High Performance Liquid Chromatograph

Shaker table for the hairy root cultures

Hoagland's medium

Ingredient	For a 100% strength solution in one liter water (mg)
Ammonium phosphate (monobasic)	115.03
Boric acid	2.86
Calcium nitrate	656.40
Cupric sulfate.5H2O	0.08
Ferric citrate	5.32
Magnesium sulfate	240.76
Manganese chloride.4H2O	1.81
Molybdenum trioxide	0.02
Potassium nitrate	606.60
Zinc sulfate.7H2O	0.22

Appendix D. Publications, presentations and patent

Journal Publications

1. Ninad P. Gujarathi, S. Ranil Wickramasinghe, and James C. Linden. *A novel, integrated bioreactor system using* Pistia stratiotes *for removal of oxytetracycline from water.* **In preparation**.
2. Ninad P. Gujarathi and James C. Linden. *Oxytetracycline oxidation by reactive oxygen species produced in the nutrient medium of* Helianthus annuus *hairy roots cultures*. Submitted to **Biotechnology and Bioengineering** (2005).
3. Ninad P. Gujarathi, Bryan J. Haney, Heidi J. Park, S. Ranil Wickramasinghe, and James C. Linden. *Hairy roots of* Helianthus annuus*: A model system to study phytoremediation of tetracycline and oxytetracycline.* **Biotechnology Progress** (In press, published on the web as an ASAP article).
4. Ninad P. Gujarathi, Bryan J. Haney, and James C. Linden. P*hytoremediation potential of* Myriophyllum aquaticum *and* Pistia stratiotes *to remove antibiotic growth promoters, tetracycline and oxytetracycline, from aqueous systems.* **International Journal of Phytoremediation** (In press, 2005).
5. Ninad Gujarathi and James Linden. *Potential for phytoremediation of antibiotic-contaminated water.* **Agronomy News**, Cooperative Extension, Colorado State University, December 2004, 24 (3): 9.
6. Binbing Han, James C. Linden, Ninad P. Gujarathi, and S. Ranil Wickramasinghe. *Population balance approach to modeling hairy root growth.* **Biotechnology Progress** 2004, 20: 872-879.

Conference and Symposium presentations

1. "Phytoremediation of Antibiotics"- a talk presented at **CHEMCON2004**, Mumbai, India, December 29, 2004.
2. "Oxytetracycline Modification in the Root Exudates of *Pistia stratiotes*"- a paper presented at **CHEMCON2004**, Mumbai, India, December 28, 2004.
3. "Phytoremediation of Antibiotics"- a poster presented at **CSU Research Colloquium Series: Environmental Research**, Colorado State University, Fort Collins, USA, November 10, 2004.
4. "Modeling Growth of Hairy Roots"- a technical paper presented at the annual **American Institute of Chemical Engineers (AIChE) Conference**, San Francisco, USA, November 18, 2003.
5. "Remediation of Antibiotic Growth Promoters Present in Run-off from Animal Feedlots using Myriophyllum" - a poster presented at the **Phytoremediation Conference**, University of Colorado, Denver, USA, September 19, 2003.

Patent

1. Linden, J. C. and Gujarathi, N. P. Aquatic plant exudates and hairy root cultures for phytoremediation of antibiotics in water and wastewater. (**Patent pending**; applied on March 16, 2005).