

EFFECTS OF TRICLOSAN EXPOSURE ON NITRIFICATION
IN ACTIVATED SLUDGE, BIOFILMS, AND PURE
CULTURES OF NITRIFYING BACTERIA

by

Kylie Brigitta Bodle

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DEDICATION

This work is dedicated to my family, who have supported me, encouraged me, and made this accomplishment possible.

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ABSTRACT

Emerging contaminants, such as pharmaceuticals or personal care products, have the potential to impact many wastewater treatment processes due to their antimicrobial properties. Nitrifying bacteria initiate the nitrogen removal process in wastewater treatment, and are particularly sensitive to inhibition by these and other contaminants. The impacts of the emerging contaminant triclosan (TCS) on two common nitrifying bacteria were evaluated under multiple growth conditions. The resilience of biofilms and suspended cell cultures of the ammonia oxidizing bacterium (AOB) *Nitrosomonas europaea* was compared during TCS exposure. Impacts of TCS on *Nitrobacter winogradskyi*, a common nitrite oxidizing bacterium (NOB), were also considered. Lastly, activated sludge biofilms and suspended cells were also exposed to TCS to further evaluate impacts on nitrification. Triclosan at part per million levels was found to reduce respiration in nitrifying biofilms, and NOB were much more impacted by TCS than AOB. Interestingly, biofilms of *N. europaea* were just as impacted by TCS as suspended cells. Triclosan adsorbed strongly to cellular material and degradation was only observed in activated sludge at low concentrations. Altogether, TCS was found to reduce nitrification by AOB and NOB, and the results suggest that its presence at high levels in wastewater treatment is likely to have negative consequences.

CHAPTER ONE – INTRODUCTION

Effective wastewater treatment is necessary for protection of both human health and natural water systems. Nitrogen removal is particularly important in wastewater treatment, as its release can cause eutrophication of water systems, leading to algal blooms, oxygen depletion, and fish die off (1). Microbial activity is used in wastewater treatment plants to remove nitrogen and other nutrients from wastewater.

Nitrification, the first step in the nitrogen removal process, consists of the microbial conversion of ammonia (NH_3) to nitrite (NO_2^-) by ammonia oxidizing bacteria (AOB). Nitrite oxidizing bacteria (NOB) then convert NO_2^- to nitrate (NO_3^-). Nitrate is eventually converted to harmless dinitrogen gas by denitrifying bacteria, thus completing the nitrogen removal cycle.

Of specific interest here are the first steps in the nitrogen removal process, or the conversion of ammonia to nitrite by AOB, followed by the conversion of nitrite to nitrate by NOB. The first step is significant because it is catalyzed in wastewater in large part by *Nitrosomonas europaea*, a well-studied microorganism on which most knowledge of AOB is based (2). *N. europaea* is ubiquitous in wastewater treatment, and is a fairly slow-growing microorganism that is sensitive to various changes in its growth environment, such as the introduction of chemical contaminants (3-8). In turn, this makes the ammonia oxidation step vulnerable to contaminants in wastewater, thereby jeopardizing the success of the entire nitrogen removal process—if ammonia oxidation cannot proceed, nitrification will not occur, and neither will denitrification.

In particular, the enzyme used for ammonia oxidation (ammonia monooxygenase or AMO) is non-specific and capable of oxidizing a wide variety of substrates other than ammonia (3-5, 9). Cometabolism by this enzyme does not support growth of *N. europaea*, and therefore can inhibit successful nitrification. AMO can oxidize multiple aliphatic and aromatic compounds, many of which are found in municipal and industrial wastewater (10-12). Consequently, wastewater contamination by these groups of compounds can be a barrier to successful nitrification due to the non-specificity of the AMO enzyme.

Another ubiquitous wastewater bacterium, *Nitrobacter winogradskyi*, can catalyze the second step in nitrification—the conversion of nitrite to nitrate. *N. winogradskyi* is less well-studied than *N. europaea*, but is still typically used as the model organism for nitrite oxidation (13). The nitrite oxidoreductase (NXR) enzyme is used by *N. winogradskyi* for nitrite consumption. Little is known about inhibition of this enzyme, but NXR belongs to a family of non-specific enzymes, and therefore potentially may cometabolize non-growth-supporting compounds in a similar manner as AMO, leading again to inhibition of the nitrification process (13, 14).

Thus, AOB and NOB are sensitive microorganisms that use enzymes that can interact with or cometabolize many different aliphatic or aromatic compounds. Emerging contaminants (ECs) such as pharmaceuticals and personal care products are typically of an aliphatic or aromatic nature and readily enter wastewater due to widespread use (15, 16). ECs therefore pose a large threat to successful nitrogen removal due to the vulnerability of AOB and NOB. Typically present in ng to µg per liter concentrations in

wastewater treatment plant (WWTP) influent and effluent, ECs have the potential to adversely impact ecology and human health, and are generally not monitored or regulated in surface waters (17). Their impacts on surface waters and wastewater treatment efficacy are poorly understood. Triclosan is one such contaminant that is used for household applications and is prevalent in wastewater.

Triclosan (TCS) is a broad-spectrum antimicrobial agent that has been incorporated into various personal care products. It is widespread in the environment—in the US, WWTP influent can contain up to 86 $\mu\text{g/l}$ TCS, surface river water up to 35 ng/l , and groundwater up to 2.1 $\mu\text{g/l}$ (15, 18, 19). At low concentrations, TCS is bacteriostatic, and at higher levels it is bactericidal (20). TCS is known to inhibit bacterial reproduction via inhibition of the enzymes responsible for cell membrane synthesis and repair (18, 21), and its aromatic structure suggests that it could also be a potent inhibitor of AMO.

Previous studies have indicated that TCS significantly inhibits nitrification in activated sludge, with as little as 1 mg/l causing nearly 20% inhibition (22). Inhibition of both AOB and NOB activity and growth has been observed (23, 24). However, detailed studies of the impacts of triclosan on specific bacterial species present in activated sludge are largely absent. In particular, the effects of TCS on pure cultures of *N. europaea* and *N. winogradskyi* are not well characterized. No studies have been conducted on the impacts of TCS on nitrifying biofilms.

The lack of information available on TCS impacts on nitrifying biofilms is particularly glaring, since biofilms can act as a major bacterial defense against antimicrobial compounds. Biofilms are dense communities of cells that are attached to a

surface and enclosed in an extracellular polymeric matrix (3). Both slower growth of cells within the biofilm and contaminant diffusion limitations increase bacterial resilience in the face of growth-inhibiting conditions (25). Wastewater treatment plants frequently use biofilm-based treatment methods for nitrification, and therefore it is important to determine if the effects of TCS on biofilm-grown cells will differ from those in planktonic treatment systems. Studies have shown that biofilms of *N. europaea* were less inhibited by the aromatic compounds nitrapyrin, toluene, and phenol than their planktonic counterparts (4, 5, 26). Since TCS is also aromatic, these studies suggest that inhibition by TCS may be reduced when cells are grown as a biofilm.

The prevalence of TCS in the environment and the lack of information on its impacts on wastewater treatment efficacy make investigating its effects important. Knowledge of its effects on two ubiquitous wastewater bacteria—*N. europaea* and *N. winogradskyi*—is largely undetermined, and therefore one objective of this study was to investigate the effect of triclosan on ammonia and nitrite oxidation by pure cultures of both of the aforementioned bacteria. The inhibition mechanism of triclosan with the AMO enzyme was also investigated. Thirdly, impacts of TCS on ammonia oxidation by biofilms and suspended cell cultures of *N. europaea* were compared to determine if attached growth had any impact on bacterial resistance.

Nitrification efficacy by planktonic activated sludge cells and biofilms during TCS exposure was also investigated in order to compare the response of mixed cultures to that of pure cultures, and oxygen consumption by nitrifying biofilms during triclosan exposure was considered. Finally, triclosan fate in all cultures and growth modes was

measured to determine if degradation was possible by any of the bacterial species of interest.

This thesis consists first of a literature review that explores current information on nitrifying biofilms and their role in wastewater treatment. Nitrification microbiology and inhibition mechanisms relevant to AOB and NOB are also examined. Finally, characteristics of triclosan and current research on its impacts are summarized, with particular attention paid to studies of its effects on nitrification in WWTPs. Experimental methods are then described, followed by results and a discussion of the results' relevance and applicability. Lastly, conclusions are presented.

CHAPTER TWO – LITERATURE REVIEW

Nitrogen Cycling

Nitrogen is an essential nutrient for all living organisms as a major component of protein and DNA. The atmosphere is composed of 78 percent dinitrogen gas, and primary producers utilize the nitrogen fixation pathway to convert atmospheric dinitrogen gas to ammonia and organic nitrogen. Ammonia can be oxidized to nitrite and subsequently nitrate during nitrification, a process performed by ammonia and nitrite oxidizing bacteria (27, 28). In denitrification, nitrate is reduced back to dinitrogen gas by way of nitrite, nitric oxide, and nitrous oxide, thus completing the conventional pathway of the nitrogen cycle. Ammonification, another dissimilatory process, occurs when organic nitrogen released from plant or animal decay or waste is converted to ammonium by bacteria. Assimilation occurs when plants or microorganisms uptake nitrogen ions such as nitrite, nitrate, and ammonium and use them for growth (29).

The conventional nitrification/denitrification pathway is well studied and the process is frequently used in wastewater treatment. However, microorganisms can use other pathways of nitrogen conversion. Discovered in 1994, anaerobic ammonia oxidation (anammox) is a shortcut of the conventional nitrogen cycle (30). Autotrophic anammox bacteria directly convert ammonium, nitrite, and bicarbonate to dinitrogen gas, thus bypassing several steps in the typical nitrification-denitrification process (31). Figure 1 summarizes the processes in the nitrogen cycle relevant to wastewater treatment.

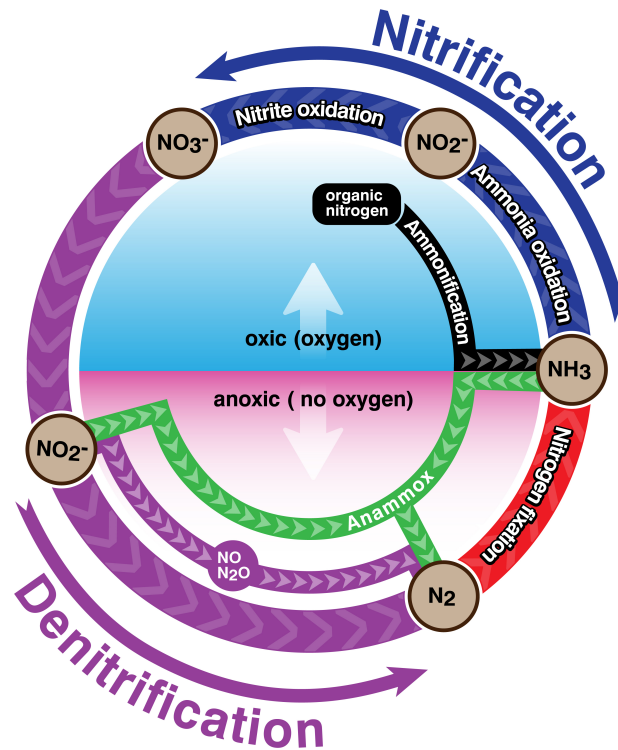


Figure 1. A simplified image of the nitrogen cycle as it applies to wastewater treatment. Illustration by Joseph Parchen.

Nitrification and Denitrification in Wastewater Treatment

Nitrogen enters wastewater via municipal sewage, industrial wastes, or urban or stormwater runoff. Municipal sewage contains high concentrations of ammonia (NH₃) and some organic nitrogen as products of ammonification and decay of organic matter. Industrial wastes and runoff typically contain higher concentrations of organic nitrogen, as well as ammonia and nitrates (1, 32). If left untreated, either form of nitrogen can cause negative impacts to natural water bodies and aquatic life—nitrate (NO₃⁻) release can result in algal blooms and subsequent oxygen depletion, thus leading to fish die off, and ammonia can be toxic to aquatic animals (1). For these reasons, nitrogen-containing

compounds must be removed in wastewater treatment plants (WWTP) to suitable levels before release to natural water systems.

Nitrogen removal at WWTPs is also regulated at the state and national level. The Environmental Protection Agency developed the National Pollutant Discharge Elimination System (NDPES) to control pollution from point sources (33). NDPES permits set effluent limits for various pollutants, as well as total maximum daily loads (TMDL), which are calculations of the maximum amount of a single pollutant that a surface water can receive and still meet water quality standards (34). Though TMDLs vary based on the receiving water body, total nitrogen limits typically range from 0.3 to 2 mg/l (34). Failure to meet nitrogen removal standards can result in large fines to WWTPs.

Wastewater treatment plants utilize microbiological activity to remove carbonaceous biological oxygen demand (BOD) and nutrients such as nitrogen and phosphorus from wastewater (35). Either suspended or attached growth processes can be used: suspended growth systems maintain microorganisms present as activated sludge for BOD and nutrient removal, and attached growth processes utilize biofilms for treatment. Attached growth systems will be discussed in more detail in the next section.

Choice of mechanical wastewater treatment system design depends on wastewater characteristics, the nature of influent contaminants, and the level to which these must be treated. Typically, when suspended growth systems are utilized, plug-flow, complete-mix, or batch reactors are employed (36). Conditions can be optimized for the desired treatment step: for successful nitrification by aerobic nitrifying bacteria to occur, aeration is necessary for oxygen addition. Denitrification by heterotrophic bacteria can only

proceed with a carbon source present and under anoxic conditions, as oxygen competes with nitrate reduction. Thus, spatial or temporal separation of the nitrification and denitrification treatment steps is required to allow for the sequential process to occur under aerobic and anoxic conditions.

One commonly used design that combines biological nitrogen removal with phosphorus removal is the Bardenpho process (33), which is particularly pertinent to this thesis—wastewater samples were obtained from the first aerobic zone of the Bardenpho WWTP in Bozeman, MT and used in subsequently described experiments. The Bardenpho process consists of five stages: fermentation, first anoxic, nitrification, second anoxic, and reaeration. A simple schematic of the process is shown in Figure 2. In the first stage, primary clarifier effluent enters the anaerobic fermentation chamber with recycled activated sludge from the secondary clarifier. The mixture of the two streams under anaerobic conditions strongly favors growth of phosphate-accumulating organisms (PAO), naturally occurring heterotrophic bacteria that uptake volatile fatty acids (VFAs) when oxygen is unavailable. Upon re-entering aerobic conditions in the second aerobic stage, the PAOs use intermediate products created from VFAs to uptake phosphorus, thereby removing it from wastewater (33, 35).

To achieve nitrogen removal, in the first anoxic stage of the Bardenpho process recycled nitrate-rich liquor from the aerobic zone is combined with fermentation chamber effluent with high chemical oxygen demand (COD). The excess COD addition from the fermentation chamber promotes consumption of the remaining oxygen, thereby providing excellent anoxic conditions for denitrification. Approximately two-thirds of influent

nitrogen is removed in this stage (37). Next, oxygen is introduced to the chamber in the first aerobic stage, and nitrification of influent ammonia occurs concurrently with phosphorus uptake. The second anoxic stage is used to reduce any remaining nitrate via heterotrophic denitrification, and a carbon source can be added here, if necessary. Finally, the second aerobic stage is used to strip any nitrogen gas from solution and to prevent phosphorus release by PAO, which could occur under anoxic conditions (33, 35). This process can produce effluent containing only 3-5 mg/l total nitrogen (35).

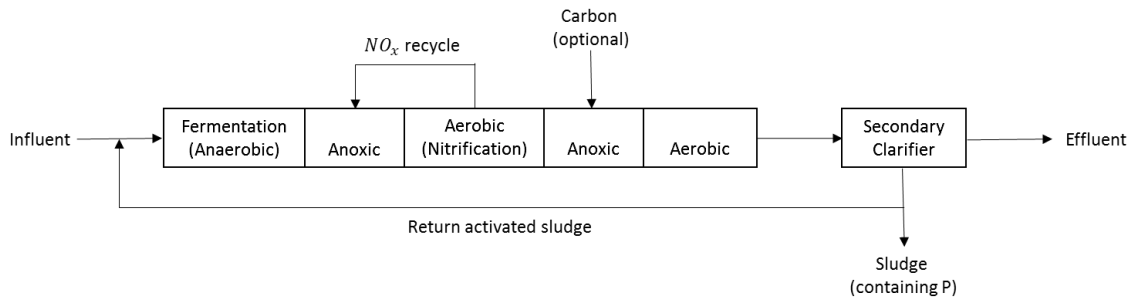


Figure 2. Five-stage Bardenpho process (35).

Biofilm-Based Wastewater Treatment Technologies

Bacterial cultures can grow either as planktonic (suspended) cells or as biofilms, dense communities of cells attached to a surface and enclosed in an extracellular polymeric substance (EPS) matrix (3). Planktonic cells tend to experience faster growth than biofilms, as they are not limited by substrate diffusion through the EPS matrix (5). Both of these qualities—faster growth and more rapid substrate diffusion—cause planktonic cells to typically be less resistant to antimicrobial compounds than biofilms (25). Previous research has found that biofilms of the nitrifying bacterium *Nitrosomonas*

europaea were less inhibited by nitrapyrin, phenol, and toluene than suspended cells, in part due to slower metabolism of biofilm cells (4, 5, 26).

In wastewater treatment plants, attached growth processes are utilized in order to obtain effective biodegradation. Faster growing microorganisms are found in the surface layers of biofilms, thereby protecting the slower growing microbes living in the subsurface layers. The retention of attached bacteria on reactor surfaces or carrier particles prevents washout of slow growing organisms, such as autotrophic nitrifying bacteria, and thus allows for shorter hydraulic retention times and higher treatment capacity (38). Because nitrifiers have a much slower growth rate than heterotrophs, nitrification is not favored until carbonaceous BOD concentrations drop and nitrifiers can outcompete heterotrophs for the dissolved oxygen (DO) at the outer layer of the biofilm (39). At this point, nitrifying organisms are then found in the outer layers of the biofilm, where oxygen is readily available. Depending on biofilm thickness, anoxic conditions at the center of the biofilm can exist due to oxygen depletion, favoring growth of denitrifying bacteria. Anaerobic conditions even deeper in the biofilm can encourage growth of fermenting and methanogenic anaerobes (39, 40).

Since the early 1900s, trickling filters have been used for wastewater treatment, and today a multitude of options exist for other forms of biofilm-based treatment. Fluidized bed, moving bed, airlift suspension, membrane biofilm, and granular sludge reactors are just a few examples of technology used for biofilm treatment (29, 38, 40). Granules, or small aggregates of microorganisms that form a biofilm unattached to a surface, are also commonly used in wastewater treatment (40).

Trickling Filtration Systems: Trickling filters (TF) use a form of non-submerged attached growth in which wastewater is applied continuously to the top of a vessel containing a rock bed or highly designed plastic packing media. Wastewater flow is gravity driven and unsaturated, thus providing high oxygen transfer. Developments in plastic packing material design have resulted in high specific surface area, and allow effective air ventilation to facilitate high levels of aerobic biofilm growth. Nitrifying biofilms growing on the packing material consume oxygen and ammonia present in the wastewater, converting it to nitrate for downstream denitrification. Trickling filtration systems can perform nitrification, remove up to 90% of the biological oxygen demand (BOD), and provide a low energy, low maintenance option for biofilm treatment (40-42). Nitrification efficiency increases as BOD₅ loading decreases, with up to 100% nitrification occurring at a loading rate of 0.08 kg BOD₅ m⁻³ d⁻¹ and 0.2 g NH₃ m⁻² d⁻¹ (41).

Unfortunately, trickling filters are sensitive to low temperatures and tend to produce effluents with higher suspended solids concentrations (40, 41, 43). Predatory macro fauna are also prevalent in these systems and can negatively impact nitrification: snails, in particular, can consume enough nitrifying bacteria to reduce treatment efficiency (42). Control of these macro fauna is possible with chemical treatment, but temperature, pH, and alkalinity adjustment tend to also inhibit nitrifier activity (41). Mechanical treatment methods such as flooding or hydraulic flushing have been used successfully, but results vary—one study observed that flushing had no impact on fly and worm removal in a pilot scale TF (44).

Fluidized Bed Bioreactors (FBR): FBRs use submerged biofilms to perform nitrification, phosphorus removal, hazardous substance removal, and even denitrification in some situations (40, 45). Influent and recycled aerated wastewater are fed in an upflow configuration through a 3-4 m deep fluidized bed of carrier media (38). Biomass that develops on the bed media removes contaminants, and treated effluent flows off the top of the reactor. The type of solid media used for biofilm attachment and growth can greatly impact nitrification efficacy; studies have found 48% ammonium removal in FBRs with sand (3.8 hour hydraulic retention time or HRT) (46), 95% removal with soil (20 hour HRT) (47), and 98% removal in beds with lava rock media (2 hour HRT) (45). Both soil and lava rock have greater effective surface areas than sand, which together indicates that carrier media with higher effective surface area generates higher nitrification efficiency, likely because increased surface area provides more opportunities for biofilm attachment.

FBRs provide long retention times for contaminant treatment and are very resistant to shock loads (40). High levels of total suspended solids (TSS) and COD removal are also possible (40). Non-biodegradable contaminants can adsorb onto the filtration media, thus providing a method for recalcitrant substance removal. Ammonia can also adsorb onto carrier media, thereby allowing a longer retention time for degradation to occur (40).

Unfortunately, the high recycle flow and aeration rates required by FBRs result in large energy requirements. Control of biomass growth can also be challenging (40). However, these reactors do not have the issues with macro fauna that are experienced in

trickling filters, and the concentration of biomass is much greater than that in conventional activated sludge reactors. Shear forces exerted by fluidization also prevent clogging of porous media and increased head loss caused by bacterial biofilm overgrowth on the media (48).

Moving Bed Biofilm Reactors (MBBR): Moving bed biofilm reactors utilize various forms of carriers to maximize active biofilm surface area. Sponges, rope, and plastic carriers of multiple shapes are all used for biofilm attachment (40). Because carriers greatly increase the effective surface area available for biofilm growth, MBBRs require much less space than traditional activated sludge reactors (40, 49). They also do not require backwashing and are less susceptible to clogging than reactors using finer carrier media (49). Depending on wastewater qualities, high fractions of ammonium removal are possible: influent ammonium in a landfill leachate was reduced by 88% using an MBBR (HRT = 1.25 days) (50), and 94% of ammonium in a municipal wastewater was removed via a MBBR with an HRT of 6 hours (51). These efficacies and HRTs are on par with those observed in some FBRs (45-47).

Process disadvantages include an initial startup period to obtain adequate biofilm growth and head loss during flow through the carriers. Aeration requirements also increase energy demands. Altogether, however, MBBRs have a high capacity for BOD removal and nitrification (40, 49).

Biofilm Airlift Suspension Reactors (BAS): A BAS reactor consists of two concentric columns: a riser and downcomer. The riser is located inside of the downcomer column, and both are filled with carrier media such as sand, activated carbon, or ceramic

particles (38, 52). Air is sparged through the riser section of the reactor, causing circulation of wastewater, carrier media, and oxygen. Both air and wastewater enter at the bottom of the reactor. A separator is located on top of the reactor to prevent carriers from leaving the reactor and to allow treated wastewater to exit. Carrier material provides a large surface area for biofilm growth, and thus high loading rates and short retention times are possible, at times as short as 0.5 hours (38, 52). These systems provide excellent BOD removal and nitrification and can also be used for denitrification by recirculating gases produced by biological growth (such as carbon dioxide) rather than air (38, 40, 52). Multiple studies of pilot scale BAS reactors have observed nearly 100% removal of influent ammonium, with HRTs varying from 1.8 to 7.2 hours (53-55).

Disadvantages to BAS reactors include long start-up times, design complexity, and biofilm sloughing from carrier material (55, 56).

Membrane Biofilm Reactors (MBR): This form of reactor utilizes membranes to deliver a gaseous substrate, such as oxygen or hydrogen, to a biofilm growing on the membrane surface (40). The membrane's lumen can be pressurized with a gas, which diffuses through the membrane wall to the attached biofilm (57). Nutrients in the bulk liquid then counter-diffuse through the biofilm, thus allowing high fluxes and efficient wastewater treatment, even in sub-optimal conditions. Oxygen concentrations are highest and BOD is lowest at the biofilm-membrane interface, and therefore nitrifiers dominate in the deepest areas of the biofilm. In the outer layers of the biofilm, oxygen concentrations are lowest, but BOD is highest, and heterotrophs dominate. Nitrate diffusing out of the biofilm can be used as an electron acceptor by heterotrophs for

denitrification (58). Ammonium is present in highest concentrations at the outer edges of the biofilm, and therefore must diffuse through the biofilm to reach nitrifiers at the base. Altogether, effective nitrification in MBRs is possible in the presence of BOD due to diffusion limitations between nitrifiers growing in the inner layers of the biofilm and heterotrophs in the outer layer, as well as heterotrophic consumption of BOD (58).

The high gas-transfer efficiency of these systems greatly reduces operating costs and also prevents stripping of volatile organic carbon, which can occur in traditional bubbling systems (57). Unfortunately, biofilms can accumulate excessively, resulting in head loss, short circuiting of flow, and mass transfer limitations, and therefore biofilm accumulation must be controlled carefully (57). This can be done using high shear conditions or gas sparging (57). MBRs can be used for concurrent carbon removal, nitrification, denitrification, or anaerobic ammonia oxidation, depending on the growth conditions supplied (40, 57). In conjunction with activated sludge systems, concomitant nitrification and denitrification are possible: planktonic heterotrophs reduce nitrate produced by membrane-attached nitrifiers, thereby achieving total nitrogen removal in a single tank (59, 60).

Granular Sludge Reactors (GSR): An aerobic GSR uses dense granular biomass aggregates to treat wastewater. This granular biomass behaves as a biofilm, with substrate diffusing into the roughly spherical shape of the granules (40). The high density of the aerobic granules leads to excellent settleability and an ability to withstand high organic loads and toxicity (61). Aerobic granules can also perform simultaneous nitrification, denitrification, and phosphorus removal, as both aerobic and anoxic zones exist inside the

same granule—aerobic, nitrifying bacteria will dominate in the outer layers of the aggregate, while denitrifiers and PAOs will dominate in the center (61, 62). Oxygen saturation levels as low as 20% have been shown to allow simultaneous removals (63, 64), and aerobic granules are capable of nitrogen removal at low temperatures (65). However, reactor start up at temperatures below 8°C has been found unfeasible (65).

Altogether, GSRs have higher treatment capacities over a smaller footprint than traditional activated sludge systems and require shorter hydraulic retention times, though post treatment is generally required to obtain low effluent suspended solids (40, 66). Filamentous bacteria can also overgrow in aerobic GSRs, unfortunately, leading to destabilization of the granules and treatment failures (67). Overgrowth can be caused by multiple different conditions, but long solids retention times, and stress conditions, such as low dissolved oxygen, high temperatures, nutrient deficiencies, and low substrate availability all have been found to favor filamentous bacterial growth (64, 67).

Microbiology of Nitrification

Biological removal of nitrogen from wastewater requires a sequence of microbially-driven processes. Ammonia oxidizing bacteria (AOB) perform the first step in nitrification, in which ammonia is converted to nitrite (NO_2^-) under aerobic conditions. This process requires two steps: the membrane-bound enzyme ammonia monooxygenase (AMO) catalyzes NH_3 oxidation to hydroxylamine (NH_2OH). NH_2OH is then oxidized to NO_2^- in the periplasmic space using the hydroxylamine oxidoreductase (HAO) enzyme (27). AMO requires an input of two electrons for oxidation of ammonia, and the entire

process results in the release of four electrons and five protons. Two of the electrons released are then returned to AMO to support further ammonia oxidation, and two are used to generate ATP and provide reductant for other cellular processes (27).

Nitrosomonas, *Nitrosococcus*, and *Nitrospira* are all common AOB genera, though *Nitrosomonas* dominates in wastewater treatment (27, 68). Figure 3 shows the reactions in the NH_3 oxidation process performed by AOB.

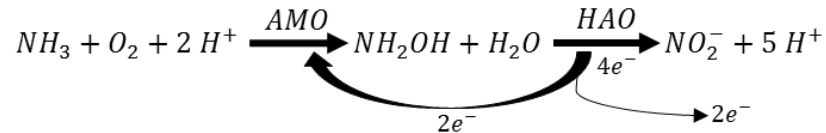
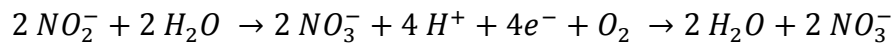


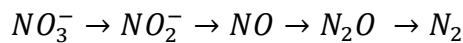
Figure 3. Process of ammonia catabolism in AOB and electron flow (27).

Nitrite oxidizing bacteria, or NOB, then convert nitrite to nitrate, thus performing the final step in the nitrification process. Several bacterial genera have been found capable of this process: *Nitrobacter*, *Nitrococcus*, *Nitrospina*, *Nitrospira*, and *Nitrotoga* (28). *Nitrobacter* and *Nitrospira* dominate in wastewater treatment under high and low substrate conditions, respectively (68). Under oxic conditions, NO_2^- is converted to NO_3^- using the nitrite-oxidizing enzyme NXR. The chemical equation for nitrite oxidation can be seen below (28):



Under anoxic conditions, nitrate is converted to inert dinitrogen (N_2) gas (denitrification) by heterotrophic bacteria, including *Achromobacter*, *Chromobacterium*, *Methanomonas*, or *Rhizobium* (35). The simplified chemical equation for this process can be seen below (35). Sequentially, nitrification and denitrification result in successful nitrogen removal from wastewater. Nitrification requires aeration: at least 4.57 g O_2 per g

NH₄-N (68). Denitrification requires COD at a ratio of roughly 4.16-4.45 g O₂ per g N when methanol is used, though this ratio is dependent on the carbon source used, temperature, and microbial populations (69).



Despite the overall success of WWTP systems, nitrous oxide (N₂O) can at times be emitted during the nitrogen removal process. Nitrous oxide is a powerful greenhouse gas with a 300-fold stronger effect than that of carbon dioxide (70). Both nitrification and denitrification processes can produce nitrous oxide, but research suggests that nitrifiers, particularly AOB, are responsible for the majority of nitrous oxide release (71-73). One survey found that the following operational parameters resulted in higher nitrous oxide production: low dissolved oxygen and high nitrite concentrations in both nitrification and denitrification stages, and low chemical oxygen demand (COD) to nitrogen ratios during denitrification (70).

Nitrifying Biofilms

Multiple populations of AOB and NOB can co-exist in nitrifying biofilms. Various studies have found that AOB and NOB populations are stratified and heterogeneous throughout biofilm layers, with most species existing in microcolonies (74-79). Both AOB and NOB are present in the aerobic region of the biofilm, though AOB tend to dominate in slightly more aerobic layers than NOB. *Nitrosomonas* (particularly *N. europaea* and *N. eutropha*) and *Nitrobacter* species are typically the dominant AOB and NOB in biofilms, respectively, though growth conditions can have a large impact on populations (74-76). Gieseke et al. found that colonies of the AOB

Nitrosococcus mobilis were surrounded by dominant cells of *Nitrosomonas* in upper layers of a wastewater biofilm, with the dominant NOB *Nitrobacter* and *Nitrospira* slightly lower (74). AOB are generally present in higher fractions than NOB, likely because of their faster growth rate (74, 78).

Oxygen and substrate concentrations have a large impact on community structure of both AOB and NOB in biofilms. Satoh et al. showed that *N. europaea* tended to dominate when ammonium concentrations were high (77), but Okabe et al. found that colony sizes decreased in the presence of organic carbon due to competition with heterotrophic organisms (78). In low oxygen conditions, *Nitrospira* outcompeted *Nitrobacter*, likely due to its lower affinity for oxygen (76). If organic carbon is available, heterotrophic bacteria are typically present in the uppermost, oxygen-rich area of biofilms because they can out-compete nitrifiers for oxygen. However, nitrifiers can efficiently scavenge oxygen, and therefore have an advantage at slightly lower oxygen concentrations (77).

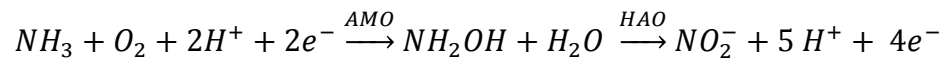
Nitrosomonas europaea

One AOB ubiquitous in microbiological wastewater treatment is *Nitrosomonas europaea*. *N. europaea* is a gram-negative, obligate chemolithoautotroph that obtains its carbon from carbon dioxide and energy for growth and metabolism from ammonia oxidation (6, 27, 80). *N. europaea* is therefore a fairly slow growing microorganism (7-8 hour doubling time (27)), and is sensitive to changes in its growth environment, such as temperature, pH, or contaminant introduction (3-8). It is a well-studied organism on

which most knowledge of AOB is based, and its genome was sequenced in 2003 (2, 80).

This makes it an excellent model organism for nitrification research.

N. europaea performs ammonia oxidation according to the pathway previously described and utilized by all AOB (27). As ammonia oxidation is the first step in the entire nitrification process, the presence of actively respiring AOB is critical for successful nitrogen removal from wastewater. The chemical equation for ammonia oxidation by *N. europaea* can be seen below (5):



Unfortunately, the AMO enzyme is non-specific and is capable of oxidizing a wide range of aliphatic and aromatic hydrocarbons (3-5, 9). It can also oxidize carbon-hydrogen bonds to alcohols, carbon-carbon double bonds to epoxides, and sulfides to sulfoxides (2, 3, 9), and therefore allows many potential opportunities for competition with and inhibition of the ammonia oxidation process during wastewater treatment. Other compounds may interact with AMO, particularly in the following ways: direct binding with the enzyme's active site, thus blocking ammonia from binding, or depletion of the reductant (e^-) supply to AMO, as oxidation of interfering compounds will not resupply reductant via subsequent oxidations (9). For these reasons, contamination of wastewater by aliphatic or aromatic compounds can result in incomplete or inefficient nitrification, leading to inefficient nitrogen removal. Aromatic contamination typically comes from industrial wastewater, but emerging contaminants, such as pharmaceuticals, antimicrobial agents, or other personal care products can also contain aromatic groups. Since these

compounds typically enter wastewater as a result of household or pharmaceutical use, it is important to understand their impacts on nitrification and AOB in particular.

Cometabolism by *N. europaea*. Cometabolism is the biological transformation of a non-growth-supporting compound by non-specific enzyme, such as AMO (10). Vanelli et al. showed that cometabolism of various halogenated methanes, ethanes, and ethenes was possible by *N. europaea* (11), and Wahman et al. demonstrated AMO cometabolism of four trihalomethanes commonly found in treated drinking water (12). Cometabolism can degrade compounds to either more or less toxic forms—for example, cometabolism of the carcinogenic groundwater contaminant trichloroethylene (TCE) by *N. europaea* results in formation of harmless carbon dioxide and chlorine ions (10). However, TCE competitively inhibits the AMO enzyme, thus interfering with ammonia oxidation. *N. europaea* was also found capable of a limited amount of TCE oxidation before irreversible inactivation of ammonia oxidation occurred (10). Altogether, cometabolism by AMO can have positive implications for contaminant remediation, but not for NH₃ treatment, and therefore growth conditions for the desired oxidation must be carefully tailored in order to focus the AMO enzyme.

Nitrobacter winogradskyi

N. winogradskyi is a nitrite oxidizing bacterium that, like *N. europaea*, is ubiquitous in wastewater treatment. Like other NOB, it performs the second step in the nitrification process and is a facultative chemolithoautotroph that derives its energy from the oxidation of nitrite to nitrate and carbon from carbon dioxide (81). *N. winogradskyi* is

typically used as the model bacterium for nitrite oxidizers, though research on it, and NOB in general, is not as well-developed as that on *N. europaea* (13). Its genome was sequenced in 2006 (81).

N. winogradskyi uses the membrane-associated enzyme nitrite oxidoreductase (NXR) to catalyze nitrite oxidation (13). Little is known about inhibition of NXR, or inhibition of *N. winogradskyi* growth in general, though the NXR enzyme belongs to a family of enzymes known to at times have broad substrate specificity (14). If this is the case for NXR, cometabolism of substrates other than nitrite may be possible, leading to inhibition of growth and nitrification.

Reversible Inhibition Mechanisms

The wide potential for inhibition of non-specific enzymes such as AMO makes the quantification of inhibitory impacts important. Enzymatic reactions can be modeled mathematically in order to quantify reaction rates and impacts of an inhibitor on a reaction rate, such as that of aliphatic or aromatic compounds on the AMO enzyme. Rate constants are altered in the presence of an inhibitor and modification of the Michaelis-Menten (M-M) equation can be used to describe the different ways in which inhibitors affect reaction rate. The M-M equation is seen below, where v is reaction rate, S is substrate concentration, and K_m is the Michaelis-Menten constant, or the substrate concentration at which the reaction rate is at half of the maximum rate, or V_{max} (82).

$$v = \frac{V_{max} S}{K_m + S}$$

Enzyme inhibition can occur via a variety of mechanisms, depending on the inhibiting compound and enzyme. Inhibitor-enzyme binding may be reversible or irreversible: irreversible inhibition occurs when the inhibiting compound forms a covalent bond with the enzyme, resulting in a permanent deactivation (83). Reversible inhibitors form complexes with the enzyme that have different catalytic properties than those of the native enzyme (82). Reversible inhibitors can be released from the enzyme, allowing recovery of enzyme activity. Several forms of reversible inhibition are possible: competitive, uncompetitive, non-competitive, or mixed (82).

Competitive inhibition occurs when the substrate and inhibitor compete for the same enzyme binding site—for example, many inhibitors of the AMO enzyme compete with ammonia for the enzyme active site (3, 9). An enzyme-inhibitor complex forms reversibly, resulting in an increase in the Michaelis-Menten constant K_m by a factor of $\left(1 + i/K_{ic}\right)$, where i is the inhibitor concentration and K_{ic} is the competitive inhibition constant (82).

Uncompetitive inhibition occurs when the inhibitor binds with the enzyme-substrate complex instead of the free enzyme. This results in a decrease in both the reaction rate V_{max} and K_m by a factor of $\left(1 + i/K_{iu}\right)^{-1}$, where K_{iu} is the uncompetitive inhibition constant (82).

Non-competitive inhibition occurs when an inhibitor is capable of binding with both the free enzyme and the enzyme-substrate complex with the same affinity (83). This form of inhibition generally only occurs with small inhibitors, such as protons, metal ions and small anions, and is a special case of mixed inhibition (82). In pure non-competitive

inhibition, only the rate of reaction varies with inhibitor concentration and K_m is unaffected: V_{max} decreases by a factor of $\left(1 + i/K_{iu}\right)^{-1}$ where K_{iu} is the enzyme-substrate-inhibitor constant (82). Mixed inhibition occurs when the inhibiting compound can bind with both the free enzyme and the enzyme-substrate complex, but binding occurs with different affinities (82, 83).

AMO has been shown to experience irreversible, competitive, and non-competitive inhibition by a variety of inhibitors. As mentioned previously, Hyman et al. found TCE to be a reversible, competitive inhibitor of the AMO enzyme during 10-minute exposure experiments (10). However, Rasche et al. observed irreversible inhibition of the AMO enzyme by TCE during one-hour exposure tests, with one exception: *N. europaea* cells exposed to the lowest TCE concentration were capable of 20% recovery of ammonia oxidizing activity after exposure, indicating some reversible inhibition or synthesis of new enzyme (7). This shows that the exposure time and dosage can affect the mechanism of inhibition of the same enzyme.

McCarty lists over 40 compounds known to competitively inhibit AMO, such as methanol, benzene, toluene, and cyclohexane (84). Keener and Arp found that the competitive character of AMO inhibition decreased as inhibitor size increased, with non-competitive inhibition occurring with more hydrophobic compounds and hydrocarbons with over two carbon atoms (3). Because AMO can be inhibited by such a variety of compounds, with structures that are ubiquitous in the environment, contamination of wastewater by these compounds can readily cause AMO inhibition. Emerging

contaminants, prevalent in wastewater and typically of an aliphatic or aromatic nature, therefore have a large potential for AMO inhibition.

Emerging Contaminant: Triclosan

Introduction to Emerging Contaminants and Triclosan Properties

As stated above, many contaminants in wastewater can interact with or be cometabolized by AOB, including compounds classified as emerging contaminants. The US Geological Survey defines emerging contaminants as “any synthetic or naturally occurring chemical... that is not commonly monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects” (17). Emerging contaminants largely enter the environment via discharge of treated wastewater (15, 16). They generally consist of pharmaceutical compounds and personal care products and are found in the ng to µg per liter range in surface waters (16). Not only are the impacts of these pervasive contaminants poorly understood in surface waters, their effects on wastewater treatment efficacy are ill-defined as well (15-17). Of large concern is the ability of these compounds to increase antibiotic resistance in bacteria, as well as partition to solids during wastewater treatment or cause toxicological responses in animals and wastewater bacteria (16). One such contaminant commonly used in household applications is triclosan.

Triclosan (TCS) is a broad-spectrum antimicrobial agent that has been used in the US since the 1960s (24). It is incorporated into various personal care products, such as

deodorants, soaps, lotions, and toothpaste. It is also used as a plastic or fabric coating in household and industrial products to prevent bacterial growth.

TCS is a halogenated biphenyl ether (Figure 4) with chemical formula $C_{12}H_7Cl_3O_2$. It is a white crystalline powder that is thermally stable, non-volatile, and has low solubility in water, though it is readily soluble in various organic solvents. Properties can be seen in Table 1 (18, 24).

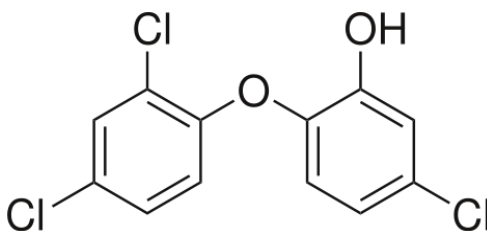


Figure 4. Triclosan structure (18).

Chemical Property	Value
IUPAC name	5-chloro-2-(2,4-dichlorophenoxy)phenol (18)
Molecular Weight	289.55 g/mol (18)
Melting Point	56-60 °C (18)
Boiling Point	280-290 °C (18)
Aqueous Solubility	10 mg/l at 20 °C, log K_{ow} = 4.76 (18)

Table 1. TCS properties and values (18).

Toxicity

TCS has a low level of toxicity to humans and animals. The oral LD50 (median lethal dose) for mice is 4,000 mg/kg (18). However, TCS has been found to be

chronically toxic to fish and algae between 34.2 and 200 µg/L, depending on species and life stage (85, 86).

Studies have also suggested that TCS can behave as a thyroid disruptor due to its structural similarity to other thyroid hormones (87). Thyroid disruptors interfere with human growth and development, particularly in children (18). Studies have also shown triclosan to have androgenic and estrogenic activities in human breast cancer cells and fish (88), and to lower testosterone levels in male rats (89, 90).

Occurrence in Wastewater and the Environment

Because triclosan is such a prevalent antimicrobial, it readily enters water bodies via municipal or industrial wastewater, as well as from landfill leachate or contaminated runoff. A US Geological Survey study reported that 58% of 139 surveyed streams contained triclosan from 0.14 to 2.3 µg/L (91). Surface river water has been found to contain 3.5 to 34.9 ng/L (18), and groundwater 7 to 2,110 ng/L (15). River, lake, and other marine sediments have also been found to contain triclosan. A study of seven WWTPs found that, of the TCS entering plants, 79% is biodegraded, 15% is adsorbed onto biosolids, and 6% is discharged into water bodies (92). Wastewater influent and effluent in the US has been found to contain 0.245-86.2 µg/L and 0.05-5.37 µg/L triclosan, respectively (18, 19). Activated sludge from WWTPs has also been found to contain 0.5-15.6 mg/kg dry weight TCS (19). When these biosolids are applied to fields for agricultural amendment, studies have found TCS present in the crops—one study found 13 to 136 nanograms of TCS present per gram of dry soybean weight (93).

Triclosan has also been detected in the human body—specifically, at a concentration of 2.4-3,790 µg/L in 75% of 2,517 urine samples (94), and from 4.1-19 ng/g in blood serum samples (95). It has been found in dairy products and meats from 0.02-0.15 ng/g and in indoor dusts at a concentration of approximately 1.1 µg/g (18, 96).

Mechanism of Action and Resistance

At low concentrations (typically 0.1-1 mg/l), triclosan acts a bacteriostatic compound, meaning that it prevents bacterial reproduction (20). It is bactericidal at higher concentrations (20). Typically, triclosan prevents bacterial reproduction by inhibiting the active sites of the fatty acid biosynthetic enzyme *enoyl-acyl* carrier protein reductase (Fab1), thereby preventing cell membrane synthesis and repair (18, 21). It has also been found to target the enoyl-reductase InhA protein in *Mycobacterium smegmatis* (20). The minimum inhibitory concentration (MIC) of TCS for *Escherichia coli* growth is 0.8 mg/l (97) and ranges from 0.025 to 1 mg/l for *Staphylococcus aureus* (98).

Triclosan has been shown to act as a tight-binding, reversible inhibitor of the Fab1 enzyme in *E. coli* (99), while other studies have observed both competitive and uncompetitive inhibition of Fab1 in *E. coli* (21). TCS inhibition has also been observed to occur reversibly in *N. europaea* (100), with several studies suggesting competitive inhibition of the AMO enzyme (24, 101, 102). It is important to note, however, that these studies do not experimentally show competitive inhibition, but rather infer it from observations of inhibitory impacts of TCS on nitrite production.

At bactericidal levels, TCS causes membrane damage or rupture. Its sorption follows a “Z pattern” (Figure 5)—initial adsorption is slight, then increases with

increasing concentration, until reaching a critical applied concentration which causes cell lysis and TCS release (103, 104). Interestingly, some bacteria are resistant to triclosan (20).

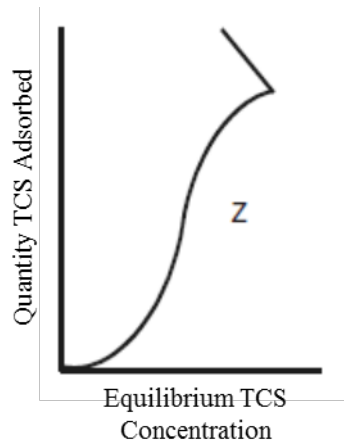


Figure 5. Sorption isotherm for triclosan uptake [20].

Degradation Products

Triclosan can be degraded to more toxic products via photo- or biodegradation. Methyl-triclosan can be formed under aerobic and anaerobic conditions, which can bioaccumulate in wildlife and humans (105-108). Photodegradation of TCS can result in formation of 2,8-dichlorodibenzo-p-dioxin (2,8-DCDD), a highly carcinogenic compound (109). Reaction with chlorine or chloramines can cause formation of the EPA priority pollutants 2,4-dichlorophenol (2,4-DCP) and 2,4,6-trichlorophenol, as well as chloroform in some cases (110-112).

Degradation of TCS in WWTPs has been observed. Studies of two WWTPs in Germany measured increases in methyl-triclosan concentrations as TCS concentrations decreased, particularly after contact with aeration basins, suggesting aerobic

microbiological TCS transformation (107, 108), though there is no detailed research on the mechanism of TCS methylation (113). Butler et al. hypothesized that the conversion was a defense mechanism against TCS toxicity, as methylation decreases the toxic effects of TCS (114). Experiments with aerated activated sludge and TCS observed that approximately one percent of TCS was converted to methyl-TCS, and 75-99% of TCS was removed by biological degradation over a 10-day period (107). Gas chromatography/mass spectrometry (GC/MS) analysis of wastewater effluent from three WWTPs in Hong Kong detected 2,4-DCP, 2,8-DCDD, and methyl triclosan at concentrations ranging from 10-20 ng/l (115). Influent TCS concentrations ranged from 300-500 ng/l (115). 2,8-DCDD was only detected in a plant using UV disinfection, and 2,4-DCP was found in plants using chlorine disinfection (115). Again, methyl triclosan was observed in activated sludge and effluent from aeration basins (115).

Regulations

Despite its persistence in the environment and potential impacts on human health and aquatic ecosystems, triclosan is not regulated in surface waters in the US, though its use as a pesticide is regulated by the EPA and non-pesticide usage is regulated by the Food and Drug Administration (18). In Canada and Japan, it has been banned, and categorized as a “dangerous irritant to the environment and aquatic life” in the European Union (18). Germany has banned its use in food plastics (18).

Impacts of Triclosan on Nitrification in WWTPs

As previously mentioned, aromatic compounds have the ability to interfere with the AMO enzyme, thus inhibiting nitrification by AOB. When these compounds are present in wastewater, successful nitrification, and therefore nitrogen removal, can become jeopardized. Previous studies have shown that nitrification is sensitive to various aromatic wastewater contaminants, such as phenol, toluene, oxytetracycline, chlortetracycline, tiamulin, and streptomycin (4, 5, 116). Because triclosan is both an aromatic compound and found ubiquitously in wastewater, it has an immense potential to adversely impact nitrification.

Previous studies have found that TCS significantly reduces nitrification in WWTPs—concentrations as low as 1 ppm have been shown to cause 19.3% reduction (22). Nitrite oxidation was completely prevented in activated sludge with TCS concentrations greater than 6 ppm (23). A TCS concentration of 2 ppm has been found to decrease ammonia oxidation in activated sludge by up to 70% (24). In activated sludge capable of degrading TCS to its byproducts, an 80% decrease in nitrifying activity was observed after exposure to 2 ppm TCS (24). Pure *N. europaea* cultures were not capable of any recovery in ammonia oxidizing activity after degradation of 2 ppm TCS (24). No studies on the impacts of triclosan on nitrifying biofilms have been reported.

Summary

The prevalence of triclosan in wastewater and the environment makes understanding its impacts an important task. Few studies have investigated its effects on

nitrifying bacteria and thereby on the efficacy of biological nitrogen removal in wastewater treatment, and no studies have examined the impact on attached growth systems of nitrifying bacteria. Different bacterial growth modes (biofilm or suspended) have the potential to experience different levels of inhibition by triclosan. The mechanism of this inhibition is also unknown—sorption, interaction with the AMO enzyme, or lysis may be responsible for inhibition of the nitrification process. Investigating these unknowns can provide knowledge about more effective wastewater treatment in the presence of TCS, thus preventing possible nitrogen release to water bodies, and perhaps lead to greater TCS regulation in the US.

The goal of the research described herein was to evaluate and compare the impacts of triclosan exposure on biofilms and suspended pure cultures of the wastewater bacterium *N. europaea*. Impacts on suspended and biofilm cultures of aerobic activated sludge were also investigated, as well as impacts on suspended cultures of *N. winogradskyi*. In particular, the inhibition of nitrifying activity was considered, with attention also given to effects of TCS on oxygen consumption in nitrifying biofilms and the mode of interaction between TCS and the AMO enzyme in pure cultures. It was hypothesized that TCS would negatively impact nitrification rates in all experiments, but that suspended cells would experience greater inhibition due to the previously mentioned inherent resistance of biofilms to antimicrobial compounds (25). It was also hypothesized that TCS exposure would decrease oxygen fluxes into biofilms, because its presence would decrease nitrification activity and thereby lessen oxygen consumption. To investigate these hypotheses, the experimental approach measured nitrification rates and

TCS loss in pure culture studies and activated sludge tests, in addition to biofilm studies performed in flow cells.

CHAPTER THREE - MATERIALS AND METHODS

Triclosan Preparation

Triclosan was purchased from Alfa Aesar at 99% purity. A 150 mM stock solution of triclosan was prepared in dimethyl sulfoxide (DMSO), which was diluted to a 1000 ppm stock solution, also in DMSO. Triclosan standards for high performance liquid chromatography (HPLC) analysis were prepared in glass vials and diluted in deionized water, batch, and biofilm growth medium using the 1000 ppm solution. The 1000 ppm solution was also used as stock solution for all exposure experiments. Standard solutions were stored in a 5°C refrigerator, and stock solutions were stored in a fume hood at 25°C.

HPLC Analysis

An Agilent 1100 Series HPLC was used to detect changes in TCS concentrations during exposure experiments. An Agilent Eclipse XDB-C18 4.6 mm x 150 mm column with 5 µm pore size was used with an Agilent Zorbax XDB-C18 guard column, dimensions 4.6 mm x 12.5 mm with 5 µm pore size. The mobile phase consisted of 70% acetonitrile and 30% nanopure water at a flow rate of 1 ml/min, and the sample injection volume was 100 µl. The column temperature was set to 30°C. An Agilent UV diode array detector was used to quantify TCS concentrations.

Triclosan had a consistent retention time of approximately 6 minutes and peak area at a wavelength of 210 nm was used to determine TCS concentration. Standards

were prepared by diluting the 1000 ppm TCS stock solution in experimental medium and stored at 5°C in amber 1.5 ml glass vials with screw caps fitted with silicon septa.

Because triclosan sorbs strongly to plastic and some experiments required sample processing in plastic microcentrifuge tubes, a calibration curve was created from both centrifuged and non-centrifuged standards (Figure 6). TCS concentrations in samples that had been exposed to plastic could then be more accurately calculated, as the loss due to sorption was standardized in the calibration curve. Centrifuged standards were spun at 9000 x g for 10 minutes and then immediately transferred to glass vials.

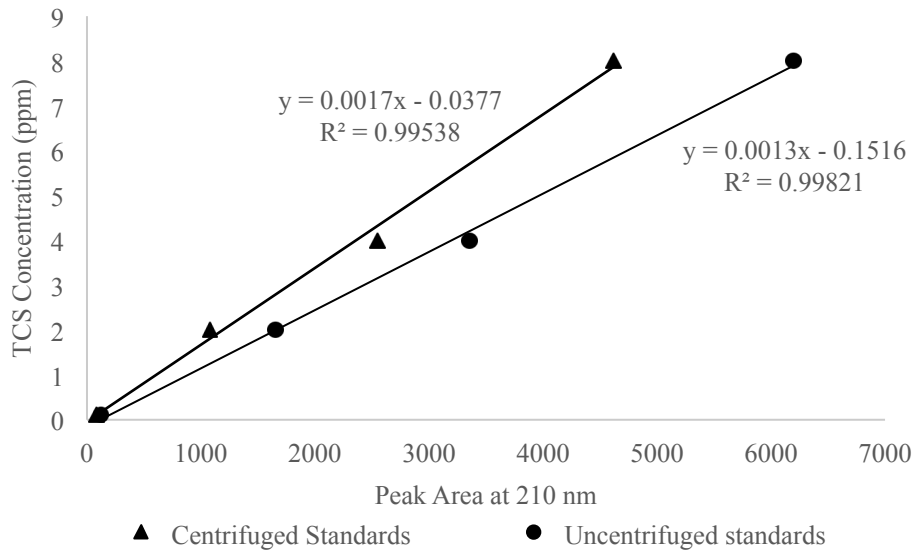


Figure 6. Triclosan concentration versus peak area at 210 nm in AOB medium.

Anion Chromatography Analysis

A Dionex ICS-1100 anion chromatograph (IC) system was used to determine nitrite and nitrate concentrations produced by AOB and NOB in activated sludge. An IonPac AS22 RFIC analytical column with 4 mm by 250 mm dimensions and an IonPac

AG22 4 by 50 mm guard column were used. The eluent had a flow rate of 1.2 ml/min and was prepared from a 1:100 dilution of Dionex AS22 Eluent Concentrate (ThermoScientific), resulting in a final eluent concentration of 4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate. Standard solutions were prepared from serial dilutions of Dionex 7 Anion Standard solution (ThermoScientific). A 25 μ l sample loop was used with a 125 μ l injection volume. Suppression was carried out at 31 mA using a Dionex AERS 500 (4 mm) suppressor. A Dionex DS6 heated conductivity cell was set to 35°C.

Activated sludge samples were filtered with 0.2 μ m syringe filters or centrifuged at 9000 x g for 10 minutes, and 600 μ l effluent or supernatant was placed in 0.5 ml Polyvials with filter caps (ThermoScientific). Standards were also measured in Polyvials with filter caps, and two to three standards were run with activated sludge samples during each IC run. Nitrite and nitrate peaks consistently appeared at approximately 5.6 and 7.7 minutes, respectively. Chromeleon 7 computer software (version 7.1.3.2425) was used to analyze nitrite and nitrate peak areas and correlate them to concentrations.

Oxygen Profile Measurements

Oxygen profiles in nitrifying biofilms were measured using a Clark-type dissolved oxygen microelectrode (described in detail in (117)) with 10-15 μ m tip diameter from Unisense. A two-point calibration was performed using medium sparged with pure dinitrogen gas for ten minutes for a zero measurement and medium at atmospheric oxygen saturation. A Unisense micromanipulator with a motor controller was used to position the microelectrode and move it through the biofilm during oxygen

profile measurements. Data was acquired using the SensorTrace Suite program from Unisense, version 2.2.100.25238. Three to five replicate profiles were taken per TCS exposure test.

Activated Sludge Experiments

Suspended Cell, Batch Growth TCS Exposure

Activated sludge samples were taken in sterile 500 ml Nalgene bottles from the aerobic zone of the treatment train at the Bozeman Wastewater Reclamation Facility. Samples were stored at 5°C overnight prior to use in experiments.

Sterile 125 ml glass Wheaton bottles with rubber septa caps were used as bioreactors. 30 ml of sterile biofilm growth medium was aseptically added to each bottle. Bottles were shaken at room temperature for the duration of the experiment. Added TCS concentrations were varied from 2-16 ppm and samples were prepared in triplicate. This range of TCS concentrations was used because it allowed visualization of a wide range of reductions in nitrification, although these concentrations are 10 to 100 times higher than those typically seen in wastewater. Control bottles had DMSO added to them at the same volume as bottles receiving the highest volume of TCS stock to account for possible impacts by DMSO.

The sludge sample bottle was mixed and allowed to reach room temperature prior to the experiment. Activated sludge was inoculated directly into the bioreactors at a volume required to achieve an optical density 5x or 10x greater than that of *N. europaea* in late exponential phase. This was done to ensure adequate microbial concentrations

were present for quantification of the impacts of TCS on nitrification. Samples were taken at inoculation (time zero), 60, and 180 minutes for TCS analysis. OD_{600} was measured at 0 and 180 minutes. Samples were taken every 30 minutes for nitrite and nitrate analysis over the 3-hour duration of the experiment. After 180 minutes, a 10-ml sample was taken for total suspended solids (TSS) and volatile suspended solids (VSS) analysis: 0.2 μm , 5.5 cm diameter glass-fiber filters (Whatman) were dried at 105°C and their weight prior to filtration measured. Ten ml of sludge was filtered through each filter, and the filter and retentate were dried for at least two hours at 105°C to obtain the TSS. Filters were then baked at 550°C for two hours, and the final weight was measured to obtain the VSS.

Nitrite and nitrate samples were filtered using a 0.2 μm syringe filter and prepared for IC analysis. Samples for TCS analysis were centrifuged at 9000 x g for 10 minutes, and the supernatant was then immediately transferred to glass vials for HPLC analysis.

Longer-term experiments were also performed to investigate effects of TCS on nitrification efficacy over a 3-day period. Samples were prepared in triplicate. 100 ml of sterile biofilm growth medium was added to 250-ml sterile Erlenmeyer flasks. After inoculation with 8.5 ml sludge, flasks were shaken at room temperature at 100 RPM. Samples for nitrite and nitrate were taken two to three times per day, and TCS analysis samples were taken once a day. TSS and VSS were measured at 0 and 3 days.

Nitrifying Biofilm Cultivation

A 3-channel flow cell reactor (FCR) (BioSurface Technologies, Bozeman, MT) was used to grow nitrifying wastewater biofilms and compare responses to triclosan with that of pure cultures. The FCR and influent and effluent tubing were sterilized in an autoclave and allowed to cool in a laminar flow hood. Seventy ml of autoclaved, 5% weight per volume granulated agar (Fischer Scientific) was then added to each channel and allowed to solidify in the flow hood. The agar provided an attachment surface for the biofilm.

Activated sludge sampled as described in the previous section was used to inoculate the FCR. Twenty ml of well-mixed activated sludge sample was added to each cell of the reactor (influent and effluent tubing were clamped off to prevent leakage) in the laminar flow hood. The FCR was then placed on a benchtop and bacteria were allowed an attachment period of 3 days.

In order to facilitate attachment and proliferation, the FCR was operated in several different modes. For the first 70 days following the 3-day attachment period, the FCR was tilted at a 10° angle and biofilm growth medium was pumped through each needle port at 0.5 ml/min. This was done to ensure that debris and biosolids from the sample were flushed out of the reactor whilst encouraging bacterial growth. The lids to each FCR channel were propped open approximately 2 cm and covered with KC400 Kinguard sterilization wrap (Kimberly-Clark) to facilitate oxygen transfer and prevent contamination.

After day 70, the FCR was laid flat and effluent from each channel was combined in one glass recycle flask. The recycle flask was stirred at 150 RPM to reintroduce oxygen to the medium, and pumped back to each channel at 20 ml/min, resulting in a residence time within the recycle vessel of approximately 5 minutes. Fresh medium continued to be pumped into the influent port at 0.5 ml/min, and fresh and recycled medium mixed at a Y-junction prior to entering each channel. This recycle system was implemented in order to homogenize the bacterial communities growing in each channel. The single pass residence time in each channel was approximately one minute, and the overall residence time was 4 hours.

After day 170, effluent from each channel was routed to individual recycle vessels in preparation for exposure experiments (Figure 7). Each recycle vessel was stirred at 150 RPM and had a residence time of approximately 7 minutes. Recycled medium was pumped back to each channel at 15 ml/min. The overall residence time for each cell and its corresponding recycle vessel was 4 hours. Sodium carbonate concentrations in the feed medium were also doubled to a final concentration of 7.55 mM to ensure that adequate carbon was available for growth in the nitrifying biofilm growth medium.

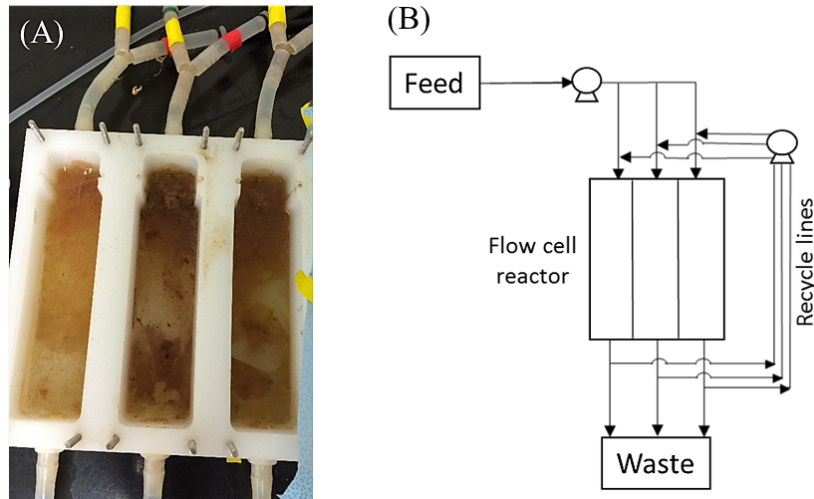


Figure 7. (A) Nitrifying biofilms grown in a flow cell reactor. (B) Final FCR set up prior to running exposure experiments.

Throughout the entire cultivation period for the nitrifying biofilms, effluent samples were periodically taken (every 3-4 days) for nitrite and nitrate production and measured via anion IC analysis.

Biofilm Exposure Experiments

Upon reaching steady-state nitrite and nitrate production levels in each channel of the FCR (approximately day 270), biofilm exposure experiments were carried out. The goal of these experiments was to quantify TCS impact on both nitrite and nitrate production and on oxygen profiles throughout the depth of the biofilm. TCS sorption to biomass was also quantified.

Oxygen profiles were measured prior to TCS exposure. One of the channels was disconnected from both the influent and effluent port, and influent and recycle flow shut off. Pumping continued as usual to the other two channels. After clamping the effluent tubing, air was blown through the influent port at a rate of approximately 100 ml/min to

ensure adequate oxygen diffusion occurred under no flow. Air was filtered through a bacterial air vent prior to entering the reactor.

The lid to the channel was then removed and two equivalent volumes (40 ml) were replaced with fresh nitrifying biofilm growth medium. The fresh medium was added to the channel with a serological pipet, washing out spent medium. This was performed aseptically and at a low flow rate to prevent biomass washout. Channels hold a volume of approximately 20 ml, and therefore 20 ml fresh medium was retained.

Immediately after flushing old medium, at least three oxygen profiles were measured at 50 μm increments throughout the depth of the biofilm (Figure 8). Oxygen measurements were taken at locations where biofilms appeared fairly homogeneous and areas with flocs of biomass were avoided to obtain uniform profiles. Profiles were typically taken where biofilms ranged from 4-8 mm thick. Before and after oxygen profiles were taken, one ml of liquid was removed for nitrite and nitrate analysis using a syringe and needle. The sample was processed for IC analysis.

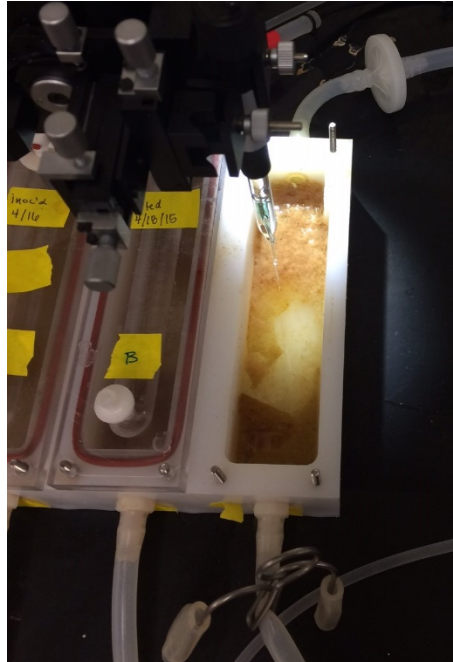


Figure 8. Oxygen microsensor setup over channel C of the nitrifying biofilm FCR.

Forty ml of fresh nitrifying biofilm medium containing 8-40 ppm TCS was then added to the channel using a glass serological pipet. Again, this was performed aseptically and at a laminar flow rate. Immediately thereafter, a 1.5 ml liquid sample was taken for IC and HPLC analysis using a syringe and needle, and at least three oxygen profiles were measured in the same location as previous control profiles were taken. After oxygen measurements had been taken (approximately 15 minutes later), a 1.5 ml liquid sample was taken from roughly the same area as the previous samples. The sample was used to quantify changes in nitrite, nitrate, and TCS concentrations over the course of the experiment. This same procedure was then repeated for the other two channels in the flow cell reactor.

Oxygen flux calculations were performed using equations derived from Fick's Law and assuming one-dimensional diffusion gradients dominated in the biofilm (118).

The oxygen flux in the boundary layer between the bulk solution and the biofilm (J_W) was determined using the following equation:

$$J_W = -D_{OW} \left(\frac{dC}{dz} \right)_{ext}$$

where D_{OW} is the diffusion coefficient for oxygen in water at 25°C and $(dC/dz)_{ext}$ is the oxygen concentration gradient in the fluid layer above the biofilm. D_{OW} is tabulated as 1.97E-5 cm²/s (119). The following equation was used to quantify triclosan reduction of oxygen flux (118):

$$\text{Percent reduction} = \left(1 - \frac{J_W (TCS)}{J_W (control)} \right) \times 100\%$$

In this way, the impact of triclosan on oxygen consumption in the biofilm could be considered. It was expected that the presence of TCS would decrease respiration, thus decreasing the flux of oxygen in the liquid layer over the biofilm.

Pure Culture Cultivation

Pure Culture Batch Growth

Nitrosomonas europaea ATCC strain 19718 was used for pure culture exposure assays. The cells were grown in sterile 2 L flasks containing 1 L of AOB growth medium at pH 7.8: 25 mM (NH₄)₂SO₄ (Amresco), 60 mM KH₂PO₄ (Alfa Aesar), 5.8 mM NaH₂PO₄ (BDH), 3.77 mM Na₂CO₃, 0.73 mM MgSO₄, 0.2 mM CaCl₂, 0.65 μM CuSO₄, 9.9 μM FeSO₄, and 16.5 μM EDTA free acid (all Fisher Scientific). Flasks were incubated at 30°C and shaken at 100 RPM. Harvesting took place after cells reached the

late exponential growth phase, or 3-4 days post-inoculation with an optical density (OD_{600}) at 600 nm and 1 cm path length of approximately 0.09.

Nitrobacter winogradskyi ATCC strain 25391 was also used for pure culture exposure experiments on the impact of TCS on NOB. Cells were grown in 2 L flasks with 1 L of NOB growth medium at pH 7.8, consisting of AOB growth medium containing 50 mM $NaNO_2$ (Fisher) in lieu of $(NH_4)_2SO_4$. Flasks were incubated at 30°C and shaken at 100 RPM. Harvesting took place after cells reached the late exponential growth phase, or 3-4 days post-inoculation with an OD_{600} of approximately 0.09.

Pure Culture Biofilm Growth

A 6-channel drip flow biofilm reactor (DFR, Figure 9) (BioSurface Technologies Inc., Bozeman, MT) was used to cultivate *N. europaea* biofilms (120). Frosted glass slide coupons were placed into each channel, and the reactor, influent, and effluent tubing were sterilized by autoclaving. The reactor was allowed to cool and placed in a laminar flow hood.

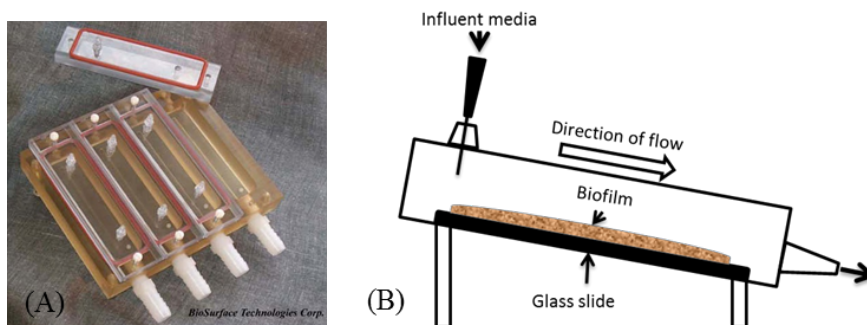


Figure 9. (A) Plastic drip flow reactor (Biosurface Technologies). (B) DFR schematic, illustrated by Ellen Lauchnor.

Two liters of batch-grown *N. europaea* cells in exponential growth phase were centrifuged in four sterile 500 ml centrifuge bottles at 5500 x g for 20 minutes. The supernatant in each bottle was poured off, and 30 ml of supernatant was added back into the culture and used to resuspend the pellet in each bottle. Pelleted cells from all bottles were then combined in a Falcon tube to a final volume of 30 ml.

In the laminar flow hood, 5 ml of the concentrated cell suspension were aseptically added to each channel of the DFR. The DFR was then placed in an incubator at 30°C, laid flat, and cells were allowed a 1-day attachment period under no flow conditions. Biofilm growth medium consisted of the AOB growth medium with 2.5 mM (NH₄)₂SO₄ and 20 mM HEPES (Fisher) buffer at pH 7.8, rather than the KH₂PO₄ and NaH₂PO₄. HEPES buffer was used due to issues with phosphate precipitation in the biofilm reactor system. After the initial attachment period, sterile biofilm growth medium was pumped through each channel at a rate of 0.25 ml/min. The biofilm growth medium was also incubated at 30°C and stirred at 300 RPM to ensure sufficient oxygen remained in solution. Reactor effluent was aseptically sampled for pH and nitrite production every 2-3 days, and biofilms were allowed to grow undisturbed until reaching steady nitrite production rates, typically 6-8 weeks.

Nitrite Production Analysis

A colorimetric assay was used for fast measurement of nitrite concentrations during cell growth and all pure culture exposure experiments. Reagents consisted of a 1% w/v solution of sulfanilamide (Sigma) in 1 M hydrochloric acid (HCl) and 0.2% w/v N-

(1-naphthyl)ethylenediamine dihydrochloride (NED, Sigma Aldrich) in deionized water. Ten μl of sample was added to 890 μl sulfanilamide solution and 100 μl NED solution and mixed. After 30 minutes, the absorbance of the sample was quantified at 540 nm using a Genesys 10-S UV Scanning UV-Vis spectrophotometer (ThermoScientific) or BioTek Synergy HT plate reader. Standard concentration solutions were made in deionized water using sodium nitrite and stored in a 5°C refrigerator. New standards and related standard curves were remade once a month.

Protein Concentration Quantification

Protein concentrations in pure cultures were quantified using a colorimetric assay. Cells were added to a 3 M NaOH solution and incubated at 60°C for 30 minutes in order for adequate lysis to occur. 3 M nitric acid was then added to each protein sample until the pH of the mixture was between six and eight. 50 μl of each protein sample was then mixed with 150 μl Coomassie Blue G-250 reagent (Thermo Scientific) and the absorbance of the resulting blue color read at 595 nm using a BioTek Synergy HT plate reader.

A standard curve for protein concentration was created using 2 mg/mL bovine serum albumin (BSA) standard from Sigma-Aldrich. BSA standards were diluted in DI water and stored at 5°C. Standard solutions were then added to an equimolar mixture of HNO_3 and NaOH (approximately 1.5 M each, pH 7), and 50 μl of this mixture added to 150 μl Coomassie Blue reagent. The resulting regression equation was used to calculate protein concentrations:

$$\text{Protein concentration } \left(\frac{\mu\text{g}}{\text{mL}} \right) = 274.83 (A_{595}) - 60.425$$

Optical density was also correlated to protein concentration using the resulting regression equation:

$$\text{Protein concentration } \left(\frac{\mu\text{g}}{\text{mL}} \right) = 95.41 (OD_{600}) + 2.29$$

Pure Culture Exposure Experiments

Suspended Cell, Batch Growth

Batch-grown *N. europaea* or *N. winogradskyi* cells were harvested for suspended cell exposure experiments in their late exponential growth phase, or 3-4 days after inoculation, $OD_{600} \sim 0.09$. Cells were centrifuged at 5500 x g for 20 minutes, washed with sterile 30 mM HEPES buffer (pH 7.8), and centrifuged again at 5500 x g for 10 minutes. The resulting pellet was resuspended in 30 ml HEPES buffer.

Sterile 125 ml glass Wheaton bottles with rubber septa caps were used as bioreactors. 30 ml of sterile medium was aseptically added to each bottle. AOB medium contained 40 mM KH_2PO_4 and 0.125 mM $(\text{NH}_4)_2\text{SO}_4$, resulting in 0.25-10 mM $\text{NH}_4\text{-N}$ at pH 7.8, and NOB medium contained 5 mM NaNO_2 instead of $(\text{NH}_4)_2\text{SO}_4$. Aliquots of the 1000 ppm TCS stock solution were added to each bottle to obtain desired TCS concentrations (0.25-8 ppm). Again, this range of TCS concentrations was chosen because it allowed visualization of a wide range of nitrification reduction values. Published data was consulted to find suitable ranges for AOB exposure to TCS, and ranges for NOB exposure to TCS were found experimentally (100). To account for possible impacts by DMSO, control bottles had DMSO added to them at the same

volume as bottles receiving the highest volume of TCS stock. All bottles were prepared in triplicate. Prepared bioreactors were then shaken at 250 RPM at 30°C for 30 minutes to ensure that medium was at 30°C prior to cell addition. A one ml sample was taken from each bottle to measure initial nitrite and pH prior to inoculation.

One ml of the resuspended, washed cells was added to an extra bottle containing batch experimental medium only. This bottle was fully mixed, and the resulting cell density (OD_{600}) was measured with a UV-Vis spectrophotometer (Genesys 10-S UV Scanning) at 600 nm. The inoculum volume required to obtain a target cell density was then calculated.

After inoculation, time zero samples were taken for OD_{600} , nitrite, and TCS measurement. One mL of sample was placed in a cuvette. Another 1 mL aliquot was placed in a plastic microcentrifuge tube for nitrite and TCS analysis and was centrifuged at 9000 x g for 10 minutes. The centrifuged supernatant was immediately transferred to glass test tubes in order to decrease TCS sorption to the plastic microcentrifuge tube. For 3-hour exposure experiments, OD_{600} was measured at 0 and 180 minutes. TCS analysis was performed on samples taken at 0, 60, and 180 minutes. Nitrite analysis was performed on samples taken every 30 minutes over the three-hour period. Bottles were incubated at 30°C and shaken at 250 RPM for the duration of the experiment. Nitrite production was assessed to measure AOB activity, and nitrite depletion was assessed to measure NOB activity.

Batch Recovery Experiments

Recovery tests were conducted on *N. europaea* or *N. winogradskyi* cells to determine if inhibition by TCS was reversible and whether enzyme inhibition kinetics could be determined. After three-hour exposure experiments with 8 ppm TCS exposure for AOB and 0.6 ppm TCS for NOB, triplicate batch cultures were recombined and centrifuged at 5500 x g for 10 minutes. The supernatant was decanted and 10 ml of 30 mM HEPES buffer, pH 7.8, was used to resuspend the cells. This washing process was repeated five times. Following the final centrifugation step, cells were resuspended in three ml HEPES buffer. The cells were further diluted into fresh AOB medium to an OD₆₀₀ of approximately 0.09.

OD₆₀₀ samples were taken at 0 and 180 minutes and samples for nitrite were taken every 30 minutes according to the procedures previously described. Bottles were incubated at 30°C and shaken at 250 RPM for the duration of the three-hour recovery experiment. A sample was taken for TCS measurement immediately following inoculation of washed cells to fresh medium to ensure that the washing process had removed all triclosan.

AMO Isolation Tests

Ammonia monooxygenase (AMO) isolation tests were conducted to determine if inhibition by triclosan was due to interaction with the AMO enzyme, or the HAO enzyme and downstream electron transport chain. Three-hour batch exposure tests were run as described previously with the addition of 5 mM hydroxylamine (Fisher), because HAO

catalyzes conversion of NH_2OH to NO_2^- . These tests allowed the effect of triclosan on HAO and downstream processes to be evaluated independent of AMO activity.

Samples for OD_{600} , nitrite, and TCS were taken every 30 minutes for three hours as described in the previous section. Nitrite production rates in cultures with TCS were compared to those in controls with DMSO to determine if HAO activity demonstrated inhibition independent of inhibition experienced by the AMO enzyme.

AOB Biofilm Exposure

AOB Biofilms were assumed to reach maturity at approximately 6-8 weeks, or after measured nitrite production rates had consistently remained over 0.01 mmol/hour for at least one week. Glass petri dishes and glass lids were sterilized in an autoclave and 20 ml of sterile biofilm test medium containing 2.5 mM $(\text{NH}_4)_2\text{SO}_4$ and 20 mM HEPES, pH 7.8, was added to each petri dish in a laminar flow hood.

Effluent tubing from the DFR was clamped to maintain sterility, and the DFR was transferred to the laminar flow hood. Coupons were removed from their respective channels and placed in each petri dish using forceps disinfected with 70% ethanol. Dishes were then transferred to a shaker table at 30°C and shaken at 60 RPM to ensure adequate oxygen transfer throughout the 4 to 5-hour length of the experiment.

For the first one to two hours, 100 μl samples were removed at 20 minute increments for nitrite analysis. These measurements provided “control” samples, as the percent reduction in ammonia oxidation experienced by biofilms was calculated by comparing nitrite production rates before and after TCS addition.

After control activity was measured, petri dishes were transferred back to the laminar flow hood. The coupons were removed from the petri dishes using ethanol-sterilized forceps and 1000 ppm TCS stock was added to the medium in a volume required to obtain the desired concentration (4-8 ppm). Control samples had DMSO added to them at the same volume as samples receiving the highest volume of TCS stock solution. Dishes were prepared in duplicate and gently mixed. Coupons were then placed back in the petri dishes and the lid replaced, and petri dishes were again shaken at 30°C and 60 RPM.

One ml samples were taken at 0, 60, and 180 minutes for TCS analysis. At 20-minute intervals, 100 µl medium was also removed for nitrite production analysis for the next three hours. Upon reaching the end of the experiment, petri dishes were returned to the laminar flow hood, and coupons were aseptically placed back in the DFR channels. The DFR was then returned to the incubator, effluent tubing unclamped, and flow started again. Biofilms were allowed to recover for at least three days between exposure experiments.

After multiple exposure tests, biofilms were destructively sampled for protein quantification. Coupons were placed in the laminar flow hood. Biofilms were then scraped from each coupon using an ethanol-sterilized razor blade and resuspended in 3 M NaOH. Protein was quantified according to the procedure described previously.

Triclosan Sorption Tests

It was found that TCS concentrations decreased over the course of batch and biofilm experiments. In order to determine if measured decreases were due to sorption or

degradation, 4L of batch grown *N. europaea* cells were prepared. Sterile 125 ml Wheaton bottles were filled with 30 ml sterile biofilm test medium. 1000 mg/l sterile N-allylthiourea (ATU, 98% purity, Alfa Aesar) stock solution was added to each bottle to achieve a final concentration of 2 mg/l. ATU is a well-documented inhibitor of the AMO enzyme (121) and was added to ensure degradation by AMO was not responsible for TCS disappearance. TCS stock solution was also added to bottles in triplicate to obtain final concentrations of 4 or 8 ppm.

Using the protein concentration vs. OD₆₀₀ standard curve, the optical density required to obtain a protein concentration analogous to the average measured protein concentration of *N. europaea* biofilms was calculated. The volume of concentrated cells required to obtain this OD₆₀₀ was then calculated and added to each bottle using an extra sample bottle as a test. Bottles were incubated at 30°C and shaken at 250 RPM for the duration of the three hour experiment. Samples for HPLC and nitrite analysis were taken prior to inoculation and at 0, 60, 120, and 180 minutes. Nitrite samples were taken to ensure that the AMO enzyme was completely inhibited. Protein samples were taken at 180 minutes according to the method described previously.

CHAPTER FOUR - RESULTS

Activated SludgeSuspended Cell Exposure Experiments

In order to test the impacts of triclosan exposure on nitrification efficacy in activated sludge, samples taken from the aerobic area of the treatment train at the Bozeman WWTP were exposed to varying concentrations of triclosan. Because the activated sludge contained mixed bacterial species in addition to other organic materials, the optical density alone could not be used to quantify bacterial concentrations, and therefore OD₆₀₀ was varied from 0.25 to 0.5, equal to TSS of 330 mg/l and 610 mg/l, respectively. Results from three-hour exposure experiments are shown in Figure 10.

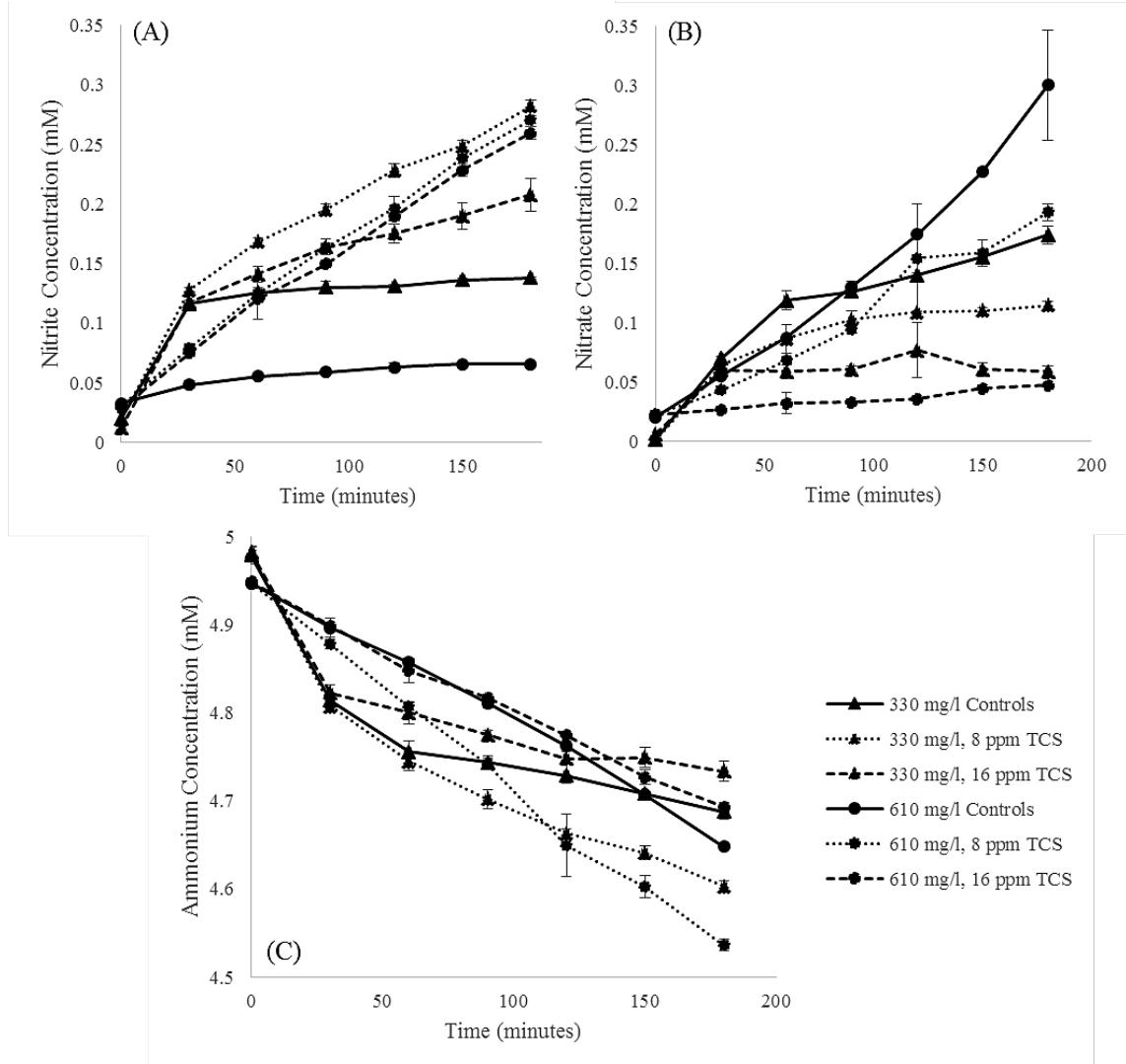


Figure 10. Nitrite (A), nitrate (B), and ammonium (C) concentrations over time in activated sludge exposed to 8 and 16 ppm TCS (dotted and dashed lines respectively). Triangles indicate sludge with an average TSS of 330 mg/l. Circles indicate sludge with an average TSS of 610 mg/l. Error bars signify standard deviation of triplicate samples, and are at times smaller than point sizes.

In control samples with an average optical density of 0.25 (330 mg/l TSS), nitrite concentrations reached a plateau around 0.12 mM in the first 30 minutes. Nitrate increased fairly linearly with time to a final concentration of approximately 0.18 mM. Control samples with 610 mg/l TSS reached a lower nitrite concentration plateau of 0.05

mM in the first 30 minutes of the experiment, and produced almost twice as much nitrate as 330 mg/l TSS control samples—approximately 0.3 mM by 180 minutes. The 610 mg/l TSS controls appear to produce a lower nitrite maximum because these samples likely had higher concentrations of NOB, and therefore higher rates of nitrite oxidation may prevent nitrite accumulation. Altogether, these results indicate successful conversion of nitrite to nitrate in the activated sludge samples without TCS exposure.

Before considering the impacts of TCS on nitrification in treated samples, it is important to note that the dissolved, or effective, TCS concentration was lower than the total added concentration due to sorption with activated sludge material (Figure 11). The largest decreases in TCS concentration occurred immediately after inoculation, and gradual decreases continued thereafter. As TSS increased, so did sorption of triclosan; thus samples with 610 mg/l TSS were exposed to dissolved TCS concentrations of 5.9 ± 0.16 and 2.6 ± 0.1 ppm TCS at time zero, rather than 16 and 8 ppm TCS. Samples with 330 mg/l TSS were exposed to 9.5 ± 0.25 and 4.3 ± 0.15 ppm dissolved TCS. For consistency, samples containing TCS will be referred to by the concentrations of TCS added prior to inoculation, although their dissolved concentrations were smaller after inoculation.

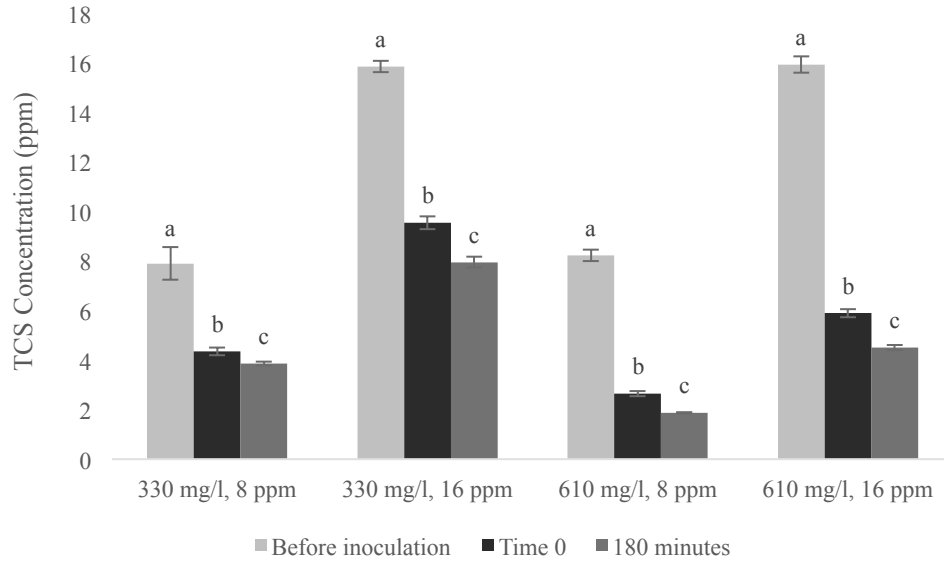


Figure 11. Triclosan concentrations over time in varying TSS sludge samples. Dissimilar letter labels at each time point indicate statistically significant decreases in TCS concentrations to 95% confidence. Error bars indicate standard deviations of triplicate samples.

Samples exposed to TCS at both TSS concentrations saw nitrite accumulation after the first 30 minutes of sampling (Figure 10A). Nitrite production rates decreased as triclosan concentrations increased, though 8 ppm TCS did not cause any reduction in nitrite production at either TSS concentration. The following equation was used to determine percent reduction:

$$\% \text{ Reduction} = 100 \times \left(\frac{r_{co} - r_i}{r_{co}} \right)$$

where r_{co} is the rate of nitrite or nitrate produced by control samples and r_i is the rate of nitrite or nitrate produced by TCS exposed samples. 16 ppm TCS caused $15 \pm 3.3\%$ and $13.7 \pm 0.3\%$ reduction of nitrite production in 330 and 610 mg/l TSS samples, respectively.

Nitrate production in 330 mg/l TSS samples was reduced by $36 \pm 2.8\%$ and $75 \pm 3\%$ in the presence of 8 and 16 ppm TCS, respectively. In samples with an average TSS of 610 mg/l, NO_3^- production was reduced by $33 \pm 6.2\%$ and $91 \pm 0.3\%$ in the presence of 8 and 16 ppm TCS, respectively. This indicates greater impacts of TCS on NOB populations than AOB: AOB successfully converted ammonia to nitrite, but NOB were incapable of fully oxidizing the available nitrite to nitrate to the extent observed in TCS-free controls.

The impact of TCS was evaluated in longer-term tests over three days with activated sludge containing a TSS of 330 mg/l. The impacts of two ppm TCS were considered in addition to those of 8 and 16 ppm. A similar pattern of reduction in nitrification efficacy was observed, with NO_2^- accumulation occurring in treated samples (Figure 12A). Higher TCS concentrations also resulted in decreased NO_2^- production rates. Nitrite production by samples exposed to 2 ppm TCS was reduced by approximately $31 \pm 2.8\%$ when compared to controls with DMSO, and samples exposed to 8 and 16 ppm TCS experienced $56 \pm 1.7\%$ and $77 \pm 0.8\%$ reduction in nitrite production, respectively. (DMSO was the solvent used to make stock solutions of TCS, as it has a low aqueous solubility). Samples with 2 ppm TCS added had a concentration of 0.9 ppm TCS after inoculation, and samples with 8 and 16 ppm TCS added had concentrations of 4.5 and 8.5 ppm, respectively, after inoculation.

Controls with DMSO added in the same volume as 16 ppm samples experienced approximately $62 \pm 0.6\%$ decrease in NO_3^- production compared to controls in medium alone, thus the TCS exposed samples with DMSO were compared to the controls with

DMSO. Nitrate production was almost completely diminished in samples containing 8 or 16 ppm TCS (Figure 12B): comparison to DMSO-containing control samples resulted in percent reduction of $94 \pm 1.1\%$ and $98 \pm 0.2\%$, respectively. Two ppm TCS reduced NO_3^- production by $25 \pm 3.7\%$. Reduction of NO_3^- production may be due to decreased nitrite availability in addition to triclosan presence, though the low concentrations of nitrite in control samples indicate that NOB can efficiently scavenge NO_2^- . Altogether, ammonium conversion occurred to the greatest extent in control samples, with conversion decreasing at TCS concentrations increased (Figure 12C).

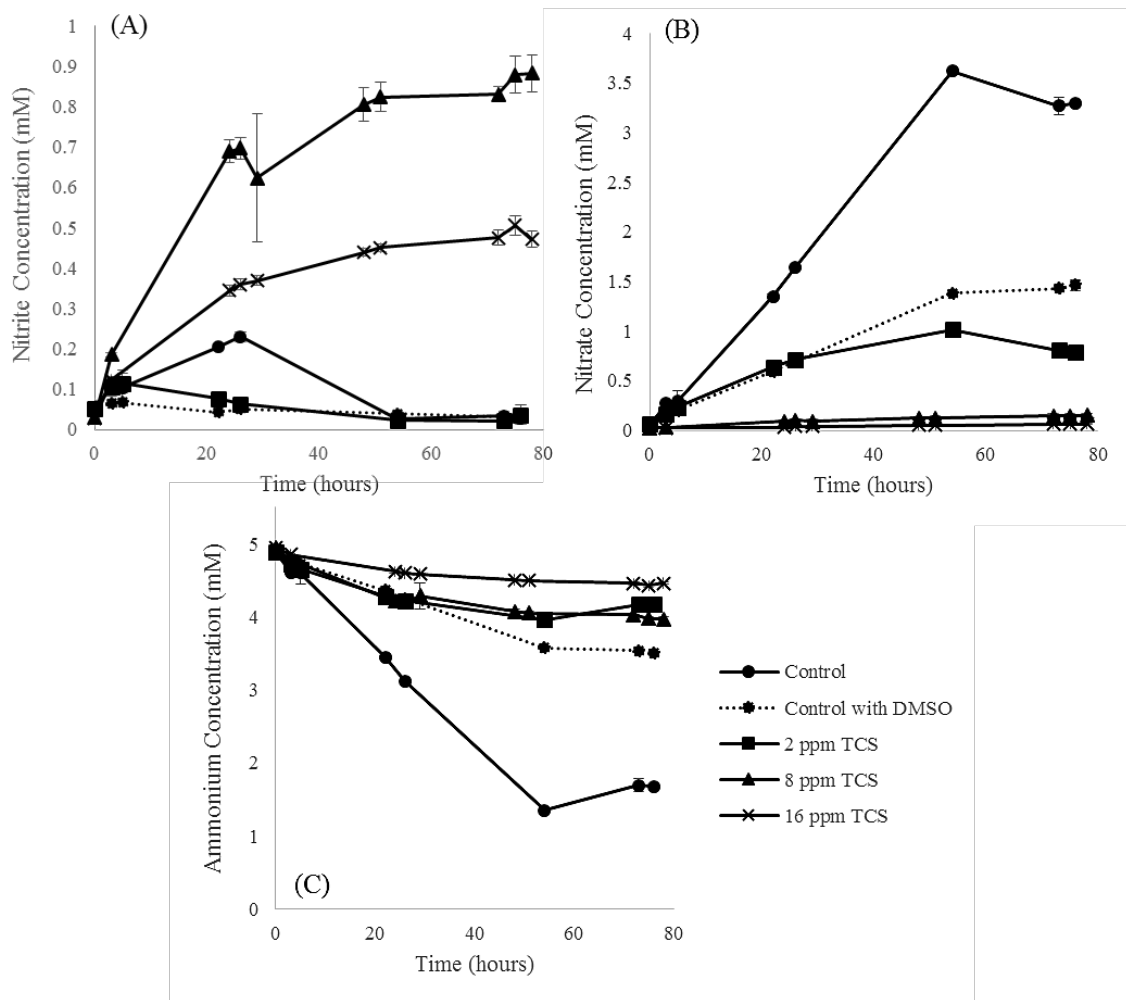


Figure 12. Triclosan impacts in long term exposure WW batch experiments: (A) Nitrite concentrations, (B) nitrate concentrations, and (C) ammonium concentrations over time. Error bars indicate standard deviations of triplicate samples, and are at times smaller than point sizes.

In samples containing 16 ppm TCS, triclosan concentrations did not decrease significantly after the initial sorption period following inoculation. However, in samples with 2 and 8 ppm TCS, concentrations decreased throughout the duration of the experiment (Figure 13). Degradation of up to 3 ppm TCS by nitrifying activated sludge has been reported (24, 101, 107), but degradation has not been reported in concentrations greater than that. For this reason, it is probable that degradation occurred in samples with

2 ppm TCS, but TCS decreases in samples containing 8 ppm are more likely due to sorption. In this study, TCS degradation was defined as disappearance of the parent compound only, and the fate or occurrence of any daughter products was not evaluated.

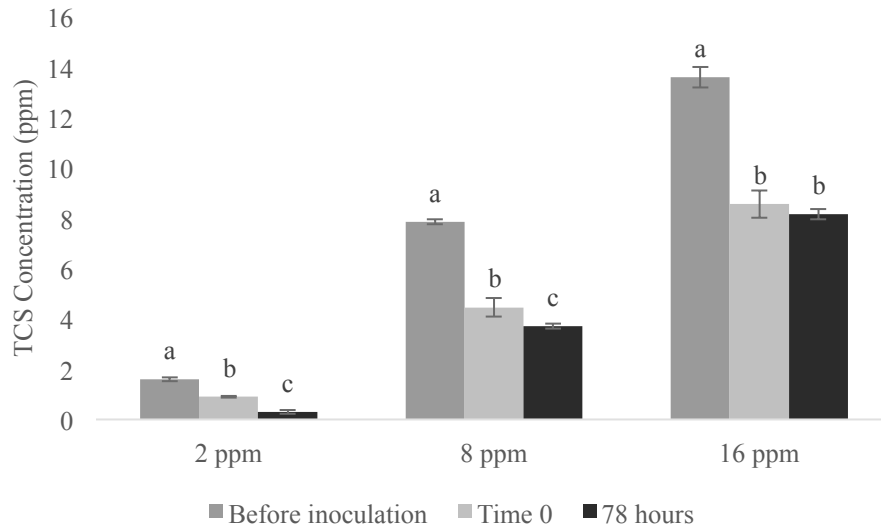


Figure 13. Changes in TCS concentration in activated sludge samples over a three day period. Different letter labels above the time points for a given sample indicate statistically significant differences ($\alpha = 0.05$). Error bars indicate standard deviations of triplicate samples.

The loss of TCS due to sorption and degradation was quantified in short- (3 hour) and longer-term (3 day) tests. TCS disappearance due to adsorption was determined by autoclaving an activated sludge sample and adding it to medium with 16 ppm TCS. Equal portions of non-killed activated sludge were inoculated into medium with 16 ppm TCS. All samples had an average TSS of 2800 mg/l, and measurements were taken for TCS over a three-hour period. An average loss of 4.40 ± 0.14 mg triclosan per g TSS was measured in autoclaved samples, and live samples saw an average loss of 4.45 ± 0.11 mg triclosan per g TSS. A one-tailed, two-sample T-test assuming equal variances could not

conclude with 95% confidence that the two averages were different ($p = 0.32$). In other words, sorption, not microbial degradation, is the reason for measured decreases in triclosan concentrations over the three-hour experimental period. It is worth noting, however, that tested TCS concentrations were significantly higher than those typically seen in WWTPs, and therefore degradation of lower concentrations may be possible.

To test if degradation was possible at lower TCS concentrations (2 ppm) and if it was due to nitrifiers, heterotrophs, or both, long-term (3 day) tests were performed on autoclaved activated sludge samples and samples inactivated with 10 ppm ATU. ATU exposure inactivates the AMO enzyme, which drives metabolism and energy production in *N. europaea*. This inactivation was performed to rule out any possibility that oxidation by the AMO enzyme was responsible for TCS disappearance. TSS was approximately 330 mg/l for all samples. The losses of TCS over time were compared in autoclaved, live, and ATU-inactivated samples (Figure 14).

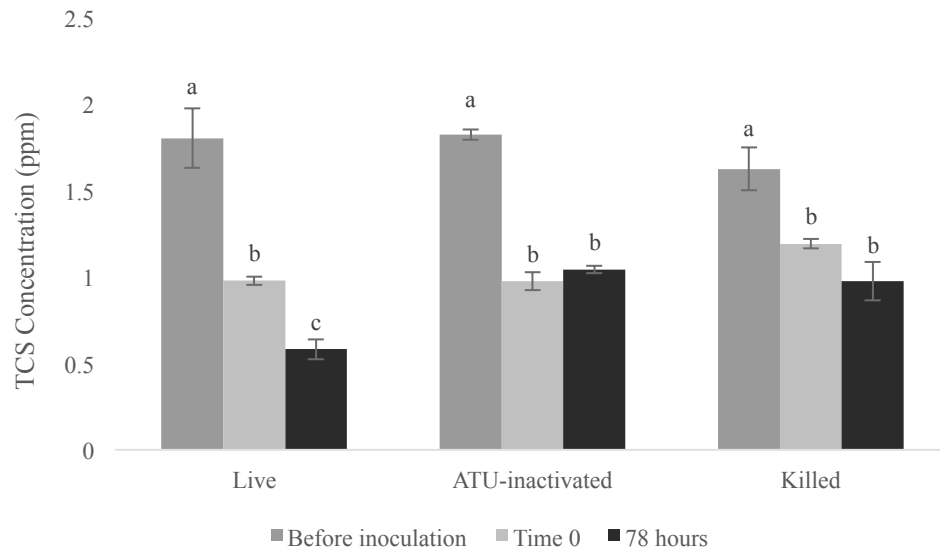


Figure 14. Losses in TCS over an approximately 3-day period in live, ATU-inactivated, and killed samples. Different letter labels above the time points for a given sample indicate statistically significant differences ($\alpha = 0.05$). Error bars indicate standard deviations of triplicate samples.

As shown in Figure 14, losses in dissolved TCS concentrations occurred to the greatest extent in live samples. TCS in live samples decreased by 1.2 ± 0.14 ppm over the 3-day experimental period. A single-factor ANOVA found that the concentrations of TCS in ATU-inactivated and killed cells at time zero and 78 hours did not change significantly—in other words, mean TCS concentrations in both sample sets remained constant after the initial sorption period. TCS losses in the two sample sets were also statistically similar, at 0.7 ± 0.1 ppm ($p = 0.07$). However, TCS concentrations in the live activated sludge did decrease significantly between 0 and 78 hours, and were significantly lower than the inactivated and killed TCS concentrations at all time points. Taken together, these results indicate that degradation did not occur in killed or ATU-inactivated samples, because active organisms should have caused increased losses.

Because heterotrophic bacteria were the only active microorganisms in ATU-inactivated samples and no degradation was observed, it can be concluded that heterotrophic bacteria were not responsible for the TCS degradation observed. Nitrifying bacteria only active in the live samples must therefore be responsible for TCS degradation.

Nitrifying Biofilm Exposure Experiments

Oxygen profiles in nitrifying biofilms were measured before and after TCS exposure to investigate the impacts of triclosan on microbial respiration. Figure 15 demonstrates typical results from these experiments. Biofilms were located approximately 200-600 μm from the air-liquid interface, with a diffusive boundary layer between the bulk fluid and biofilm located from 0-200 μm where diffusion was assumed to dominate. Though biofilms were typically 4 mm thick, oxygen concentrations generally reached zero at shallower depths. The 0 μm depth measurement indicates the top of the boundary layer. Liquid depths changed slightly after TCS addition, however, and therefore maximum oxygen concentrations did not always occur at 0 μm . Generally, as TCS concentrations increased, the slope of the oxygen gradient decreased, indicating lower respiration.

Because TCS adsorbs strongly to plastic, effective TCS concentrations are used to describe results in this section only. This was the amount of dissolved TCS measured immediately after addition to the reactor. Control experiments also found that triclosan adsorbed to the agar base used for biofilm attachment. At the time of exposure experiments, however, the agar base was fully covered by the biofilm, and therefore losses due to agar-TCS adsorption were considered minimal. These losses were also

accounted for by measurements taken immediately after addition of TCS medium to the reactor.

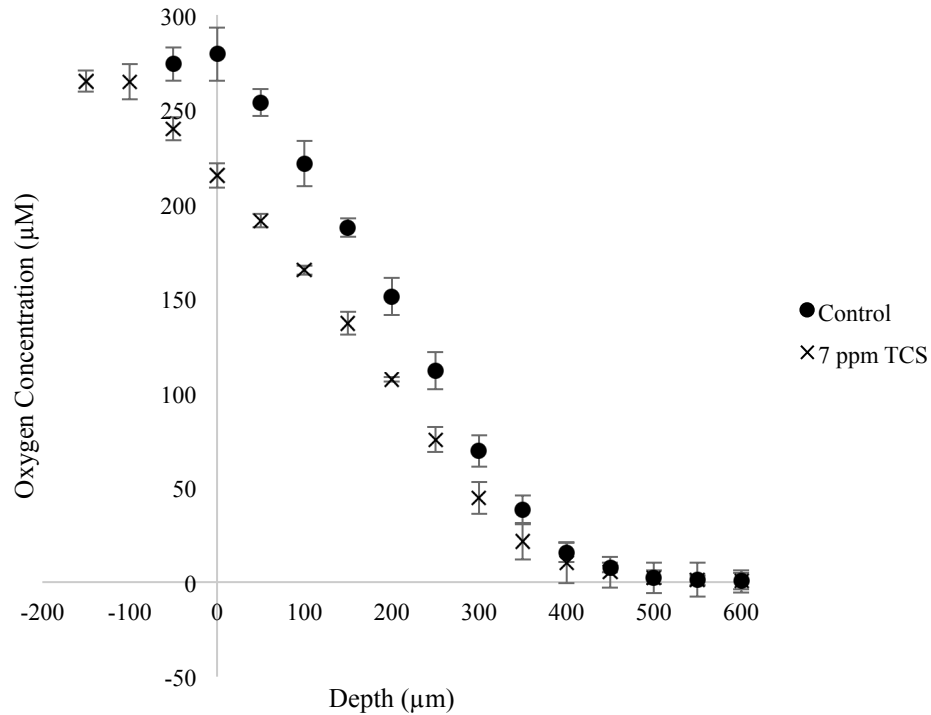


Figure 15. Oxygen profiles before (“Control”) and after addition of an effective concentration of 7 ppm TCS. Liquid depths changed slightly after TCS addition, and therefore maximum O_2 concentrations do not consistently occur at a depth of 0 μm . Error bars indicate standard deviations of triplicate oxygen measurements at a single location.

The flux of oxygen through the liquid boundary layer over the biofilm decreased as TCS concentrations increased, indicating diminished oxygen consumption by the biofilm. This increased the percent reduction of oxygen flux when compared with untreated biofilms, as is visualized in Figure 16. As TCS concentrations increased, reduction also increased linearly until approximately 10 ppm TCS. Reduction then appeared to plateau around 35%. Below 4.6 ppm TCS, no reduction was seen.

Ten ppm ATU was also added to the reactor to gauge the fraction of respiration due to nitrifying bacteria activity. Assuming that 10 ppm ATU inhibited respiration from all nitrifying bacteria present in the biofilm, it can be concluded that approximately 25% of all respiration is due to the activity of nitrifying bacteria (Figure 16).

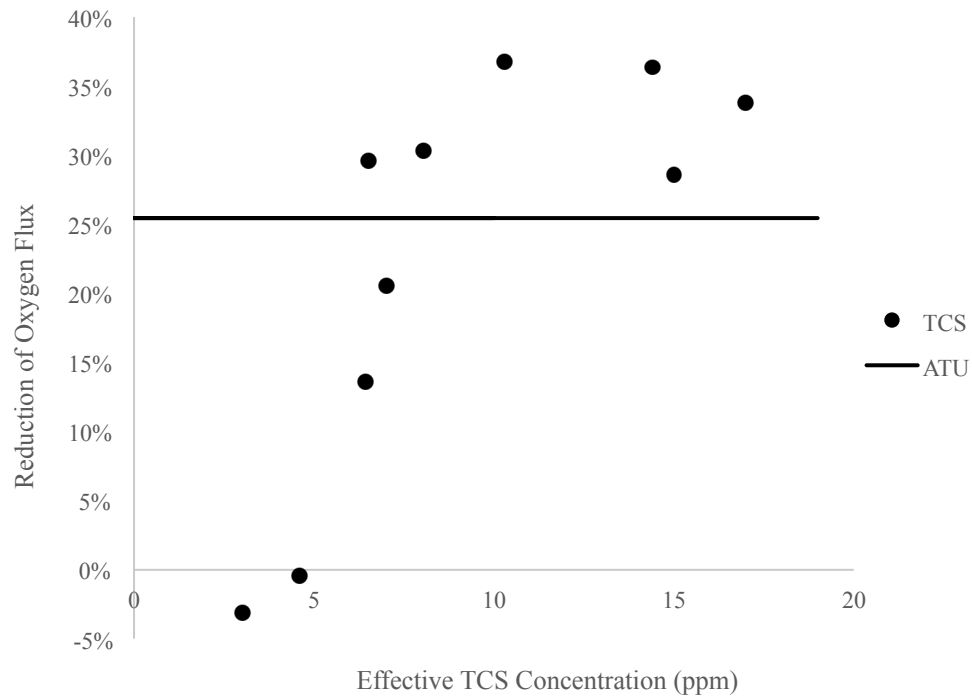


Figure 16. Percent reduction of oxygen flux versus effective TCS concentration.

Altogether, TCS is here shown to reduce oxygen consumption by both heterotrophs and nitrifiers in activated sludge biofilms. At approximately 7.5 ppm TCS, 25% reduction of oxygen flux is seen. Over this concentration of TCS, it is clear that reduction of both heterotrophic respiration and nitrification occurs. This demonstrates that triclosan has the ability to reduce the activity of non-nitrifying bacteria in activated sludge.

Pure Culture, Suspended Cell Exposure Experiments

Nitrosomonas europaea

Pure culture exposure experiments were conducted to isolate TCS impacts on both AOB and NOB. Planktonic, batch-grown *N. europaea* cells were exposed to various TCS concentrations. Nitrite concentrations increased linearly over time as cells oxidized ammonium (Figure 17). Control experiments were performed with and without DMSO. Both control experiments produced nitrite at similar rates, indicating that the presence of DMSO did not influence nitrite production in the pure cultures. In experiments with higher TCS concentrations, nitrite production rates were decreased (Figure 17), indicating that higher TCS concentrations result in greater reduction of ammonia oxidation (Figure 18).

The percent reduction of ammonia oxidation by *N. europaea* was calculated by comparing the average nitrite production rates of *N. europaea* batch tests with only DMSO to that of TCS-containing samples. Controls with DMSO were used for these calculations to ensure that percent reduction results were based solely on triclosan presence. The following equation was again used to determine percent reduction:

$$\% \text{ Reduction} = 100 \times \left(\frac{r_{co} - r_i}{r_{co}} \right)$$

where r_{co} is the rate of nitrite produced by control samples and r_i is the rate of nitrite produced by TCS exposed samples. Control samples with DMSO typically produced nitrite at a rate of $82 \pm 9.6 \text{ mmol NO}_2^- \text{ g protein}^{-1} \text{ hr}^{-1}$. Percent reduction in ammonia oxidation rates varied from approximately $6 \pm 2\%$ at 2 ppm TCS to $44 \pm 4\%$ at 8 ppm

TCS. Samples containing 4 ppm TCS experienced $18 \pm 4\%$ reduction, and ammonia oxidation in samples with 6 ppm TCS was reduced by $40 \pm 4\%$. Sorption to cells in pure cultures occurred to a much smaller extent than it did in activated sludge tests, with TCS concentrations after inoculation decreased by approximately 0.5-1 ppm.

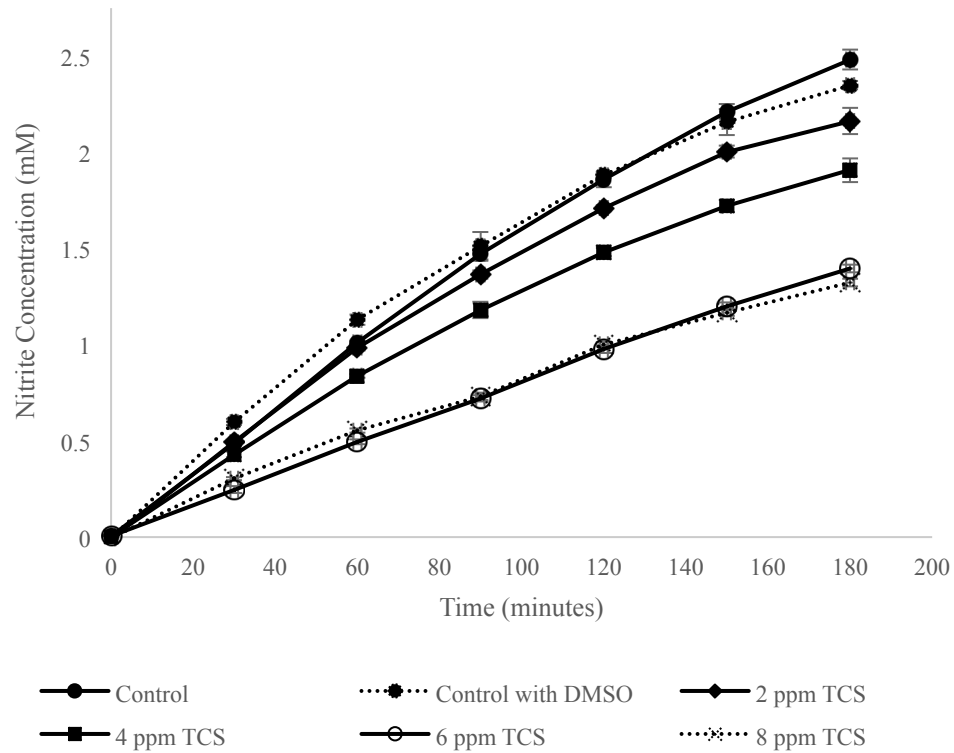


Figure 17. Nitrite production over time with 5 mM NH₄⁺. Error bars indicate standard deviation of triplicate samples and are at times smaller than points.

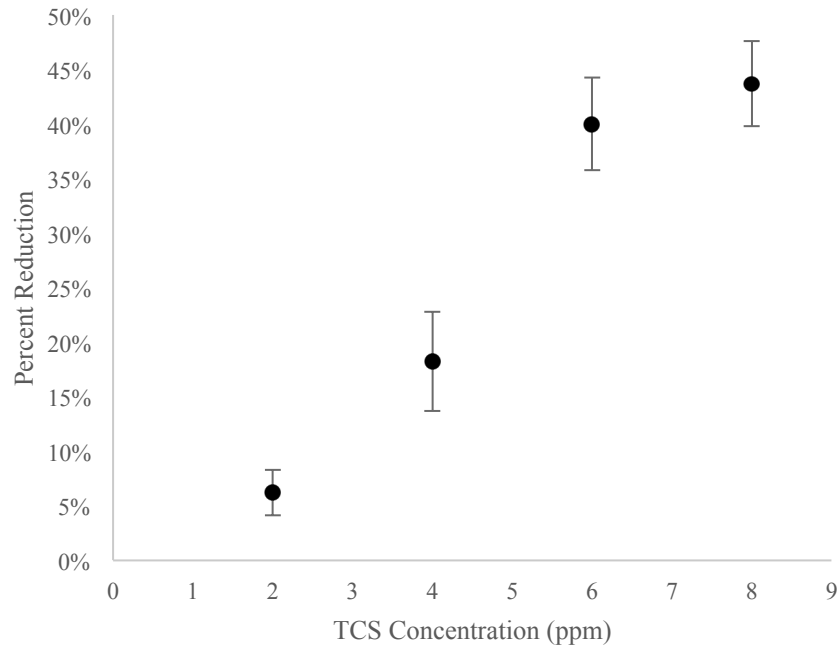


Figure 18. Percent reduction in ammonia oxidation versus TCS concentration with 5 mM NH_4^+ as substrate. Error bars indicate standard deviation of triplicate samples.

Triclosan exposure experiments with different ammonium concentrations were also performed to determine if there was any relationship between the ammonia oxidation rate per cell and extent of TCS impacts. As substrate concentrations increased, ammonia oxidation rates also increased in both control samples and samples exposed to 4 ppm TCS (Figure 19). However, in samples exposed to 8 ppm TCS, ammonia oxidation rates peaked at 2.5 mM and decreased at higher concentrations. At lower substrate concentrations of 0.5 to 1 mM NH_4^+ , samples saw similar rates of ammonia oxidation. Thereafter, nitrite production in TCS-treated samples occurred at rates slower than that of controls, with higher TCS concentrations resulting in lower ammonia oxidation rates.

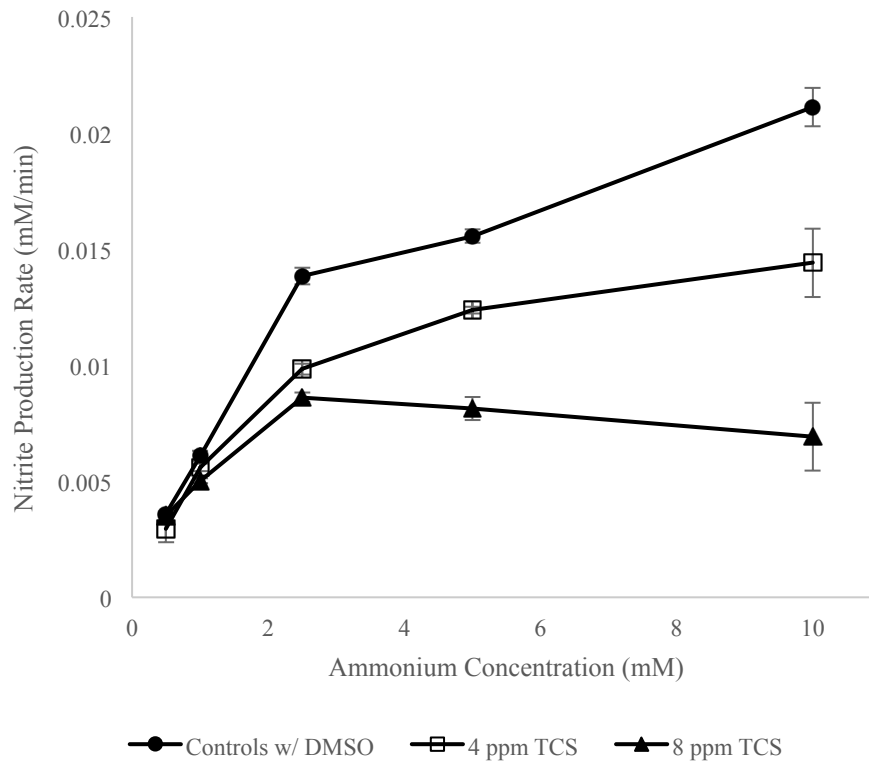


Figure 19. Results of batch exposure experiments with different ammonium concentrations. Error bars indicate standard deviation of triplicate samples and are at times smaller than points.

Altogether, this indicates that greater ammonia concentrations correlate with higher percent reduction (Figure 20). When 1 mM ammonium was present, cells experienced approximately $8 \pm 1.5\%$ reduction in nitrite production when exposed to 8 ppm triclosan, but when exposed to the same TCS concentration and 10 mM ammonium, over $67 \pm 5\%$ reduction of nitrite production was seen. A similar pattern was observed in cells exposed to 4 ppm TCS. *N. europaea* cells exposed to both 4 and 8 ppm TCS experienced no reduction in ammonia oxidation when only 0.5 mM ammonium was available.

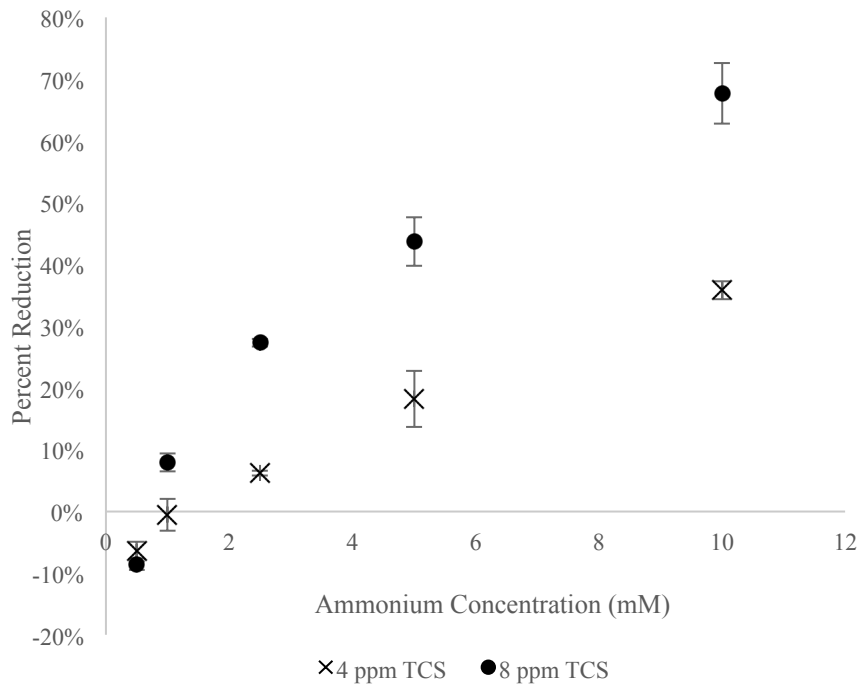


Figure 20. Percent reduction versus substrate concentration at 4 and 8 ppm TCS. Error bars indicate standard deviation of triplicate samples and are at times smaller than points.

N. europaea Recovery Experiments

To determine if TCS inhibition was reversible, recovery experiments were conducted after the three-hour exposure tests (Figure 21). For the first three hours, samples were treated with TCS (or DMSO, in the case of the controls). After washing, cells were resuspended in fresh AOB medium for the next three hours and sampled periodically for nitrite.

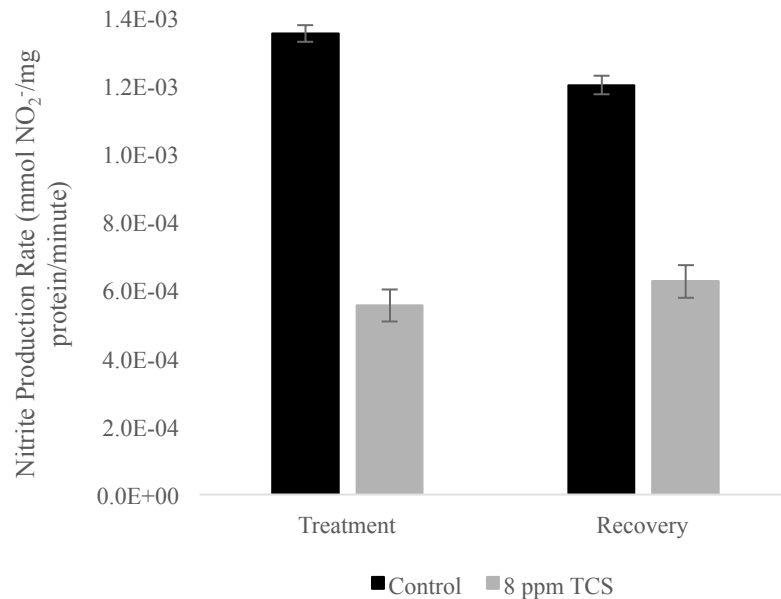


Figure 21. Nitrite production during (treatment) and after (recovery) TCS exposure. Error bars indicate standard deviation of triplicate samples.

As can be seen above, cells exposed to 8 ppm TCS did not recover after exposure. Nitrite production is reduced by $59 \pm 4\%$ over the first three hours of exposure. After washing, previously exposed cells still experience $48 \pm 5\%$ reduction in comparison to the washed control cells. Despite five cycles of washing, 0.35 ± 0.06 ppm TCS was still measured in previously exposed samples at the beginning of the recovery period. However, this concentration is much smaller than that present during the TCS exposure—8 ppm—and therefore ammonia oxidation was expected to increase considerably if recovery was possible. A t-test concluded with 95% confidence that the nitrite production rates before and after TCS exposure were not statistically different ($p = 0.07$). It can therefore be concluded that either recovery did not occur after considerable decreases in TCS concentrations, or that 8 ppm TCS caused cell death, resulting in irreversible losses in ammonia oxidation activity.

AMO Isolation Tests

Exposure experiments were run with 5 mM hydroxylamine (NH_2OH) and without $(\text{NH}_4)_2\text{SO}_4$ in order to determine if triclosan had an inhibitory effect on the hydroxylamine oxidoreductase (HAO) enzyme. Because NH_2OH is the product of AMO oxidation of NH_3 , presence of AMO inhibitors prevents its formation and consequently the formation of nitrite. Its addition in place of ammonia should therefore allow uninhibited nitrite production when strict AMO inhibitors are present.

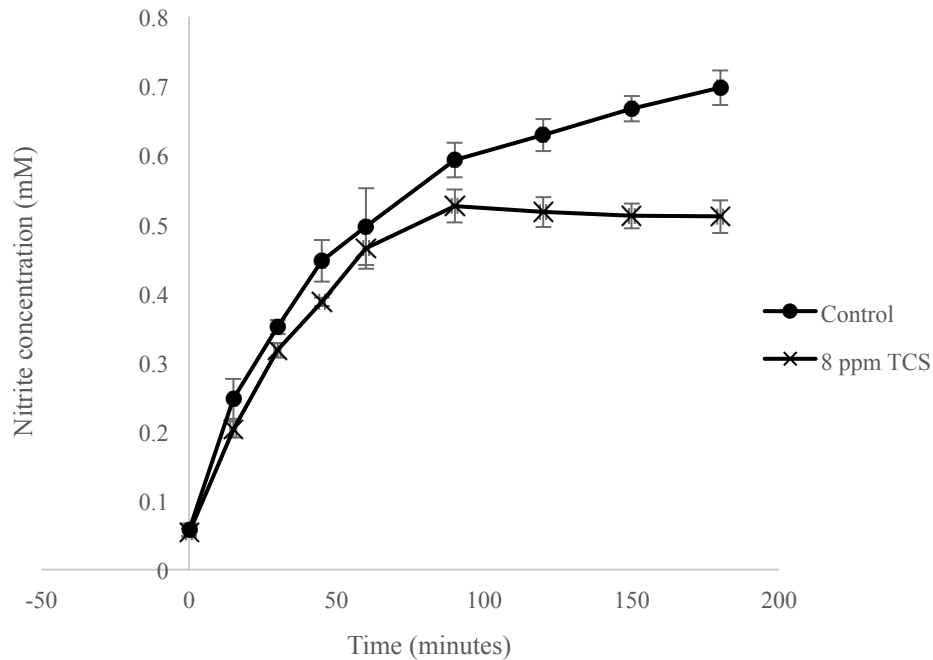


Figure 22. Nitrite production over time in batch *N. europaea* cultures grown on hydroxylamine instead of ammonia substrate. Error bars indicate standard deviation of triplicate samples and are at times smaller than points.

As shown in Figure 22, TCS also appears to act as a delayed HAO inhibitor. In the first 90 minutes, nitrite production rates in treated and untreated samples were not statistically different ($p = 0.06$). However, after this time period, treated samples stopped

producing nitrite entirely while controls continue to do so, albeit at a slightly slower rate. This suggests that triclosan exposure causes broad inhibition or toxicity to the cell beyond just the AMO enzyme, potentially resulting in cell death or inactivation.

Nitrobacter winogradskyi

Exposure and Recovery. To confirm the previous observation that nitrite consumption by NOB was more impacted by TCS than AOB, pure suspended cultures of *N. winogradskyi* were exposed to varying TCS concentrations (Figure 23) and the percent reduction in nitrite consumption was calculated.

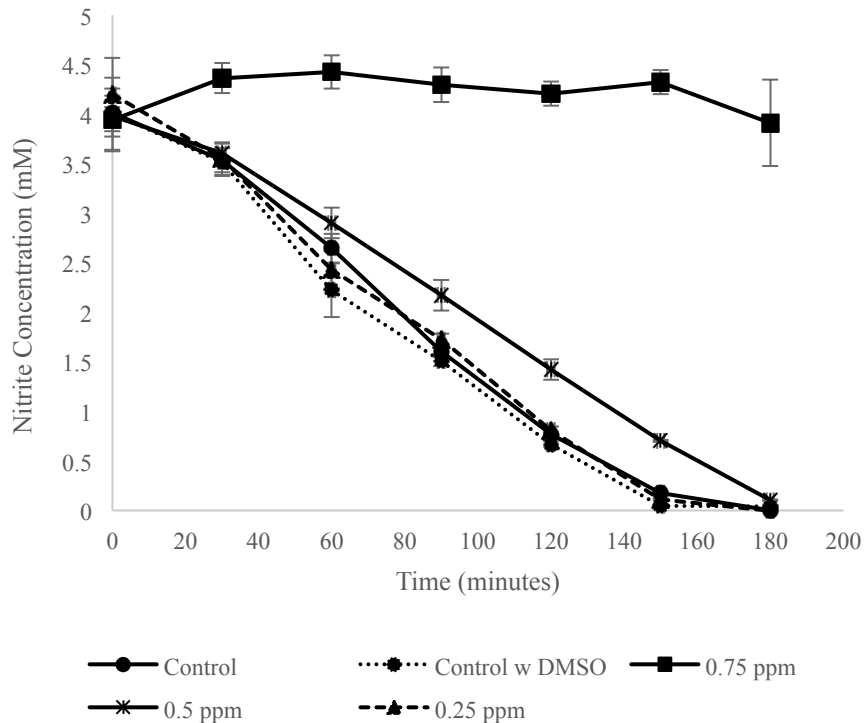


Figure 23. Nitrite consumption over time in pure suspended cultures of *N. winogradskyi* exposed to varying TCS concentrations. Error bars indicate standard deviation of triplicate samples and are at times smaller than points.

In controls with and without DMSO, nitrite consumption occurred at a roughly constant rate of $1.44 \text{ mmol L}^{-1} \text{ hour}^{-1}$ throughout the experiment. Samples with only 0.25 ppm triclosan performed nitrite oxidation at approximately the same rate as the controls. Increasing the TCS concentration to just 0.5 ppm decreased the nitrite oxidation rate to $1.35 \text{ mmol L}^{-1} \text{ hour}^{-1}$, resulting in $7.2 \pm 5\%$ reduction. At concentrations at or above 0.75 ppm TCS, no oxidation of nitrite was observed, which was confirmed by a one-tailed t-test assuming equal variance at a 95% confidence level ($p = 0.4$). Results from tests at 2-8 ppm TCS concentrations are not shown. These results indicate that the previous observation is correct: NOB are more impacted by triclosan than AOB, as they experience greater loss of activity at lower TCS concentrations.

Recovery tests were performed on *N. winogradskyi* cells after three hours of 0.6 ppm TCS exposure. Cells were washed and resuspended in fresh NOB medium. Rates of nitrite oxidation during and after treatment are seen in Figure 24.

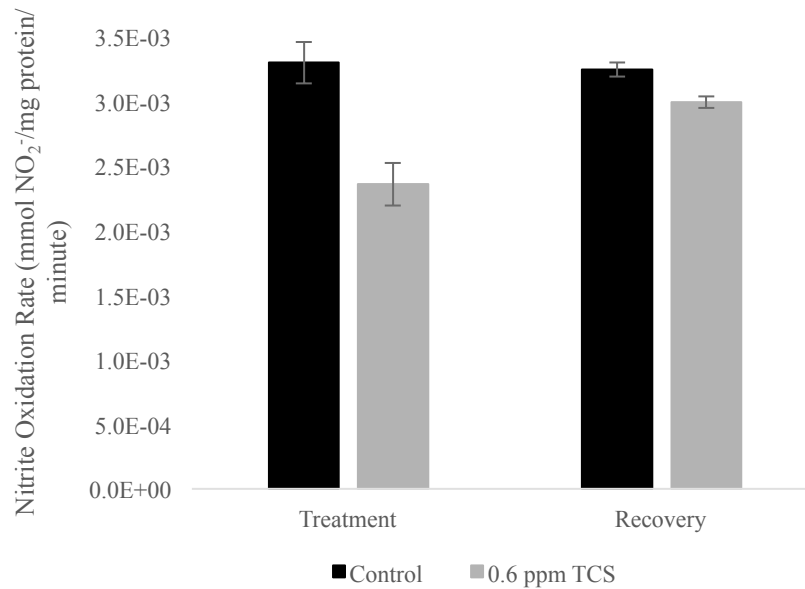


Figure 24. Nitrite oxidation rates by *N. winogradskyi* during treatment with 0.6 ppm TCS and after washing (recovery). Error bars indicate standard deviation of triplicate samples.

During treatment, nitrite oxidation decreased by $29 \pm 3\%$. After washing, nitrite oxidation was reduced by $8 \pm 2\%$. Using a t-test, the oxidation rates of treated and washed cells were compared. It was concluded with 95% confidence that cells oxidized nitrite faster after washing ($p = 1.5E-3$), indicative of recovery from TCS exposure. This may be because cells were exposed to a low TCS concentration—recovery may not be possible at higher concentrations, as was seen with cultures of *N. europaea* during exposure to 8 ppm TCS.

N. europaea Biofilm Exposure Experiments

The impacts of triclosan on biofilms of *N. europaea* were tested to compare biofilm response to that of suspended cells. *N. europaea* biofilms were grown in a drip flow reactor for 6-8 weeks according to methods described previously. Table 2

summarizes the average overall nitrite production rates of AOB in the DFR the week prior to starting 4 ppm TCS exposure tests. Each cell can be considered a replicate.

Cell	Average nitrite production rate (mmol hr ⁻¹ mg protein ⁻¹)
A	0.016 ± 0.003
B	0.009 ± 0.003
C	0.019 ± 0.003
D	0.009 ± 0.001
E	0.014 ± 0.003
F	0.016 ± 0.005
Average	0.014 ± 0.004

Table 2. Average overall nitrite production rates by AOB in the drip flow reactor in the week prior to 4 ppm TCS exposure experiments.

To evaluate effects of triclosan on pure *N. europaea* biofilms, batch experiments were performed on the biofilms with 4 and 8 ppm TCS exposure. For the batch experiments, coupons were removed from the DFR and placed in glass petri dishes with biofilm growth medium, which reduced the oxygen transfer efficiency to the biofilms, resulting in lower ammonia oxidation rate in comparison to those in the DFR. Ammonia oxidation rates in the reactor were also likely higher due to growth of biomass in areas other than the coupon that were not quantified. Thus, the rates of nitrite production in the DFR could not be used for comparison to the batch experiments. Instead, the nitrite production rates of individual biofilms were compared in the batch tests before and after TCS exposure, where TCS was added at 120 minutes after the start of the test. Prior to TCS addition, control nitrite production rates were constant, though they varied between different biofilm samples. After addition of TCS at 120 minutes, nitrite production rates

decreased in TCS amended biofilms. Nitrite concentrations increased linearly over time during the experiments, regardless of whether TCS was present, allowing for calculation of constant nitrite production rates. Higher TCS concentrations caused greater reductions in nitrite production. Percent reduction was calculated using the following equation:

$$\% \text{ Reduction} = 100 \times \left(\frac{r_{pre} - r_{post}}{r_{pre}} \right)$$

where r_{pre} is the rate of nitrite production in a biofilm prior to exposure to TCS, and r_{post} is the nitrite production rate in the biofilm after TCS exposure.

Rates of nitrite production before and after 4 ppm TCS treatment are shown in Figure 25. R-squared values for nitrite production rates during exposure tests ranged from 0.96-0.99 (Figure 25). Biofilms experienced approximately 22-32% reduction in ammonia oxidation during TCS exposure, with an average of $25 \pm 4\%$. Control samples exposed to DMSO experienced no negative effects.

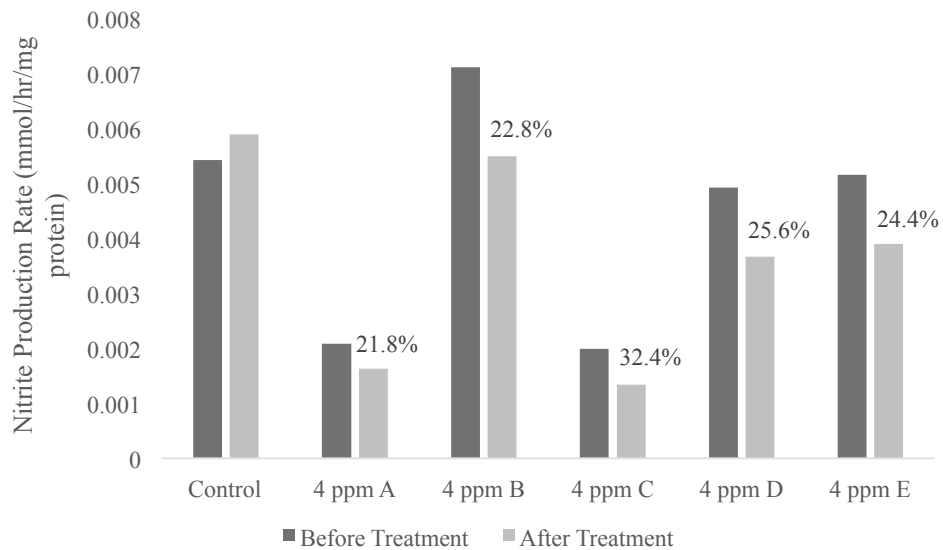


Figure 25. Nitrite production rates on a protein basis in biofilms before and after 4 ppm TCS exposure and corresponding percent reduction values.

Biofilms were similarly exposed to 8 ppm TCS. Rates before and after treatment are shown in Figure 26. After correcting for the impact of DMSO on the control biofilm, percent reduction of ammonia oxidation ranged from 31-40%, with an average value of $35 \pm 4\%$. R-squared values were 0.96-0.99.

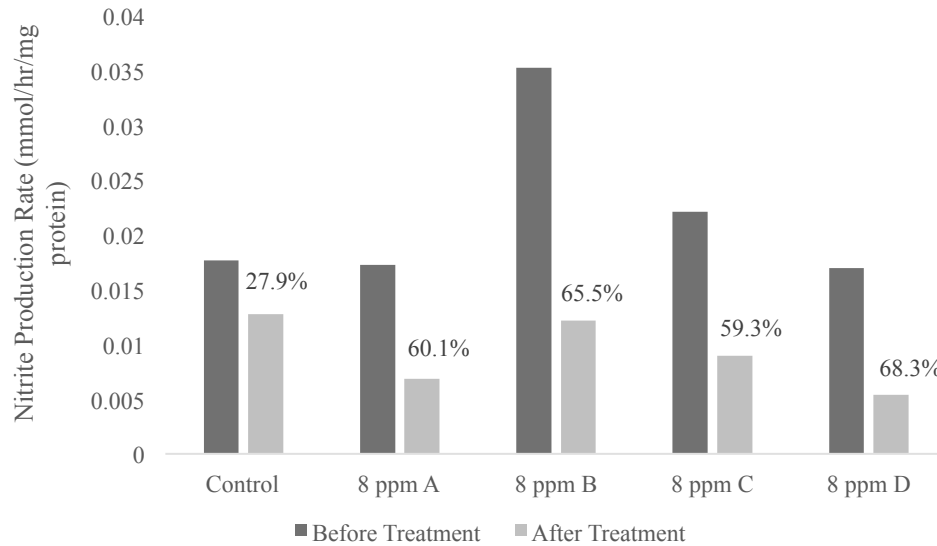


Figure 26. Nitrite production rates on a protein basis in biofilms before and after 8 ppm TCS exposure (or DMSO, in the case of the control) and corresponding total percent reduction values.

Biofilms exposed to 8 ppm TCS had much lower protein content than those exposed to 4 ppm TCS, which resulted in thinner biofilms and higher overall nitrite production on a per protein basis. On average, overall nitrite production in each cell of the DFR was $0.049 \pm 0.023 \text{ mmol NO}_2^- \text{ mg protein}^{-1} \text{ hr}^{-1}$. Again, due to the experimental method, ammonia oxidation rates by the biofilms were lower in the petri dishes during the batch experiments.

The results of ammonia oxidation reduction in multiple biofilm experiments were averaged for 4 and 8 ppm TCS exposure and compared with the results of TCS exposure calculated from suspended cell experiments (Figure 27). These results indicate that ammonia oxidation in the biofilms was inhibited or inactivated to a similar extent as suspended cells during exposure to similar triclosan concentrations, despite higher overall nitrite production rates in suspended cell exposure experiments, in which controls with DMSO typically produced nitrite at a rate of $0.082 \pm 0.009 \text{ mmol NO}_2^- \text{ mg protein}^{-1} \text{ hr}^{-1}$.

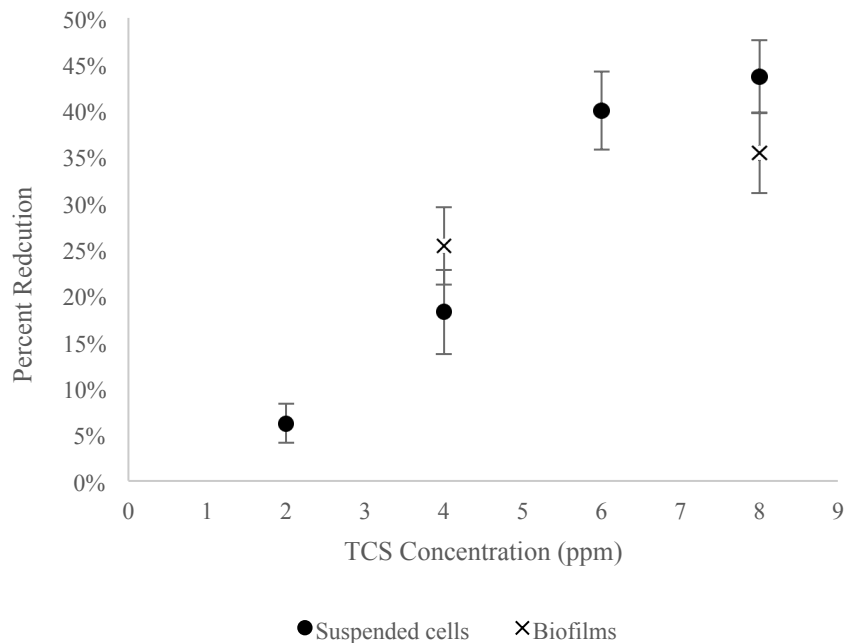


Figure 27. Percent reduction in ammonia oxidation experienced by AOB biofilms and suspended cells. Error bars indicate standard deviation of triplicate samples in suspended cells, and standard deviation of five and four replicate samples in biofilms exposed to 4 and 8 ppm TCS, respectively.

Ammonia oxidation in *N. europaea* biofilms exposed to 4 and 8 ppm TCS experienced $25.4 \pm 4.2\%$ and $35.4 \pm 4.3\%$ reduction, respectively. In contrast, ammonia oxidation in suspended cell cultures experienced $18.2 \pm 4.5\%$ reduction at 4 ppm TCS

and $43.7 \pm 3.9\%$ reduction at 8 ppm. A one-sided t-test assuming equal variance found that suspended cells exposed to 4 ppm TCS were less inactivated than biofilms with 95% confidence ($p = 2.5E-5$), and that suspended cells exposed to 8 ppm TCS were more inactivated than biofilms ($p = 0.02$). This presents contrasting information about whether biofilms are more resistant to TCS exposure than suspended cells: at lower TCS concentrations, the data suggest that suspended cells are less inactivated than biofilms. At higher concentrations, however, suspended cells are more inactivated by triclosan than biofilms.

Triclosan Fate Studies

Triclosan Fate in Suspended Cell Experiments

N. europaea. Triclosan concentrations were measured throughout the course of suspended cell exposure experiments to determine if loss was occurring. The largest changes in TCS concentration occurred immediately following inoculation, and concentrations decreased slightly thereafter (Figure 28). A one-tailed, two-sample T-test assuming equal variances with a level of significance (α) of 0.05 was conducted to compare TCS concentrations over time in the batch tests. Statistical analysis was performed on triplicate samples for each TCS concentration at different time intervals.

Figure 28 summarizes TCS concentrations throughout batch tests and results from t-tests. Bars at each time interval are labeled with different letters to indicate statistically significant changes: for example, 2 ppm TCS measurements did not decrease significantly before and after inoculation, and so data is labeled “a” and “ab”, with the

shared letter “a” indicating statistically equal concentrations. Again, statistically significant decreases did not occur between inoculation and 180 minutes, so data labels share the common letter “b”. However, significant changes did occur in the concentrations measured before inoculation and at 180 minutes, and therefore data labels share no common letter. From this, it can be concluded that decreases in 2 ppm TCS occurred very slowly throughout the length of the experiment.

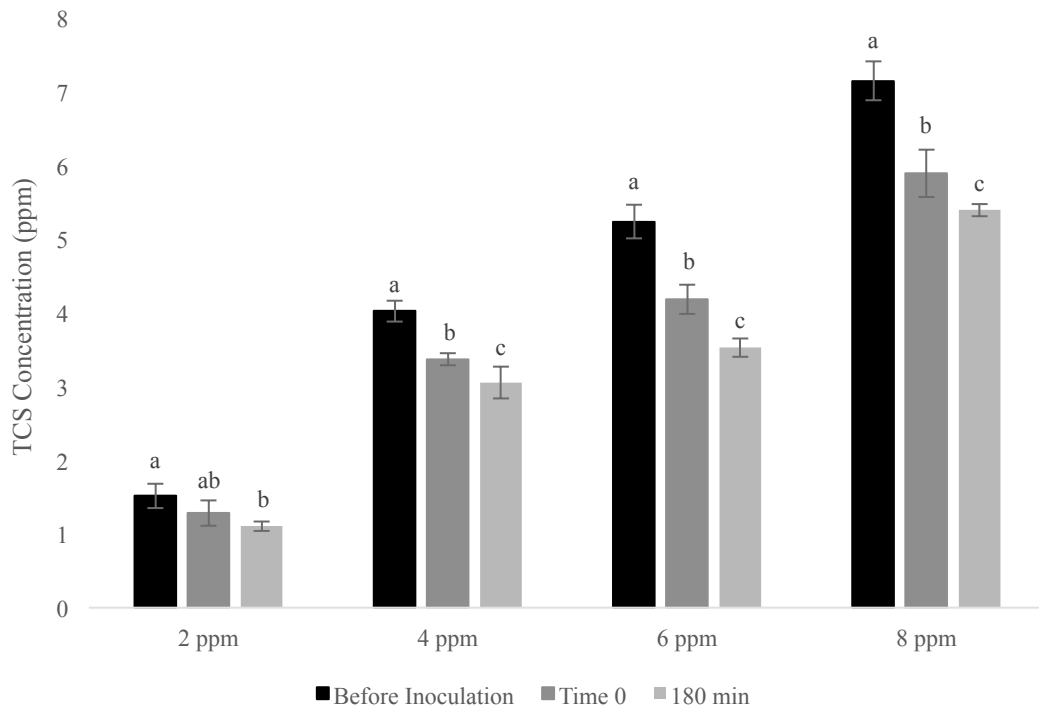


Figure 28. Changes in measured TCS concentrations over time during batch exposure experiments with *N. europaea*. Error bars indicate standard deviation of triplicate samples.

With 95% confidence, this test found that mean TCS concentrations after 180 minutes of the batch experiments were significantly less than concentrations prior to

inoculation, or that over the course of the entire experiment, triclosan concentrations decreased significantly for all samples.

In samples containing 4, 6, and 8 ppm TCS, the mean TCS concentration after inoculation was less than that prior to inoculation. The mean concentration at the end of the experiment (180 minutes) was also less than that measured immediately after inoculation. This indicates that all 4, 6, and 8 ppm samples saw significant decreases in triclosan immediately after inoculation and throughout the three-hour experiment. To determine the reason for TCS loss, ATU-inactivated cells with similar protein concentrations were exposed to triclosan, and TCS concentrations were measured over a three-hour period.

It was found that active and inactive cells exposed to 2 and 8 ppm TCS saw statistically equal losses of TCS per gram protein over the duration of the experiment (Figure 29). If degradation were occurring, active cultures should have seen greater losses of TCS than inactive ones. Since degradation was not observed in cultures exposed to the lowest and highest tested TCS concentrations, it can be concluded that adsorption was the reason for all TCS losses seen in pure suspended cultures of *N. europaea*.

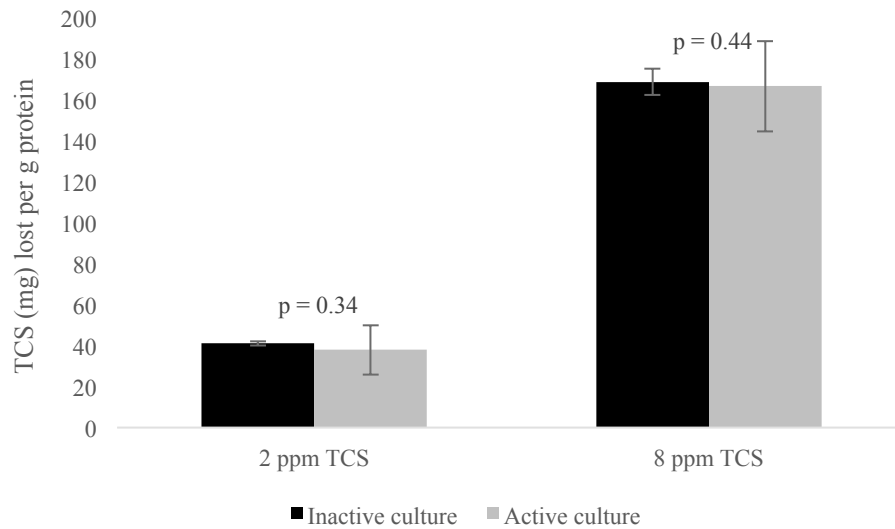


Figure 29. Losses of TCS per g protein in active and ATU-inactivated suspended cell cultures over a three-hour period. P-values indicate results from a one-sided, two-sample t-test assuming equal variances at 95% confidence. Error bars indicate standard deviation of triplicate samples.

Nitrobacter winogradskyi. Triclosan concentrations in pure culture NOB

experiments followed the same pattern observed in previous experiments with AOB—immediately following inoculation, they dropped significantly, and then continued to decrease at a slower rate as the experiment progressed. The mass of TCS lost per gram protein in killed and active cultures exposed to 0.25 ppm TCS was compared using a one-tailed t-test assuming equal variance, and it was found that the two values were not statistically different (Figure 30). This indicates that TCS losses in NOB exposure experiments are also due to sorption, not degradation. Furthermore, because cultures exposed to the lowest TCS concentration were incapable of degradation, it can be concluded that cultures exposed to higher concentrations also experienced adsorption.

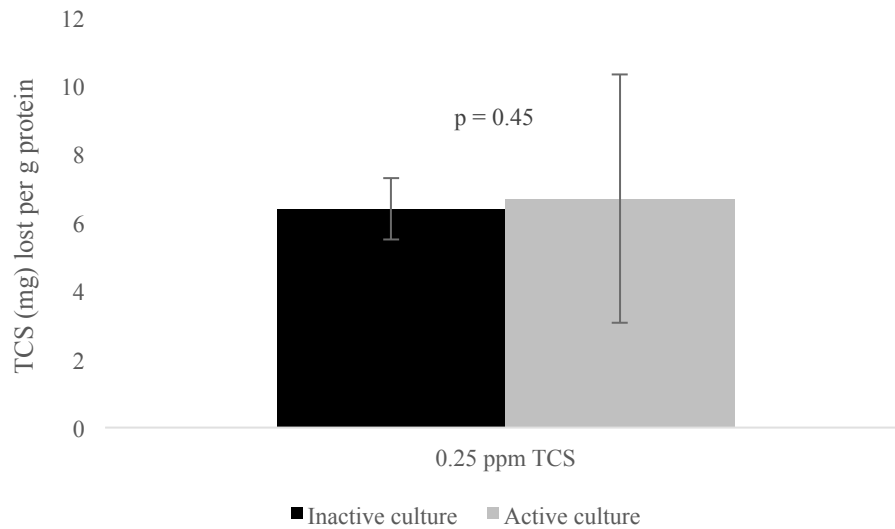


Figure 30. TCS losses per gram protein in active and inactive *N. winogradskyi* cultures over a three-hour period. P-values indicate results from a one-sided, two-sample t-test assuming equal variances at 95% confidence. Error bars indicate standard deviation of triplicate samples.

Triclosan Fate in *N. europaea* Biofilms

Triclosan concentrations were measured throughout the duration of biofilm exposure experiments to determine if any losses occurred. All biofilms had an average protein content of 1.14 ± 0.18 mg. Concentrations did not decrease immediately after inoculation of the biofilm coupon to TCS-treated medium, but instead declined most over the first hour of the experiment, with smaller decreases occurring thereafter (Figure 31).

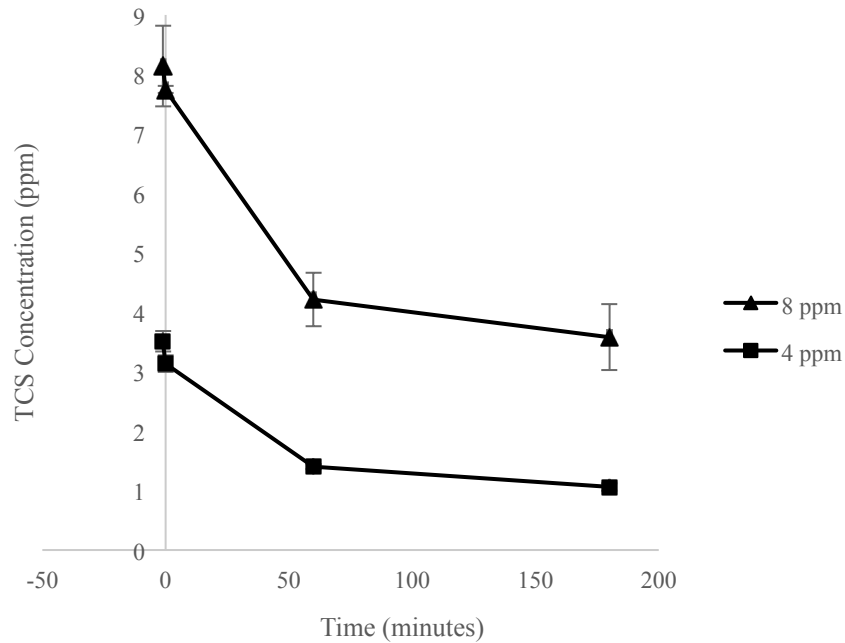


Figure 31. Measured TCS concentrations in biofilm exposure experiments over time. Error bars indicate standard deviation of five replicate samples and are at times smaller than points.

To determine if sorption or degradation mechanisms were responsible for the TCS decreases observed, batch grown, suspended *N. europaea* cells at a protein concentration analogous to that of biofilms (approximately 130 mg L^{-1}) were inactivated with ATU and exposed to 4 or 8 ppm TCS. Triclosan was also added to abiotic controls to determine if any chemical interactions were responsible for decreases in concentration. Results of these experiments are summarized in Figure 32 and compared with results from biofilm experiments.

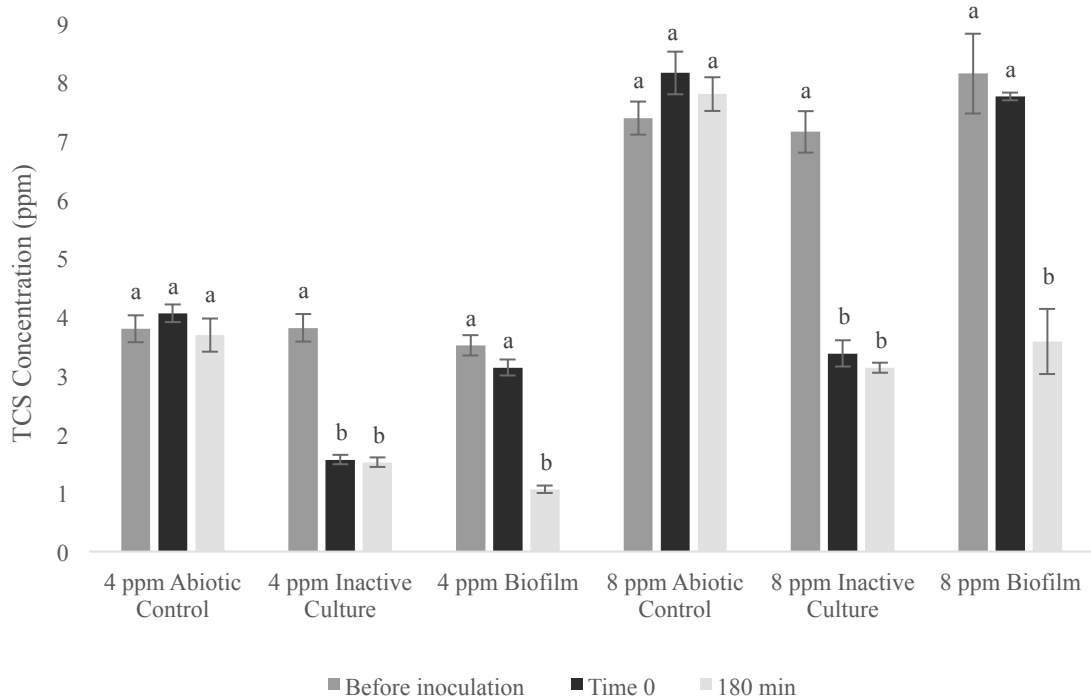


Figure 32. Results of measured triclosan concentrations over time in abiotic control samples, ATU-inactivated biofilm representative cultures, and active biofilms. Inactive cultures represent the biomass protein concentration in biofilms. Similar labels indicate statistically equal concentrations at each time interval; changes in letter indicate decreases in concentration with 95% confidence. Error bars indicate standard deviation of triplicate samples.

TCS concentrations decreased in all samples containing cells, despite cellular inactivity. Samples containing both *N. europaea* cells and ATU saw statistically significant changes immediately following inoculation at 4 ppm ($p = 4.7E-5$) and 8 ppm ($p = 5E-5$), and concentrations remained constant thereafter. TCS concentrations in abiotic control samples did not change over the three-hour experiment. A one-tailed, two-sample t-test assuming equal variances on TCS concentrations in abiotic controls failed to conclude with 95% confidence that concentrations decreased over the course of the experiment for 4 ppm TCS ($p = 0.32$) and 8 ppm TCS ($p = 0.12$). This demonstrates that sorption to cellular material is responsible for TCS losses, not abiotic interactions

between TCS and medium constituents. However, in contrast to the suspended cells, TCS in active biofilm samples did not decrease immediately after inoculation of the biofilm coupon to treated medium. Instead, triclosan concentrations decreased significantly over the experimental period.

The loss of TCS in the inactivated cultures can be attributed to adsorption of TCS to biomass. To identify if degradation in the biofilms caused greater TCS losses than sorption to inactive cells, the mass of TCS lost in inactive cultures was then compared to that lost in active biofilms (Figure 33).

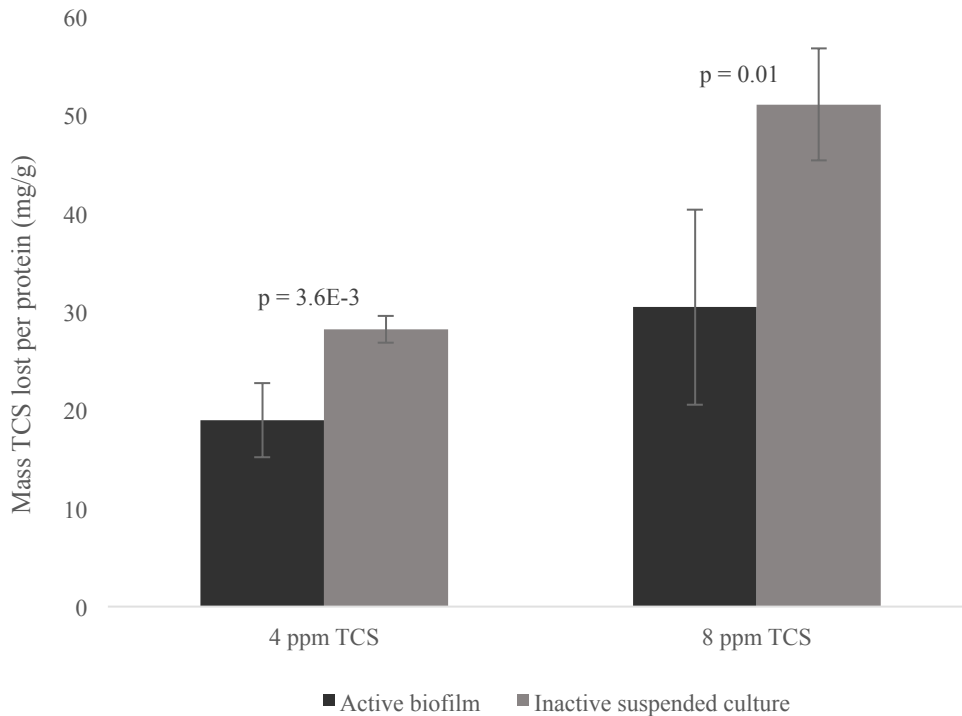


Figure 33. Milligrams triclosan lost per gram protein in active biofilms and suspended, inactivated cultures. P-values indicate results from a one-sided, two-sample t-test assuming equal variances at 95% confidence. Error bars indicate standard deviation of triplicate samples.

Inactive cultures exposed to 4 and 8 ppm TCS saw 28 ± 1.4 and 51 ± 5.8 mg TCS lost per g protein, respectively. In contrast, biofilms exposed to 4 ppm TCS only saw 19 ± 3.8 mg TCS lost per g protein, and those exposed to 8 ppm saw 30 ± 9.9 mg TCS lost per g protein. Because greater losses of TCS per protein were observed in inactive cultures, it can be concluded that adsorption, not degradation, is the dominant cause of TCS loss in biofilms. If degradation were occurring, active biofilms would produce greater TCS losses. This indicates that TCS diffusion into the biofilm likely occurred over the course of the three-hour biofilm experiment, in contrast to degradation.

Diffusion limitation in biofilms may also explain why TCS losses are smaller than those in suspended cultures. Additionally, because biofilms contain many other forms of protein in addition to that of cells, such as extracellular polymeric substances (122), the protein assay may overestimate bacterial cell concentrations, thus decreasing the calculated mass of TCS lost on a per cell basis in the biofilm. However, this artifact does not influence the time-dependent decrease in TCS observed in the biofilm experiments.

Diffusion Component of TCS Loss

After observing that TCS sorption occurred slowly over the first three hours of biofilm exposure experiments, rather than immediately after inoculation (Figure 31), calculations were performed to determine the time for diffusion of TCS into the biofilm. The Wilke-Chang correlation for binary liquids with small concentrations of A in B (123) was used to approximate the diffusivity of triclosan in water:

$$D_{AB} = 7.4 \times 10^{-8} \frac{(\varphi_B M_B)^{0.5} T}{\mu V_A^{0.6}}$$

where V_A is the molar volume of solute A (triclosan) in $\text{cm}^3/\text{g-mole}$ as liquid at its normal boiling point, φ_B is an association parameter of the solvent (water), M_B is the molar mass of the solvent in g/mole , μ is the viscosity of the solution in centipoise, and T is the absolute temperature in Kelvin (123). The viscosity of the solution was approximated as that of water at 30°C because TCS was present in such small concentrations. φ_B was 2.6 based on recommended values from the literature (123). V_A was $194.3 \text{ cm}^3/\text{g-mole}$ (124). Using these values, the diffusivity of triclosan in water at 30°C was found to be approximately $814 \mu\text{m}^2/\text{s}$. This is close to the value of $608 \mu\text{m}^2/\text{s}$ at 25°C reported by Corbin et al. (125).

The time required for TCS in the bulk fluid covering the biofilm to reach 90% of the bulk concentration at the base of a flat, uniformly thick biofilm was calculated using the following equation from Stewart (126):

$$t_{90} = 1.03 \frac{L_f^2}{D_e}$$

where L_f is the biofilm thickness and D_e is the effective diffusion coefficient in the biofilm (126). D_e was taken to be 25% of the aqueous diffusion coefficient, D_{AB} , based on published approximations (125). The biofilm thickness was estimated at $500 \mu\text{m}$ based on measurements from Lauchnor (127). Altogether, this resulted in a calculated time requirement of approximately 21 minutes for 90% of the triclosan present in the bulk fluid to diffuse to the base of the biofilm. However, this model does not account for biomass sorption, which we know to be a major factor during TCS exposure to AOB. Biofilms also are not uniformly thick, which may increase or decrease the diffusion time requirement for TCS in certain areas. Furthermore, this equation does not account for the

decrease in TCS concentration in the bulk fluid with time due to sorption. For these reasons it is safe to assume that greater than 21 minutes were required for 90% of TCS diffusion to the substratum.

Altogether, adsorption was responsible for triclosan loss in pure culture experiments. Neither *N. europaea* nor *N. winogradskyi* were capable of degradation of TCS, which suggests that the degradation observed in activated sludge cultures was due to the activity of different nitrifying bacteria than those tested, or that other bacteria are needed to promote AOB-driven TCS degradation by eliminating potentially toxic daughter products. *N. europaea* biofilms were also incapable of TCS degradation. Suspended cell cultures saw immediate sorption of TCS after inoculation, with slight decreases occurring thereafter, while a diffusion period was required for sorption to biofilms.

CHAPTER FIVE – DISCUSSION

Nitrification Loss in Activated Sludge

Nitrite oxidation by NOB in activated sludge appeared to be more reduced by triclosan than ammonia oxidation by AOB. This conclusion was drawn because nitrite accumulated in 8 and 16 ppm TCS-treated samples, indicating that NOB were incapable of consuming the available nitrite. Nitrate production was also severely reduced in samples containing TCS, with increasing TCS concentrations corresponding to decreasing nitrate production. Other studies have also found NOB to be more sensitive to TCS exposure than AOB, with no nitrite oxidation observed in the presence of over 2 ppm TCS (23, 128).

Ammonia oxidation was also reduced due to TCS exposure, though AOB were still capable of nitrite production in the presence of up to 16 ppm TCS. Increasing TCS concentrations correlated with decreasing nitrite production rates. This pattern was observed during both long- and short-term TCS exposure. Extent of ammonia oxidation loss appeared to increase with exposure time—for example, ammonia oxidation rates in activated sludge with an average TSS of 330 mg/l were only reduced 15% by 16 ppm TCS during 3-hour experiments, but loss of activity increased to 77% over 3 days of exposure. Likewise, ammonia oxidation in sludge exposed to 8 ppm TCS was not impacted at all over 3 hours, but was reduced by 56% during a 3-day exposure period. This was likely due to the slow death of AOB over time, resulting in decreased ammonia oxidation rates. Nitrite accumulation in 3-day experiments may have also resulted in

feedback inhibition of AOB. Additionally, the bacteriostatic properties of TCS may have prevented AOB replication in treated samples, while AOB in controls continued to proliferate.

Interestingly, increased TSS in activated sludge tests appeared to correlate with increased loss of nitrification. Samples with 610 mg/l TSS experienced greater reductions in both ammonia and nitrite oxidation than samples with an average TSS of 330 mg/l during three hour experiments. This may be because samples with higher TSS had greater populations of active bacteria, thus resulting in greater potential inactivation. This hypothesis agrees with results from published studies, which have demonstrated that increased bacterial activity results in increased inhibition (5, 25, 129).

Impacts of TCS on Respiration in Nitrifying Biofilms

Activated sludge biofilms saw significant reduction in oxygen consumption during TCS exposure. Reduction in oxygen consumption appeared to plateau at 35% with 7 ppm TCS, and no reduction was observed below 4.6 ppm. This concentration is much higher than that typically seen in wastewater, however, and therefore it is possible that nitrifying biofilms in WWTPs may not see any reduction of oxygen flux during exposure to lower TCS concentrations. It is also important to note that biofilms were heterogeneous in both thickness and bacterial populations, and therefore oxygen consumption at the point of profile measurement was not necessarily representative of oxygen consumption throughout the entire biofilm. For this reason, it is difficult to quantify the reduction in oxygen consumption experienced throughout the entire biofilm,

since the amount of reduction seen at different TCS concentrations is likely correlated with biofilm thickness and activity. Also, because oxygen consumption was measured rather than nitrification efficacy, activated sludge biofilms cannot be directly compared to suspended cell sludge tests. Biofilms were grown for over a year as well, and therefore bacterial populations were likely different than those in suspended cell activated sludge.

Triclosan was shown to reduce respiration by both nitrifiers and heterotrophs in these biofilms, with no nitrifier respiration occurring at roughly 7.5 ppm TCS. Stasinakis et al. demonstrated that TCS also inhibited oxygen consumption by heterotrophs in suspended cell activated sludge, with 10 ppm TCS causing 20% inhibition of specific oxygen uptake rate in 5 day old sludge (22). Increased sludge age, exposure time, and TCS concentration also resulted in higher inhibition (22). Altogether, this demonstrates that TCS reduces oxygen consumption by both suspended cells and biofilms of activated sludge.

Impacts of Triclosan on *Nitrosomonas europaea*

Pure cultures of *N. europaea* experienced less reduction in nitrite production by TCS than published data—Hughes reported 40% inhibition of nitrite production in the presence of just 1 ppm TCS, and Roh et al. reported 70% inhibition in the presence of 2 ppm TCS (24, 100). This is likely due to variations in cell concentration, as inhibition tends to decrease as cell density increases.

Ammonia oxidation in activated sludge was much less affected by TCS than in pure cultures. During a 3-hour exposure period, AOB in activated sludge were not

impacted by 8 ppm TCS, and experienced approximately 15.5% decrease in nitrite production during exposure to 16 ppm. In contrast, ammonia oxidation in pure cultures exposed to 8 ppm TCS was reduced by 44%. This is likely due to smaller populations of AOB in activated sludge, allowing other organisms and organic matter present in the sludge to shield AOB from TCS. Sorption of TCS to these materials also decreased dissolved concentrations. For example, after an initial sorption period, the concentration of TCS in sludge originally exposed to 8 ppm was approximately 4 ppm. Conversely, the dissolved concentration of TCS in pure cultures initially exposed to 8 ppm was 5.9 ppm. In other words, AOB in activated sludge were exposed to lower concentrations of TCS than pure cultures, which may have decreased the reduction observed. The AOB in sludge may have also been less active than those in pure cultures, though it is difficult to confirm this because populations are unknown.

Reductions in ammonia oxidation rates increased with substrate concentration in pure cultures (Figure 20). This may be due to increased ammonia oxidation rates per cell in cultures with higher substrate concentrations, consequently resulting in increased uptake of triclosan or more effective antimicrobial activity against more actively respiring cells. Other research has documented a similar trend: Lee et al. found that increased ammonia oxidation rates correlated with increased TCS degradation in pure cultures of *N. europaea*, indicative of increased uptake (101). The ratio of TCS to total ammonium may also play a role in TCS toxicity. However, because influent total ammonium in domestic wastewater typically only varies from 1-2.5 mM NH₃ (1), the reduction in ammonia oxidation measured at total ammonium concentrations over 2.5

mM is unlikely to occur. Influent ammonia concentrations in industrial wastewaters varies from 0.5-3.2 mM NH₃ (1), and therefore reductions may not even occur when influent NH₃ is below 1 mM according to our results. Since wastewater typically contains far smaller concentrations of TCS as well (18), reductions in ammonia oxidation are expected to be less than the fractions observed here—however, TCS build up in treatment systems could increase impacts on AOB, or decreases in nitrite oxidation by NOB could cause nitrite accumulation and lead to product inhibition of AOB.

Enzyme Inhibition Mechanisms

Inhibition did not appear to be reversible in pure cultures of *N. europaea*, given that ammonia oxidation rates did not increase after exposure. This may be due to the high concentration of TCS used (8 ppm) during the treatment portion of the test, as other publications have observed recovery after exposure to 1 ppm TCS (100). However, because *N. europaea* cells exposed to 2 ppm TCS experienced just 6% reduction in nitrite production in the experiments described herein, recovery tests using a lower TCS concentration would have been unreliable.

Other research has hypothesized that triclosan inhibits the AMO enzyme in a competitive fashion (24, 101, 102), which is a form of reversible inhibition. However, all of these publications used 2 ppm TCS and none showed conclusive evidence of competitive inhibition. For this reason, the irreversible inhibition observed here appears to be a valid result of 8 ppm TCS exposure. Exposure to lower TCS concentrations may result in behavior consistent with a reversible inhibition model.

The lack of recovery of *N. europaea* cells after 8 ppm TCS exposure is also reasonable when the mechanism of action of triclosan is considered. At bactericidal levels, TCS causes membrane damage (103, 104). This could be occurring during 8 ppm exposure, resulting in an irreversible loss of cell activity. The concentration at which TCS becomes bactericidal for *N. europaea* has not been reported, but these results indicate that it may be between 1-8 ppm. Eight ppm triclosan also was found to have an inhibitory effect on AMO independent respiration, including the hydroxylamine oxidoreductase (HAO) enzyme and downstream electron transport, which could be another reason for the irreversible inhibition seen here. Research on HAO inhibition is limited, however, and few inhibiting compounds are known. Of those, phenylhydrazine is aromatic, and brachialactone is an aliphatic hydrocarbon (130-132). Thus, the exposure to TCS may be eliciting a similar inhibitory response as these compounds with similar functional groups.

Inhibitory impacts on HAO-catalyzed conversion of hydroxylamine to nitrite appeared to occur in a delayed fashion, with nitrite production occurring at the same rate as controls over the first 90 minutes of TCS exposure. Thereafter, nitrite production stopped entirely in treated samples (Figure 22). There are multiple possible reasons for this: triclosan could be killing cells slowly over time, and nitrite production occurs linearly until a population limit is reached. TCS may also not be directly impacting the HAO enzyme, and is instead interfering with electron transfer in some other way. Lastly, TCS binding with HAO could be a time-dependent process, causing inhibitory effects to not be immediately evident after inoculation.

Biofilm vs. Suspended Cell Reductions in Nitrification

Triclosan reduced ammonia oxidation in biofilms of *N. europaea* to almost the same degree as their suspended cell counterparts (Figure 27). As mentioned previously, this is unexpected, given that previous research has shown biofilms of *N. europaea* to be more resistant to contaminants (4, 26). Powell and Prosser showed that established biofilms of *N. europaea* were more resistant to the nitrification inhibitor nitrapyrin than suspended cells, and that detached biofilm cells were also more resistant, likely due to protection from retained extracellular polymeric material (26). Lauchnor et al. demonstrated that the concentration of phenol or toluene necessary for 50% inhibition was 5 to 7 times higher in *N. europaea* biofilms than suspended cells (4). However, they later demonstrated that the reduced activity of biofilms was responsible for the lower inhibition observed, and that suspended cells with decreased activity could potentially become as resistant to phenol inhibition as biofilms (5).

Using this information, the rates of ammonia oxidation in suspended cells and biofilms unexposed to TCS were compared. In the presence of 5 mM NH_4^+ , suspended cells produced nitrite at approximately $82 \pm 9.6 \text{ mmol NO}_2^- \text{ g protein}^{-1} \text{ hr}^{-1}$. Rates of production in biofilms were more varied, but all were much slower, from 4.5 to 22 $\text{mmol NO}_2^- \text{ g protein}^{-1} \text{ hr}^{-1}$. After exposure to 4 ppm TCS, ammonia oxidation in biofilms with an average activity of $4.5 \text{ mmol NO}_2^- \text{ g protein}^{-1} \text{ hr}^{-1}$ was reduced by $25 \pm 4.2\%$, while oxidation by suspended cells was only reduced by $18 \pm 4.5\%$. Suspended cell exposure to 8 ppm TCS resulted in $43.7 \pm 4\%$ reduction, while oxidation in biofilms with an average activity of $21.8 \pm 8 \text{ mmol NO}_2^- \text{ g protein}^{-1} \text{ hr}^{-1}$ was reduced by $35 \pm 4\%$. In other words,

ammonia oxidation by biofilms was significantly more reduced at 4 ppm TCS and less at 8 ppm.

Altogether, this presents conflicting results regarding the relative impacts of TCS on biofilms and suspended cells of *N. europaea*. These results suggest that at higher TCS concentrations, ammonia oxidation in biofilms may be slightly less reduced than suspended cells. However, at concentrations of 4 ppm or lower, biofilms were more impacted by TCS than suspended cells, even though the specific nitrite oxidation rate was much lower in the biofilms. This conclusion is in contrast with the studies in the literature on *N. europaea* biofilms discussed above (4, 5, 26). However, these published studies performed biofilm exposure experiments under flow conditions, unlike experiments described here, and therefore the change in experimental conditions may account for the differing results observed. The specific biofilm activity may also affect the magnitude of TCS impacts—prior to TCS addition, the biofilms in the 8 ppm TCS experiment were approximately five times as active as those in the 4 ppm TCS experiment. This difference was due to the shorter time period that the biofilms had been growing prior to each experiment. Less biomass was also present in the biofilms exposed to 8 ppm, indicating possible differences in TCS sorption and bioavailability.

Regardless, this finding suggests that AOB in biofilms used for batch wastewater treatment may not be more resistant to TCS, particularly when outer aerobic layers of the biofilm are in direct contact with TCS-containing wastewater. Membrane biofilm reactors, however, may provide a method of biofilm treatment in which AOB escape TCS impacts: in this system, AOB are located in the inner layers of the film, near the

membrane, and heterotrophs are located in the outer layers (133). Heterotrophic bacteria could therefore potentially protect the AOB from triclosan exposure.

Triclosan Impacts on *Nitrobacter winogradskyi*

Nitrite oxidation in activated sludge was typically less impacted by TCS than it was in pure cultures of *N. winogradskyi*. Although activated sludge samples were initially exposed to 8 ppm TCS in three hour exposure experiments, the effective dissolved concentration after adsorption occurred was approximately 2.6 ppm. These samples experienced 35% reduction in nitrite oxidation. In contrast, pure *N. winogradskyi* cultures exposed to just 0.75 ppm TCS experienced 98% reduction in nitrite oxidation.

The difference in reduction of nitrite oxidation in pure and wastewater cultures is likely due in part to variations in NOB concentrations, as well as sorption of TCS to materials other than NOB. As was hypothesized for AOB, organisms and organic matter present in activated sludge likely also shielded NOB whilst also decreasing dissolved TCS concentrations. Lower available substrate nitrite concentrations in activated sludge experiments may have also decreased oxidation rates, thereby lessening the impacts of TCS. Our results have shown this to be true for AOB (Figure 20). This has positive implications for both AOB and NOB in wastewater treatment: if other material in activated sludge can shield nitrifiers from inhibitory compounds, then the effects of such compounds may be less evident.

NOB, in particular, are at risk during TCS exposure, since they are vulnerable to much smaller concentrations of TCS than AOB. The reason for reduced nitrite oxidation

by NOB in the presence of TCS is not well-documented, but some research suggests that the nitrite oxidoreductase (NXR) enzyme used by NOB may be susceptible to toxicity or inhibition by contaminants. The NXR enzyme belongs to the dimethyl sulfoxide reductase family of enzymes (13), some of which have been found to have a broad substrate specificity (14). Though this does not conclusively prove that NXR inhibition is the reason for the impacts seen here, it gives one possible explanation. The other bacteriostatic or bactericidal qualities of TCS are also certain to reduce nitrite oxidation by NOB.

Pure cultures of *N. winogradskyi* were capable of recovery after exposure to 0.6 ppm TCS. This suggests that short term exposures of activated sludge to triclosan do not have a lasting impact on nitrite oxidation efficacy, if the TCS concentration is small enough. Since most influent TCS concentrations are on the ppb level, recovery should be possible after short term exposure in WWTPs. However, a more likely scenario in municipal WWTPs is consistent exposure to contaminants such as TCS, due to ongoing household use.

Triclosan Fate and Mode of Action

Adsorption

Measured triclosan losses were overwhelmingly found to be due to adsorption rather than degradation, resulting in decreases in the concentration of dissolved TCS. Interestingly, this did not appear to decrease TCS impacts in three hour activated sludge exposure experiments. Nitrate production in sludge initially exposed to 16 ppm TCS with an average TSS of 330 mg/l was reduced by $75 \pm 3\%$. The effective TCS concentration in

these experiments was approximately 9.5 ppm. In sludge samples with a higher TSS of 610 mg/l, nitrate production was reduced by $90 \pm 0.3\%$, although effective TCS was only 5.9 ppm after an initial exposure to 16 ppm. In other words, the dissolved concentration after sorption occurred did not correlate with reductions in nitrification in activated sludge. Instead, sludge with a lower effective TCS concentration experienced greater nitrification reduction than sludge with higher dissolved TCS levels.

A similar pattern was observed with initial exposure to 8 ppm TCS: nitrate production in samples with an average TSS of 330 mg/l was reduced by $36 \pm 3\%$ with an effective TCS concentration of 4.3 ppm. Nitrate production in sludge with a higher TSS of 610 mg/l was reduced by $33 \pm 6\%$, although the effective TCS concentration was just 2.6 ppm. The reduction in nitrate production experienced by the two sample sets was not found different to 95% confidence ($p = 0.21$). Again, sludge exposed to a smaller effective TCS concentration was just as impacted as sludge exposed to a higher concentration, post-sorption. This is surprising because lower dissolved concentrations of TCS would be expected to decrease impacts.

Interestingly, when the losses of TCS per gram biomass were considered, sorption in samples with different TSS concentrations was not statistically different to 95% confidence. In sludge exposed to 8 ppm TCS, an average of 10.3 ± 1.6 mg TCS was sorbed per g TSS, and in samples exposed to 16 ppm TCS, an average of 17.8 ± 1.6 mg TCS was sorbed per g TSS. This suggests that sorption is dependent on the TCS concentration added, not the amount of biomass material available for sorption. Altogether, it is difficult to draw conclusions from the previous observations, and it

should be noted that the data is limited and correlations cannot be determined from only two experimental conditions.

In pure cultures of *N. europaea*, triclosan losses in both biofilms and inactive suspended cultures with analogous protein concentrations were found similar on a gram protein basis, with inactive cells seeing slightly greater reductions (Figure 32). This demonstrates that adsorption, not degradation, was responsible for TCS loss. Though sorption did not occur instantaneously in biofilms (as it did in suspended cells), losses were still similar after allowing an appropriate diffusion period. Adsorption may also explain why TCS was still measured after washing suspended cultures of *N. europaea*, and furthermore why these cultures were incapable of recovery after TCS exposure: if TCS remained bound to cell walls, it may have continued to exert an inhibitory effect.

Some studies have shown that adsorption of antibiotics to the cell walls of *E. coli* was followed by changes in membrane potential, thus leading to antibiotic uptake (134, 135). Best et al. also found that adsorption of the antibiotics vancomycin and ristocetin to cell walls was responsible for inhibition of peptidoglycan synthesis in *Bacillus subtilis* (136). Villalain et al. observed that intercalation of triclosan into cell walls of the gram negative bacteria *Porphyromonas gingivalis* did not cause cell lysis, but resulted in perturbed membranes and a secondary antibacterial effect independent of fatty acid synthesis (137). Since nitrifiers are also gram-negative microorganisms, it's possible that a similar mechanism is taking place during TCS sorption to cell walls.

Biodegradation

Nitrifiers in pure cultures were not capable of degradation of TCS, even at the lowest tested concentrations (2 ppm for AOB; 0.25 ppm for NOB). Interestingly, degradation was observed in activated sludge initially exposed to 2 ppm TCS. After isolation of both heterotrophic and nitrifier activity in activated sludge, only nitrifiers were responsible for the TCS losses measured. However, because pure cultures of both *Nitrosomonas europaea* and *Nitrobacter winogradskyi* were incapable of degradation, it may be possible that a nitrifying organism other than the two tested is responsible, or that growth conditions in pure cultures were not as conducive to cometabolism as those in activated sludge. Other publications have documented TCS degradation by both nitrifiers and heterotrophs, with heterotrophic bacteria typically degrading greater fractions of TCS (24, 101, 102). Roh et al. showed that both nitrifying activated sludge and *N. europaea* could degrade triclosan, and used medium containing micronutrients for pure culture degradation tests (24). It is possible that there were micronutrients present in activated sludge that were not added during pure culture experiments, thus allowing for degradation of TCS in sludge. Other bacteria present in activated sludge may have also helped nitrifiers degrade TCS by consuming or further degrading toxic byproducts of nitrifier cometabolism. These byproducts may have prevented cometabolism in pure cultures.

CHAPTER SIX – CONCLUSIONS

Triclosan reduced nitrification by *Nitrosomonas europaea*, *Nitrobacter winogradskyi*, and a consortium of activated sludge microorganisms. In all cases, increased triclosan concentrations were related to greater reductions in nitrifying activity. Pure culture studies of TCS impacts on *N. europaea* also found that increasing substrate concentrations resulted in greater reduction of ammonia oxidation, likely because increased ammonia uptake co-occurred with increased uptake of TCS.

Both the AMO and HAO enzymes used by *N. europaea* were negatively impacted by TCS. Ammonia oxidation was irreversibly inhibited by 8 ppm TCS exposure, though exposure to lower concentrations may have resulted in a reversible enzyme inhibition mechanism. Future work could investigate this possibility. AMO-independent respiration by HAO and downstream electron transport processes were also inhibited by triclosan.

Surprisingly, ammonia oxidation by suspended cells and biofilms of *N. europaea* was reduced to nearly the same extent by triclosan exposure. Although suspended cells had the highest activity, they were less impacted by 4 ppm TCS than biofilms, and only 9% more impacted by 8 ppm TCS exposure in the presence of 5 mM total NH₄. This shows that the biofilm phenotype does not offer any protection from TCS in pure cultures of *N. europaea*.

Pure culture biofilms and suspended cells of *N. europaea* did not degrade triclosan. All losses were due to adsorption to cellular material, and sorption to biofilms was less than that to inactive biofilm-representative suspended cells, likely due to diffusion limitations. The sorption of TCS to cell material may explain why *N. europaea*

was incapable of recovery after TCS exposure—remaining sorbed TCS may have continued to exert an inhibitory effect. Further research could explore the hypothesis that sorption is a mechanism of triclosan inhibition.

Pure cultures of *Nitrobacter winogradskyi* and NOB in general were much more impacted by triclosan than AOB. Pure cultures of *N. winogradskyi* could not oxidize nitrite in the presence of 0.75 ppm TCS or higher, and nitrite oxidation in activated sludge was also severely reduced, with exposure to just 2 ppm TCS resulting in 25% reduction. Inhibition of pure cultures was found reversible, however, with recovery possible after 0.6 ppm TCS treatment. Although exposure to higher concentrations may have resulted in irreversible inhibition, TCS concentrations in wastewater are typically on the ppb level and therefore NOB recovery should occur. Pure cultures of *N. winogradskyi* were also incapable of TCS degradation.

Despite the increased sensitivity of NOB in general, NOB in activated sludge were more resistant to TCS impacts than pure cultures. This was likely due to the lower fraction of nitrite oxidizers in activated sludge, and suggests that other organisms and materials in sludge may provide protection from TCS. Lower substrate concentrations in the activated sludge experiments also could have resulted in decreased TCS impacts, as was shown in pure cultures of *N. europaea*. Further investigations should be conducted to strengthen this conclusion.

Despite determining that neither *N. europaea* nor *N. winogradskyi* could degrade triclosan, nitrifiers in activated sludge were responsible for degradation of 2 ppm TCS. This indicates that growth conditions and/or other bacteria present in activated sludge aid

in TCS degradation. Future studies aimed at isolating both the bacteria responsible for the degradation observed here and the specific nutrients present in activated sludge that encourage degradation could provide useful information—WWTPs experiencing issues with TCS contamination could potentially enrich conditions for growth of these bacteria to effectively remove TCS.

At TCS concentrations greater than 2 ppm, adsorption was again responsible for all losses of TCS in activated sludge. Interestingly, sorption was not found to increase with TSS, and decreased concentrations of dissolved TCS did not correlate with decreased impacts. However, these trends are based on results from two experimental conditions, and therefore further research should be conducted to determine if these trends are credible.

Triclosan exposure decreased respiration by heterotrophic and nitrifying bacteria present in nitrifying biofilms. Approximately 7.5 ppm TCS stopped respiration by all nitrifying bacteria. These results demonstrate that TCS can decrease the activity of a variety of different aerobic organisms. However, reduction of respiration was concentration dependent, and no impacts were measured below 4.6 ppm TCS. This effect is undoubtedly dependent on biofilm thickness, bacterial populations, and growth conditions, and therefore further research should be conducted to determine which variables increase and decrease TCS impacts. Microbial community analyses of the bacterial populations in nitrifying biofilms most affected by TCS would also offer valuable information.

Altogether, studies on the effects of triclosan on nitrifying bacteria offered valuable information. This research shows that triclosan reduced nitrification by both AOB and NOB, with far greater impacts on NOB. Respiration by heterotrophs in a nitrifying biofilm was also negatively impacted. Ammonia oxidation by biofilm-grown *N. europaea* cells was no more resistant to TCS exposure than suspended cells, though future investigations should determine if AOB in mixed activated sludge biofilms are more resistant to TCS exposure than either suspended sludge or pure culture biofilms. Because triclosan removal in WWTPs is still both unregulated and nebulous, there is the potential for accumulation of TCS in treatment systems. This could further reduce nitrification, leading to degradation of water quality. Additionally, TCS exposure could decrease AOB and NOB populations over time, furthering breakdowns in nitrogen removal. The negative impacts of triclosan, in addition to those of other emerging contaminants, are likely to present a large challenge to effective wastewater treatment in the coming years.

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