AN ABSTRACT OF THE THESIS OF

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Title: Inhibition of the Ammonia Oxidizing Bacteria *Nitrosomonas europaea* by the Emerging Contaminant Triclosan

Abstract approved:

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Lewis Semprini

Identifying the inhibition of ammonia oxidizing bacteria (AOB) by emerging organic contaminants is crucial due to the importance of AOB in wastewater treatment, the widespread use of antibacterial agents such as triclosan (TCS) in consumer products, and the sensitivity of *N. europaea* to inhibitors. Triclosan inhibition of nitrification by AOB *N. europaea* cells was determined via suspended cell batch reactor experiments using cells grown in batch and in a chemostat. Specific oxygen uptake rate tests (SOURs) were performed on both the ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) enzymes to determine the enzyme inhibition mechanism of TCS. Inhibition of long term growth of *N. europaea* cells and development of antibacterial resistance when exposed to low concentrations of TCS were also studied via suspended cell batch growth experiments. A colorimetric nitrite assay was used to quantify nitrite production and UHPLC-MS analysis was used to analyze for the cometabolic transformation of TCS.

Three hour inhibition tests showed that *N. europaea* cells are inhibited by TCS at concentrations ranging from 0.1 to 8 ppm TCS, but activity partially recovered when

washed and re-suspended in fresh media.   Triclosan inhibition results show a non-linear increase in nitrification inhibition with increasing TCS concentration with 40% inhibition occurring at 1 ppm TCS and 90% inhibition at 8 ppm TCS. The recovery of previously inhibited cells, relative to control cells, fit a semi-log plot, with cell inactivation being dependent on TCS concentration and the time of exposure, similar to models used for disinfection. Results of the exposure of chemostat-grown *N. europaea* cells to 1 ppm TCS showed less inhibition than observed with batch-grown cells. SOURs results indicate that the AMO enzyme is directly inhibited by TCS, either through competition for its active site or interactions of TCS with AMO, and that the HAO enzyme is not inhibited by TCS. Results of suspended cell growth experiments indicate that growth of *N. europaea* is inhibited by approximately 30% at 0.01 ppm TCS; where concentrations at or exceeding 0.05 ppm TCS significantly inhibit cell growth.   Resistance tests showed no development in resistance to 0.01 ppm TCS over three growth cycles or approximately 67 generations of growth. UHPLC-MS analyses indicate that TCS is not transformed in the presence of *N. europaea* over a three hour direct exposure period or during long term growth of several days.

Inhibition of the Ammonia Oxidizing Bacteria *Nitrosomonas europaea* by the Emerging
Contaminant Triclosan

by
Jamie L. Hughes

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented May 21, 2013
Commencement June 2013

Master of Science thesis of Jamie L. Hughes presented on May 21, 2013.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Jamie L. Hughes, Author

ACKNOWLEDGEMENTS

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Chapter 1: Introduction

1.1.Background and Motivation

As world populations grow and potable water becomes scarce, incentives for maintaining adequate surface water quality escalate. Therefore, the utilization and efficiency of wastewater treatment is integral to the health and well-being of receiving waters upon which expanding populations rely on as a drinking water source. Biological wastewater treatment has been used for decades to remove potentially harmful contaminants from wastewater prior to its discharge into the environment. The utilization of microbial activity to degrade contaminants, such as nitrogen, is an especially significant process due to the harmful effects that excess nutrients have on water quality and aquatic life due to eutrophication (1).

The removal of nitrogen during biological treatment is done through nitrification, the oxidation of ammonia (NH_3) to nitrate (NO_3^-), and denitrification of NO_3^- to dinitrogen gas (N_2). The process of nitrification is carried out in two steps; the oxidation of NH_3 to nitrite (NO_2^-), which is performed by ammonia oxidizing bacteria (AOB), and the oxidation of NO_2^- to nitrate (NO_3^-), which is carried out by nitrite oxidizing bacteria (NOB). This sequential nitrification process is part of the biogeochemical nitrogen cycle in natural ecosystems that is essential for life on earth (2, 3). The role of AOB in biological wastewater treatment is the focus of this research as it is the step that is most

sensitive to disruption and therefore integral to understanding potential risks posed by emerging contaminants.

Nitrosomonas europaea, a species of AOB, performs the first step in nitrification and is essential for nitrogen removal in wastewater treatment plants (WWTP) (4). *N. europaea* is an obligate chemo-lithoautotroph that derives all of the reductant required for energy and biosynthesis from the oxidation of NH_3 to NO_2^- and all of its carbon for growth from carbon dioxide (CO_2) (5). As a result, AOB are typically slow growing (4) and sensitive to a variety of compounds including heavy metals (6, 7), temperature and pH shifts (8) and organic compounds (9, 10, 11). *Nitrosomonas europaea* has been used extensively as a model organism due to the ease of its cultivation relative to other obligate ammonia oxidizing bacteria (4, 8) and because it is considered a target microorganism for the study of microbial inhibition caused by chemicals in wastewater (12).

In an age of mounting mysophobia, the use of antibacterial agents in personal care and consumer products has increased drastically. Triclosan (TCS), an antibacterial agent commonly found in consumer and personal care products such as soaps, toothpastes, detergents, fabrics and plastics (13), has become the most widely used bisphenol in the last 30 years (14). There is concern that the use and subsequent disposal of household products containing TCS will result in the accumulation of TCS in WWTP. This buildup of TCS could affect the efficiency of the functionally significant AOB, such as *N. europaea*, to oxidize NH_3 to NO_2^-, and lead to the release of excess nitrogen into the environment.

This research focuses on determining the inhibition and toxicity of TCS to the AOB, *N. europaea*, in order to define potential adverse effects that this emerging contaminant may pose on the efficiency of the wastewater treatment process.

1.2. Problem Statement

Because of the increasing addition of TCS to consumer products and the potential accumulation of TCS in WWTP, it is essential to understand the adverse impacts of TCS on the functionally important AOB since WWTP rely heavily on microbial activity for successful treatment (12). Triclosan could potentially inhibit the ability of AOB, such as *N. europaea*, in the wastewater treatment process to oxidize NH_3 to NO_2^- resulting in the discharge of nitrogen into receiving water bodies. Heavy nitrogen loadings to surface waters could negatively affect aquatic life and water quality due to algal bloom accumulation and subsequent dissolved oxygen depletion.

1.3. Significance

Understanding the interactions between *N. europaea* and TCS is necessary in order to uncover potential risks posed by this commonly-used antimicrobial to the efficiency of the wastewater treatment process and the health of receiving water bodies. Determining if TCS inhibits the ability of AOB to oxidize ammonia is significant. The role of *N. europaea* in removing nitrogen during the biological treatment phase is critical for successful wastewater treatment. Inhibition of the microbial process could result in excess nutrient discharge to the environment and negative ecological impacts. Determining if low concentrations of TCS inhibit the growth of *N. europaea* is also significant. The ability of *N. europaea* to grow in WWTP is critical to the successful removal of nitrogen from wastewater. Determining if the exposure of *N. europaea* to

TCS will result in its transformation is also significant. The transformation of TCS into another, potentially more toxic compound, could result in greater adverse environmental effects than those posed by the parent compound. Determining if *N. europaea* cells grown in a chemostat are less inhibited than batch-grown cells is also significant. With a constant influent flow of ammonia in wastewater, the process by which cells grow in WWTP more closely resembles that of chemostatic growth. Greater resistance demonstrated by cells grown in a chemostat will provide insight into the behavior of AOB inhibited by pharmaceuticals in WWTP.

1.4. Objectives

The main goal of this research was to determine the inhibition and toxicity of the emerging contaminant TCS to the ammonia oxidizing bacterium, *Nitrosomonas europaea*. The specific objectives pursued in order to achieve this goal include:

1) Determine if inhibition of ammonia oxidation by *N. europaea* occurs when exposed to TCS.

2) Determine if short term exposure of *N. europaea* to TCS fits a disinfection model.

3) Determine which enzyme of *N. europaea* is inhibited by TCS.

4) Determine if TCS is cometabolically transformed by *N. europaea*.

5) If TCS is cometabolically transformed by *N. europaea*, determine its degradation products.

6) Compare the inhibition of ammonia oxidation by *N. europaea* cells grown in batch versus *N. europaea* cells grown in a chemostat when exposed to TCS.

7) Determine if the growth of *N. europaea* is inhibited by low concentrations of TCS.

Chapter 2: Literature Review

2.1. Nitrogen Cycling

The biogeochemical nitrogen cycle refers to the cycling and transformation of the many forms of nitrogen that exist in the environment and it is essential for life on earth (2, 3). The transformation of nitrogen compounds within the nitrogen cycle can occur through several mechanisms including ammonification, fixation, synthesis, nitrification and denitrification. These nitrogen transformations are all carried out by specific microorganisms that naturally occur in the environment. Nitrification is the biological oxidation of NH_3 to NO_3^- which is carried out by chemoautotrophic bacteria and denitrification is the biological reduction of NO_3^- to N_2 performed by heterotrophic bacteria (1).

2.2. Biological Nitrification in Wastewater Treatment

Ammonia is an abundant contaminant in wastewater due to its many sources including municipal and industrial wastes, leachates, atmospheric deposition and surface runoff (1). The excessive accumulation of ammonia in surface waters can cause harmful environmental effects including ammonia toxicity to aquatic animal life, adverse public health effects, algal bloom accumulation and subsequent depletion of dissolved oxygen. As a result, the U.S. Environmental Protection Agency (EPA) has established limits for the discharge of nitrogen from WWTP (1). The use of microbial processes including nitrification and denitrification to remove ammonia during wastewater treatment is commonly used to meet wastewater nitrogen effluent standards.

Ammonia removal during wastewater treatment is carried out through nitrification and subsequent denitrification. The process of nitrification is a two-step process in which NH_3 is oxidized to NO_2^- by ammonia oxidizing bacteria (AOB) and NO_2^- is oxidized to NO_3^- by nitrite oxidizing bacteria (NOB). Denitrification is the reduction of NO_3^- to N_2 under anoxic conditions by heterotrophic bacteria (2, 15). The initial NH_3 oxidation performed by AOB is the most critical step in biological nitrogen removal.

2.3. *Nitrosomonas europaea*

2.3.1. *Ammonia Oxidation*

Ammonia oxidizing bacteria (AOB) are obligate chemo-lithoautotrophs that utilize the oxidation of NH_3 to NO_2^- to derive reductant for energy and biosynthesis. AOB are autotrophs that satisfy all of their carbon requirements for growth through CO_2 fixation (5). *N. europaea*, a species of AOB, performs the first step in the nitrification process and is critical to the removal of ammonia from WWTP (4).   The biological removal of nitrogen by *N. europaea*, and other AOB, is carried out in two steps; each catalyzed by different enzymes. The membrane-bound ammonia monooxygenase enzyme (AMO) catalyzes the oxidation of NH_3 to the intermediate product, hydroxylamine (NH_2OH), and the multi heme-containing periplasmic hydroxylamine oxidoreductase enzyme (HAO) catalyzes the oxidation of NH_2OH to NO_2^- (3, 16, 17, 18). The entire two-step reaction yields four electrons; two of which are subsequently transferred to AMO to activate O_2 and maintain steady-state rates of ammonia oxidation. The remaining two electrons are used for other reductant-requiring cellular processes including biosynthesis and ATP generation (2, 3, 5). The stoichiometric equation for ammonia oxidation carried out by AOB is as follows:

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$$
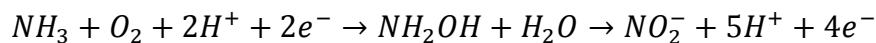
AMO is capable of oxidizing a broad range of non-growth substrates cometabolically including aliphatic hydrocarbons, aromatic hydrocarbons, and chlorinated compounds (3, 9, 16, 17, 18, 19). Over 40 compounds have been shown to be non-growth substrates of AMO and can competitively inhibit NH_3 oxidation. The requirements for reducing equivalents for monooxygenase activity, typically supplied by the oxidation of hydroxylamine, results in the need for co-oxidation of NH_3 during alternate substrate oxidation (3). Alternative substrates can influence AMO activity by three distinct mechanisms: (1) direct binding and interaction with AMO; (2) interference with the reductant supply to AMO; and (3) oxidation of substrates to highly reactive products that covalently bind and inactivate AMO (17).

2.3.2. *Sensitivity and Importance*

The energy-intensive process of deriving carbon for metabolic growth from CO_2 results in low yields and the slow growth of AOB when compared to heterotrophic bacteria (4). The slow growth of AOB makes them more susceptible to environmental perturbations and inhibition by chemical compounds. AOB are sensitive to a variety of compounds including heavy metals (6, 7), temperature and pH shifts (4, 8) and organic solvents including monoaromatic hydrocarbons (9, 10), polyaromatic hydrocarbons (16), and chlorinated hydrocarbons (11, 17, 18). However, AOB such as *N. europaea*, have been shown to be more resistant to inhibition by phenol when grown in chemostat type conditions than when grown in batch cultures (20).

N. europaea is well-accepted as an excellent model AOB for nitrification inhibition experimentation due to its ease of cultivation (4) and the availability of a wide range of physiological and transcriptional methods that aid in the characterization of nitrification inhibition mechanisms (2, 4, 21). Its well-defined NH_3 metabolism and heightened sensitivity to a diverse range of chemical compounds make it ideal for studying nitrification inhibition by pharmaceutically active compounds.

2.4. Emerging Organic Contaminant of Interest: Triclosan

2.4.1. Introduction

As public concern over the transmission of disease heightens, the use of antimicrobials is anticipated to increase. Triclosan, a broad spectrum antimicrobial, has been used throughout North America, Europe, and Asia as an ingredient in disinfectants, soaps, detergents, deodorants, toothpastes, and mouthwash in addition to fabrics and plastics and innumerable other personal care, industrial, and household products (22). This halogenated biphenyl ether and newly emerging contaminant was invented over 40 years ago and has been used increasingly over the past 25 years (23, 24, 25). Between 1992 and 1999, a majority of 700 antibacterial consumer products on the market contained TCS as an active ingredient (26). Globally, production of TCS has exceeded 1500 tons per year with Europe being responsible for 350 tons (27).

Unlike other organochlorine compounds, the use of TCS in consumer products is not highly regulated, as the antimicrobial has a low acute toxicity and is generally accepted as well tolerated and safe (22, 24, 28).

Triclosan contains functional groups of ethers and phenols and has a molecular weight of 289.54 g mol^{-1} (Figure 1). Triclosan is a white powdered solid that primarily

exists in the neutral and ionized (negatively charged) state in aquatic ecosystems due to
its pKa (Table 1) (29, 30). Although a hydroxyl functional group is present, TCS is
relatively hydrophobic with a very low solubility in water (10 mg L^{-1}) and fairly high
octanol-water partitioning coefficient indicating a potential preference for sorption and
moderate bioaccumulation in the environment. Its low vapor pressure also suggests that
it is unlikely to volatilize in the environment. The thermal stability of TCS is why certain
manufacturers have chosen the antimicrobial for the incorporation into plastics and fibers
(22).
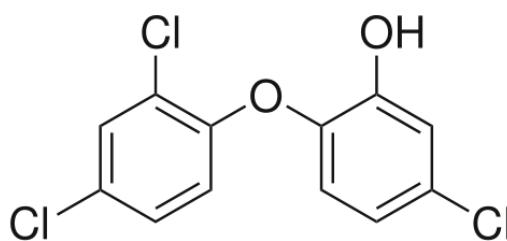
Figure 1. Molecular structure of TCS.

Table 1. Chemical properties of TCS.

Chemical Property	Value
Chemical Name IUPAC Name	Triclosan (TCS) 5-chloro-2-(2,4-dichlorophenoxy) phenol
Molecular Formula	$C_{12}H_7Cl_3O_2$
Molecular Weight	289.54 g mol^{-1}
Appearance	white powdered solid
Odor	slightly aromatic
Boiling Point	280 - 290°C
Melting Point	55-57°C
Log K_{ow}	4.76
Solubility at 20°C	10 mg L^{-1}
Vapor Pressure at 20°C	4 x 10^{-6} mm Hg
pKa	7.9

2.4.2. Triclosan in Wastewater Treatment

Concentrations of TCS in personal care products typically range from 0.1-0.45% of the product weight (31, 32, 33) with a majority of these consumer products (96%) eventually being rinsed down the drain (34) where they ultimately end up in WWTP. Triclosan has been detected in wastewater in the U.S. at concentrations up to 26.8 µg L^{-1} (27, 33, 35, 36, 37, 38). However, studies have shown that during wastewater treatment, approximately 79% of TCS is biodegraded, 15% is sorbed onto biosolids, and 6% is released into receiving water bodies (27, 33). Triclosan removal rates through wastewater treatment range from 58-98% depending on the plant type (trickling filter, activated sludge, rotating biological contactors) (39). The high removal rates of TCS in biosolids during wastewater treatment are due in part to its low solubility in water.

Despite relatively high TCS removal rates during wastewater treatment, a small percentage of TCS usually makes it through the plant untreated. Detected concentrations of TCS in treated wastewater range from 0.042 to 0.213 µg L^{-1} in Switzerland (27) and 0.03 to 2.7 µg L^{-1} in the U.S. (33, 35, 36, 37). These detectable concentrations of TCS are then discharged with the treated wastewater effluent into receiving water bodies such as streams and rivers.

2.4.3. Occurrence and Toxicity in the Environment

Triclosan is one of the most frequently detected pollutants; found in 57.6% of the 139 tested U.S. streams and rivers (40). Triclosan concentrations in natural streams and rivers are typically on the order of ng L^{-1} (27, 32, 33) but concentrations up to 2.3 µg L^{-1} have been detected in U.S. rivers (40). Unfortunately, the prevalence of TCS in

waterways is only likely to increase as consumer demand and ensuing disposal of antimicrobials is anticipated to grow.

Triclosan is considered acutely toxic to fish and other aquatic organisms (29, 41, 42) and may act as an environmental anti-estrogen or androgen (43). The antimicrobial has been detected in fish at concentrations ranging from 0.44 to 120 mg kg^{-1} after being exposed to wastewater effluent (44). The neutral form of TCS is considered to be the most toxic to aquatic organisms as molecules are believed to be less likely to bioaccumulate in the ionized state (29). Due to the TCS pKa of 7.9, environmental conditions will have a significant influence on its predominant state and consequent toxicity. At a neutral water pH of 7.0, much of the TCS would be in the neutral form. Sorption and ionization of this antimicrobial in the environment could also potentially temper the toxic effects instigated by TCS in its neutral form.

2.4.4. Mechanisms of Cellular Inhibition

A bacteriostatic agent is one that inhibits bacterial growth without harming the bacteria otherwise. Triclosan has been shown to primarily demonstrate bacteriostatic activity against Gram-positive bacteria but it is also considered effective against Gram-negative bacteria (14, 24). Originally, TCS was thought to act as a broad-spectrum non-specific biocide by affecting membrane structure and function (24). However, the mechanisms of action involved in cellular attack vary depending on the TCS concentration. At higher concentrations, TCS acts as a biocide with widespread targets but at lower concentrations, TCS targets specific cellular components of bacteria (23).

At sub-lethal concentrations, TCS blocks lipid biosynthesis by interacting with the NADP binding site of the enzyme enoyl-[acyl carrier protein] (ACP) reductase (23,

38, 45), FabI in *Escherichia coli* (13), *Pseudomonas aeruginosa* (46) and *Staphylococcus aureus* (47) or its homologue, InhA, in *Mycobacterium tuberculosis* (48) and *Mycobacterium smegmatis* (49). Triclosan acts as a site-directed, picomolar inhibitor by mimicking the enzyme's natural substrate (45). Inhibition of the fatty acid biosynthetic pathway, in turn, affects the many processes depending on lipid synthesis including phospholipid, lipopolysaccharide and lipoprotein synthesis. The originally reported effects of TCS on membrane structure and function are in reality secondary effects attributable to the disruption of fatty acid biosynthesis (26).

Concentrations of TCS that inhibit the activity of the enoyl-(ACP) enzyme in bacteria are typically lower than concentrations found in personal care products (50). Activity of the FabI enzyme in *P. aeruginosa* was inhibited by 90% when exposed to 0.15 mg L^{-1} (46). The minimum inhibitory concentration (MIC) of TCS for growth is 0.25 mg L^{-1} for *S. aureus* and 0.5 mg L^{-1} for *E. coli* (47, 51).

2.4.5. *Degradation Products and Cometabolism*

Biodegradation and photolysis are well known transformation processes that lead to the conversion of TCS into stable and bioaccumulative compounds (52, 53, 54). Triclosan can be transformed by biological processes into methyl-TCS (55, 56) and the photodegradation of TCS in aqueous solutions can lead to the formation of dioxins such as 2,8-dichlorodibenzodioxin (DCDD) and other dioxin derivatives (53, 57).

There is evidence that TCS, like other phenols, in water or in consumer products, may react with free chlorine to produce chloroform and other toxic and endocrine disrupting chlorinated products, including 2,4-dichlorophenol, 2,4,6-trichlorophenol, and

tetra- and *penta*-chlorinated hydroxylated diphenyl ethers over a range of pH values (58, 59).

Studies have shown that several strains of bacteria are able to cometabolically degrade TCS. A consortium of six different bacteria found in activated sludge from a wastewater treatment plant was able to grow on TCS as a sole source of carbon and energy (60). However, breakdown products of the degradation of TCS by the bacterial consortium were not detected via gas chromatography mass spectrometry (GC-MS) or high pressure liquid chromatography (HPLC) (60). In a different study, the cometabolic degradation of TCS and bisphenol A by *Nitrosomonas europaea* was demonstrated with TCS concentrations of 0.5 to 2 mg L^{-1} (61). The limited investigations of TCS transformation potential by *N. europaea* indicated a more thorough effort of evaluation was needed in this study.

2.4.6. *Toxicity in Humans, Efficacy and Antimicrobial Resistance*

Although TCS is not regulated in consumer products as it is generally accepted as safe (24), studies are uncovering several issues linked with its use. Potential health issues associated with the antimicrobial include antibiotic resistance, skin irritations, endocrine disruption, increasing rates of allergies, and the formation of carcinogenic by-products (26, 44, 57). However, at concentrations present in consumer products, TCS is not considered acutely toxic to humans, carcinogenic, or irritating to the skin and eyes (14).

Triclosan is often favored in personal care products over other antimicrobial compounds due to its mild nature and reduction of nosocomial infections in healthcare settings (24). However, the efficacy of TCS in antibacterial soaps is considered equivocal. Triclosan does not appear to reduce bacterial counts on hands to a greater

extent than plain soap unless it is used repeatedly and at higher concentrations that what is found in consumer antibacterial soaps (31, 62).

Typical concentrations of TCS in personal care products are considered substantially higher than the MIC for most bacteria. Therefore, the development of TCS resistance in bacteria has been considered unlikely under normal conditions (50). However, recent studies have shown that bacterial resistance may develop naturally through decreased susceptibility or it may be acquired through genetic changes including cell mutations or the acquisition of genetic material. Bacteria have been shown to use multiple mechanisms to develop resistance to TCS including target mutations, increased target expression, active efflux and degradative enzymes (23, 26).

Bacteria, such as *Streptococcus pneumoniae*, that possess FabK, an enoyl-ACP reductase that is not affected by TCS, instead of the susceptible FabI, are intrinsically resistant to higher levels of TCS (63). Among Gram-negative bacteria, *Pseudomonas aeruginosa* is unique in that it contains both FabI and FabK in its genome and can develop a natural resistance to TCS through the possession of a mechanism for active efflux of the drug (26). *P. aeruginosa* expressing a single efflux pump during plated growth exhibited a TCS MIC of 130 mg L^{-1} in an aqueous environment of pH 7.0, compared to the wild-type which was inhibited by 50% when exposed to 0.06 mg L^{-1} TCS (26, 46).

Studies have shown that the sustained use of TCS could select for bacterial cross-resistance to antibiotics (51). The acquisition of cross-resistance to antibiotics facilitated by TCS has been demonstrated in a variety of different strains including *Pseudomonas aeruginosa* (64), *Salmonella enterica* (65) and *Escherichia coli* (13, 51). Mutations or

overexpressions of the FabI gene, which is responsible for encoding the enoyl-ACP reductase, prevents TCS blockage of lipid synthesis in *E. coli* (66). Mutation of the target FabI enzyme in *E. coli* over four cycles of growth on a plate initiated the development of cross-resistance resulting in the increase of the TCS MIC from 0.5 to over 2000 mg L^{-1} (51).

2.5.Summary

The increasing addition of TCS in personal care products and its ensuing accumulation in WWTP renders understanding the adverse impacts of TCS on important wastewater AOB, such as *N. europaea*, essential for the continuing success of the wastewater treatment process. This research focused on the inhibition of the functionally important *N. europaea* by lethal and sublethal concentrations of TCS allegedly responsible for inhibiting fatty acid synthesis in bacteria. The cometabolic degradation of low concentrations of TCS, previously accomplished by other bacterial strains as well as *N. europaea*, was investigated in order to verify this phenomenon in *N. europaea* and determine potential breakdown products. Inhibition of the growth of *N. europaea* was studied in order to determine TCS concentrations that inhibited cell growth. As mentioned previously, TCS has been shown to target cell wall synthesis and inhibit bacterial growth. As TCS resistance has been demonstrated by several bacterial strains, the intrinsic development of resistance over many generations of *N. europaea* growth was also investigated.

Chapter 3: Materials and Methods

3.1. Nitrosomonas europaea Batch Growth

Nitrosomonas europaea (ATCC 19718) cells were generously donated by the Dan Arp laboratory. The cells were grown in 4 L flasks containing 2 L autoclaved AOB growth media (pH 7.8) comprised of the following: 25 mM $(NH_4)_2SO_4$, 40 mM KH_2PO_4, 3.77 mM Na_2CO_3, 750 µM $MgSO_4$, 270 µM $CaCl_2$, 18 µM $FeSO_4$, 17 µM EDTA free acid, and 1 µM $CuSO_4$. All chemicals used in the AOB growth media were purchased from VWR (Visalia, CA, USA). The flasks were placed in the dark at 30°C and shaken at 100 RPM until the cells reached the late exponential growth phase (~3-4 days with an OD_{600} ~0.072) prior to harvesting as previously described [19].

3.2. Nitrosomonas europaea Growth in Chemostat Reactors

N. europaea was cultivated in 3 L of minimal growth media in a 7 L water-jacketed Bioflo 110 bioreactor (New Brunswick Scientific, Edison, NJ, USA) [19]. The bioreactor was operated in the dark at 30°C with agitation set to 500 RPM. Dissolved O_2 levels were kept at air saturation levels (8 mg L^{-1}) with the addition of filtered compressed air at 200 ml min^{-1}. The chemostat media contained the following: 12.5 mM $(NH_4)_2SO_4$, 25 mM NH_4OH, 10 µM KH_2PO_4, 730 µM $MgSO_4$, 200 µM $CaCl_2$, 9.9 µM $FeSO_4$, 0.65 µM $CuSO_4$ and 30 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) as a pH buffer. The pH of the reactor medium was maintained at 7.8 by continuous monitoring with the metered addition of 0.5 M Na_2CO_3 which also supplied *N. europaea* with an inorganic carbon source. Prior to inoculation, the bioreactor and minimal medium were autoclaved at 121°C for 40 minutes.

The bioreactor was operated in batch mode until *N. europaea* cells lowered the NH_4^+ concentration below 5 mM. The bioreactor was then switched to a continuous culturing reactor. All conditions were kept as described previously except the agitation was raised to 700 RPM briefly and then slowly decreased to 350 RPM. The influent feed rate was set to approximately 530 ml d^{-1}, to achieve a cell and hydraulic residence time of approximately 6 days.

For TCS inhibition experiments, completely mixed liquid samples were taken aseptically from a port in the bioreactor vessel into glass sample vials. The cells were centrifuged and washed using the same procedure as the cells grown in batch. Suspended cell batch experiments with TCS inhibition were carried out with chemostat-grown cells in the same manner as with batch-grown cells.

3.3. Triclosan Preparation

Triclosan (purity >96%) was purchased from TCI America, Inc. (Portland, OR, USA). Stock solutions of TCS were prepared in 100% Dimethyl sulfoxide (DMSO). Standard concentrations were prepared in deionized water (DI) when used for calibration of the ultra high pressure liquid chromatograph (UHPLC) via dilutions of a 1000 ppm TCS stock solution. A 100 ppm TCS in DMSO solution was also prepared and used as the stock solution from which the desired concentrations were made for suspended cell batch inhibition experiments. All TCS stock solutions were stored in a 5°C refrigerator.

3.4. Suspended Cell Batch Inhibition Experiments

Suspended cell batch inhibition tests with batch-grown cells were conducted in order to determine the inhibition and potential direct toxicity of TCS to *N. europaea*. Suspended cell batch inhibition tests with chemostat-grown cells were conducted in order

to compare TCS toxicity between chemostat and batch-grown cells. Inhibition experiments with the batch culture were conducted with cells that had reached the late exponential growth phase (~3-4 days with an OD_{600} ~0.072) prior to harvesting. Inhibition experiments with the chemostat culture were conducted with cells that had reached steady state growth (OD_{600} ~0.10). *N. europaea* cells were harvested via two cycles of centrifugation at 9000 RPM followed by decanting of the supernatant, washing with 30 mM HEPES buffer (pH 7.8) and subsequent re-suspension in 30 ml 30 mM HEPES buffer. Glass bioreactor bottles (125 ml) (Wheaton, VWR, Visalia, CA, USA) were filled with 30 ml 40 mM KH_2PO_4 media (pH 7.8) with 2.5 mM $(NH_4)_2SO_4$. Triclosan was added to inhibition treatment bottles at the desired test concentration. The control bottles contained KH_2PO_4 media and $(NH_4)_2SO_4$. Bottles containing TCS and KH_2PO_4 media without cell addition were used as blanks for UHPLC analysis. The bottles were capped with screw caps and shaken at 250 RPM for 1 hour in the dark at 30°C prior to cell addition to ensure TCS was completely dissolved.

Following centrifugation, washing and re-suspension, 500 µL *N. europaea* cells were pipetted into a spare 125 ml batch bottle containing the 30 ml KH_2PO_4 media and $(NH_4)_2SO_4$. After shaking the bottle at 250 RPM for at least 10 minutes, the cell density of the concentrated *N. europaea* cells was measured via UV-Vis absorbance at 600 nm using a Beckman Coulter DU 530 Life Science UV/Visible spectrophotometer (Beckman Coulter Inc., Brea, CA, USA). The volume of cells needed in each 125 ml bottle to achieve a cell density equal to that present during harvesting (OD_{600} ~0.072 or 6 mg protein L^{-1}) was determined using the following equation:

$$Cell\ volume\ needed\ (\mu L) = \frac{Target\ OD_{600}}{Measured\ OD_{600}} * Cell\ volume\ added(\mu L) \qquad (3.1)$$

The calculated volume of *N. europaea* cells were then added to each bottle and shaken at 250 RPM in the dark at 30°C for the duration of the experiment. Nitrite production and TCS transformation were determined by extracting and analyzing 1 ml samples at 45-minute intervals for 3 hour inhibition studies. Samples for nitrite production analysis were extracted and analyzed every 2 to 3 hours for 10 hour inhibition studies and every 2 to 10 hours for 24 hour inhibition studies. Samples extracted for TCS transformation analysis via UHPLC were centrifuged at 9000 RPM for 10 minutes and the resulting supernatant was diluted 10 times with DI water and stored in a 5°C refrigerator in 1.5 ml glass vials sealed with screw caps fitted with polytetrafluoroethylene (PTFE) silicon septa (National Scientific Company, Rockwood, TN, USA). Cell density was also measured at the end of the inhibition experiments via UV-Vis absorbance at 600 nm. The OD_{600} measurement was correlated to protein concentrations by a BSA generated standard curve (19). Protein concentration was calculated using the following regression equation:

$$Protein\left(\frac{mg}{L}\right) = -106.58\ (measured\ OD_{600})^2 + 117.15\ (measured\ OD_{600}) - 1.57 \qquad (3.2)$$

3.5. Suspended Cell Batch Recovery Experiments

Recovery tests were conducted on previously inhibited *N. europaea* cells in order to determine if TCS exposure resulted in cell death or simply cell inhibition. Following suspended cell batch inhibition tests, cells were washed to remove nitrate and TCS via centrifugation at 9000 RPM for 10 minutes, followed by decanting of the supernatant and re-suspension in 30 ml 30 mM HEPES buffer (pH 7.8). The washing process of

centrifugation, decanting and re-suspension with HEPES buffer was repeated three times to ensure cells were rinsed of TCS. Washed cells were then placed in fresh KH_2PO_4 media with no TCS added and the procedures used for cell density and nitrite production measurements during the inhibition test were repeated for the 3 hour recovery test.

3.6. Long Term Suspended Cell Growth Experiments

Long term growth inhibition experiments were conducted in order to determine if low concentrations of TCS inhibit the growth of *N. europaea*. These experiments were performed using cell densities of 1 and 10% of the density of cells used during suspended cell batch inhibition tests (OD_{600} ~0.072). The 1 and 10% cell densities were used to ensure the unlimited supply of ammonia substrate for growth and provide the potential for cell growth. *N. europaea* cells were harvested via two cycles of centrifugation at 9000 RPM, followed by decanting of the supernatant and re-suspension in 30 ml 30 mM HEPES buffer (pH 7.8). All decanting and re-suspension of cells was done under the laminar flow hood to prevent microbial contamination. Prior to use, glass bottles (500 ml) utilized to construct the bioreactors, were autoclaved for 1 hour. The bioreactors were filled with 250 ml of AOB growth media as described above. Triclosan was added to inhibition treatment bottles and the control bottles contained only the AOB growth media. Bottles containing TCS and growth media without cell addition were used as controls for UHPLC analysis. Cell density of the concentrated *N. europaea* cells was measured at 600 nm. *N. europaea* cells were then added to each bottle at concentrations of 0.6 and 0.06 mg protein L^{-1}, or 10 and 1% of the cell concentration used in suspended cell batch tests, respectively. The bottles were shaken at 100 RPM for 7 days in the dark

at 30°C. No adjustments were made to maintain a constant pH during cell growth aside

from the maintenance provided by the buffered media.

Nitrite production and cell density were measured by extracting and analyzing 1

ml samples at 4-hour and 8-hour intervals for the growth reactors containing 10% and 1%

cell density, respectively. The pH was also measured daily and TCS transformation was

determined by extracting and analyzing 1 ml samples every other day. The bioreactor

sampling was done aseptically under the laminar flow hood.

3.7. Nitrite Production Analysis

Nitrite production of *N. europaea* cells was determined using a colorimetric nitrite

assay in which a 10 µL sample from the reactor was added to 890 µL 1% (w/v) in 1 M

HCl sulfanilamide and 100 µL 0.2% (w/v) N-(1-naphthyl)ethylenediamine

dihydrochloride as previously described (19). The intensity of the resulting pink hue was

quantified via UV-Vis absorbance at 540 nm using a Beckman Coulter DU 530 Life

Science UV/Visible spectrophotometer (Beckman Coulter Inc., Brea, CA, USA). NO_2^-

concentrations were correlated to cell protein concentrations using a BSA generated

standard curve (19). NO_2^- concentration was calculated using the following regression

equation:

$$NO_2^- \ (mM) = 0.192 \ (measured \ A_{540})^2 + 1.856 \ (measured \ A_{540}) \qquad (3.3)$$

Nitrite concentrations were normalized to the measured protein concentrations to

show nitrification inhibition by TCS for suspended cell batch experiments. Nitrite

production on a cell protein basis was determined by dividing the nitrite concentration

calculated from equation 3.3 by the concentration of cell protein calculated from equation 3.2.

3.8. Specific Oxygen Uptake Rate Analysis

Specific oxygen uptake rate tests were performed during suspended cell batch inhibition studies in order to determine which enzyme in *N. europaea* is inhibited by TCS. The oxygen consumption rates for the ammonia monooxygenase enzyme (AMO) and the hydroxylamine oxidoreductase enzyme (HAO) expressed by *N. europaea* were quantified using specific oxygen uptake rate tests (SOURs) (67). The SOUR assay provides immediate rate measurements of O_2 consumption that can be converted to NH_3 oxidation or NO_2^- production rates based on the following stoichiometry: $NH_3 + 1.5O_2 \rightarrow NO_2^-$ (21). A Clark-type oxygen microelectrode probe (Yellow Springs Instrument Co. model no. 5331) secured in a 30°C water-jacketed glass chamber (Gilson Medical Electronics, Inc.) and connected to a YSI 5300 biological oxygen meter (Yellow Springs Instrument Co.) and TracerDAQ computer strip chart recorder program were used to measure oxygen concentrations of both AMO and HAO.

The AMO-SOUR was conducted by filling the 1.8 ml glass chamber with *N. europaea* cells in their test solution and 100 µL 45 mM $(NH_4)_2SO_4$ as a substrate to ensure the maximum AMO-SOUR is measured. The resulting slope recorded by the TracerDAQ program during the AMO-SOUR is the rate of oxygen uptake of the combined AMO and HAO enzymes performing the two steps in the oxidation of ammonia to nitrite. To measure the HAO-SOUR, 40 µL 20 mM allylthiourea (ATU) was added to the Gilson glass chamber to block AMO activity, responsible for performing the first step of oxidizing ammonia to hydroxylamine, and 100 µL 75 mM NH_2OH was

added as an alternative substrate for HAO. The resulting slope recorded by the

TracerDAQ program during the HAO-SOUR is the rate of oxygen uptake by the HAO

enzyme as it performs the second step of oxidizing hydroxylamine to nitrite. Triclosan

inhibition of either step performed by the AMO or HAO enzyme would result in a slower

rate of oxygen uptake. The percent activity was determined by dividing the AMO-SOUR

of TCS exposed cells by that of the control cells for each reactor using the following

equation: % Activity $= \left(\frac{AMO-SOUR_{inh}}{AMO-SOUR_{cont}} \right) * 100\%$. A similar equation was used to

calculate the % activity of the HAO enzyme.

3.9. UHPLC-MS Analysis

The transformation of TCS was investigated using Ultra High Pressure Liquid

Chromatography (UHPLC) coupled with mass spectrometry (MS) in order to determine

if TCS was being cometabolically transformed by *N. europaea*. A Dionex Ultimate 3000

UHPLC with C-18 column 5 µm, I.D. 100 mm (BHK Laboratories, CA, USA) and

attached guard column (Western Analytical Products, CA, USA) was used with a

constant 75% acetonitrile, 25% 1 mM ammonium acetate mobile phase (Table 2).

Samples extracted for TCS transformation analysis via UHPLC were centrifuged at 9000

RPM for 10 minutes, diluted 10 times with DI water and stored for future use in a 5°C

refrigerator in 1.5 ml glass vials sealed with screw caps fitted with

polytetrafluoroethylene (PTFE) silicon septa (National Scientific Company, TN, USA).

A 20 µL sample injection volume with a mobile phase flow rate of 0.25 ml min^{-1} was

used during sample analysis. The column compartment temperature was set to 35°C and

the post-cooler temperature was set to 23°C during sample analysis. Triclosan was

detected using a Bruker mass spectrometer (Bruker Corporation, CA, USA) with a negative polarity and mass of 286.944 g mol^{-1}. Observed detection limits for the analysis of TCS with a Bruker micrOTOF MS system were approximately 1 ppb TCS. Electron spray ionization low concentration tuning mix (Agilent Technologies, CA, USA) injected using a kd Scientific syringe pump was used to calibrate the mass spectrometer prior to running standard concentrations and TCS samples.

Table 2. Parameters for TCS detection method using a Dionex UHPLC and Bruker micrOTOF MS

UHPLC Parameters	
Retention Time (min)	6 – 12
Injection Volume (µL)	20 - 50
Flowrate (ml min^{-1})	0.25 – 0.5
Eluent Type: A, B	1mM ammonium acetate , acetonitrile
Constant Eluent Ratio: %A, %B	25, 75
Column Type	BHK Laboratories I.D. 100mm, 5µm, C-18
Column Compartment Temp. (°C)	23
Post-Cooler Temperature (°C)	35
MS Parameters	
Compound Mass	286.944
Width	±0.01
Polarity	Negative
Nebulizer (bar)	2.5
Dry Gas (L min^{-1})	8.0
Calibration Parameters	
Number of Segments	3
Seg. 1 Limit (min), Divert Valve	0.05 min, Waste
Seg. 2 Limit (min), Divert Valve	0.3 min, Source
Seg. 3 Limit (min), Divert Valve	Retention Time, Waste
Nebulizer (bar)	0.4
Dry Gas (L min^{-1})	4.0
kd Syringe Pump Parameters	
Syringe Type	Hamilton
Syringe Size	500 µL
Rate	180 ml h^{-1}
kd Syringe Pump Parameters	
Syringe Type	Scientific Glass Engineering
Syringe Size	5 ml
Volume	0.00 ml
Rate	0.6 ml h^{-1}

Sodium formate (2.9 mM) was injected throughout sample analysis to ensure consistent calibration of samples with TCS standards. External TCS standard concentration solutions prepared in DI were run for calibration prior to sample analysis (Figure 2). Triclosan detected using UHPLC-MS analysis exhibited a reliable extracted ion chromatograph peak and mass spectra with a retention time through the column of approximately 6-7 minutes (Figure 3). The mass spectra of TCS reliably exhibited a peak of 286.94 g mol^{-1} despite having a molecular weight of 289.54 g mol^{-1}. This is most likely due to the negative polarity used to detect TCS. A chemical library called SmartFormula included in the Bruker micrOTOF MS post processing program consistently verified the mass spectra footprint as TCS with a score of 100.0.
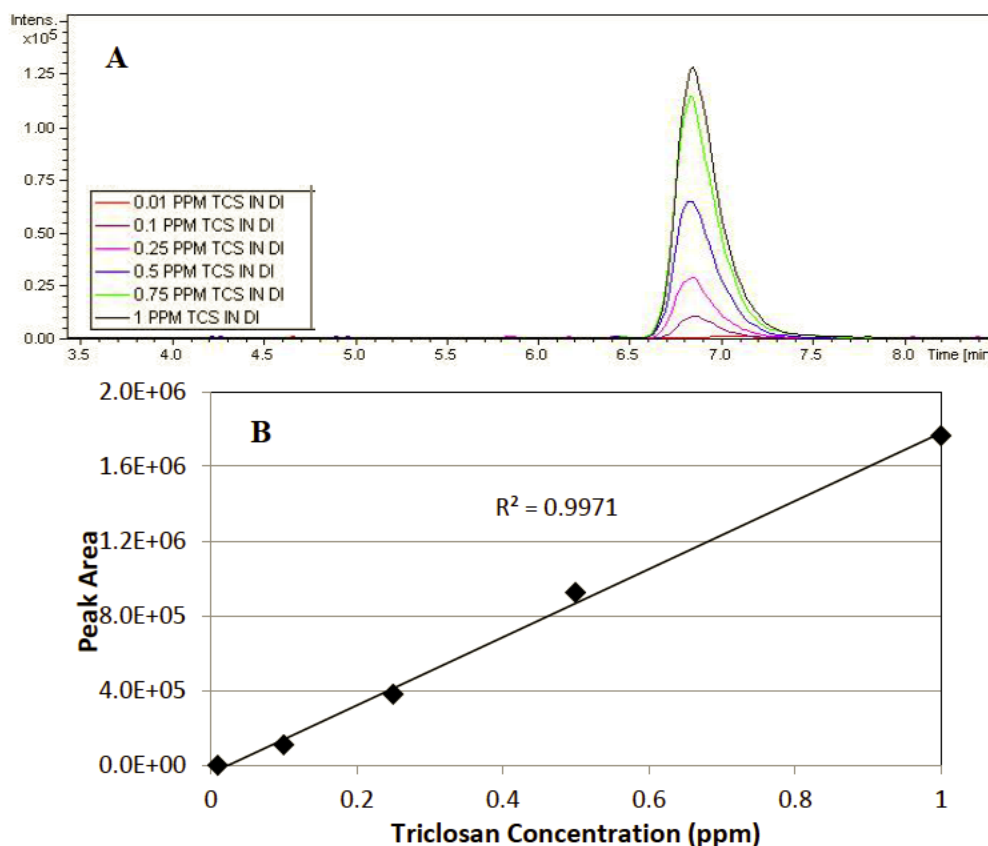
Figure 2. (A) Curves for standard concentrations of TCS detected using UHPLC-MS and (B) calibration curve of TCS standard concentrations.
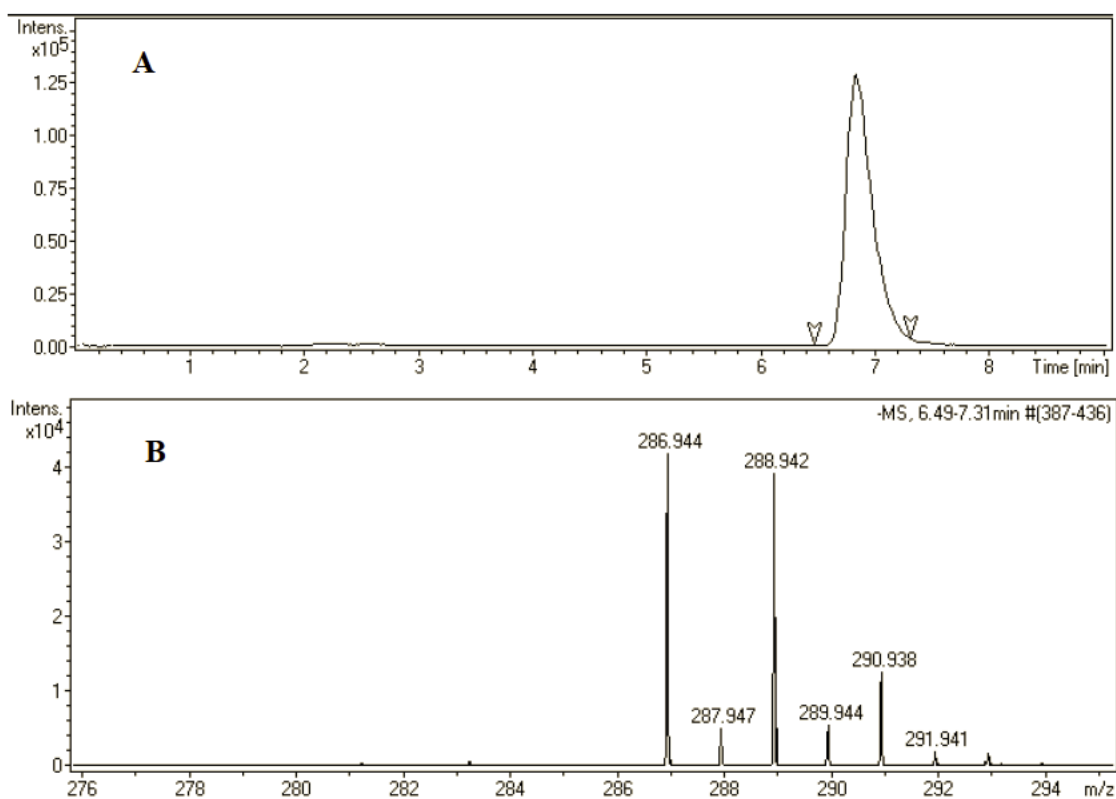
Figure 3. (A) Extracted ion chromatograph and **(B)** mass spectra of 1 ppm TCS in DI detected with UHPLC-MS.

Chapter 4: Results and Discussion

4.1.Suspended Cell Inhibition Tests

Suspended cell batch inhibition tests were conducted in order to determine the inhibition and potential direct toxicity of TCS to *N. europaea*. Nitrite production was normalized to measured cell protein concentrations in order to demonstrate nitrification inhibition throughout the batch experiments. During three hour suspended cell batch TCS inhibition tests, nitrite production increased with time for both exposed and control cells, with treated cells showing reduced amounts of nitrite production as TCS concentrations increased (Figure 4). A fairly linear increase in nitrite concentration with time was observed in both the control cells and cells exposed to TCS. The results indicate that the rates of nitrite production remained fairly constant with time even in cells that were strongly inhibited.
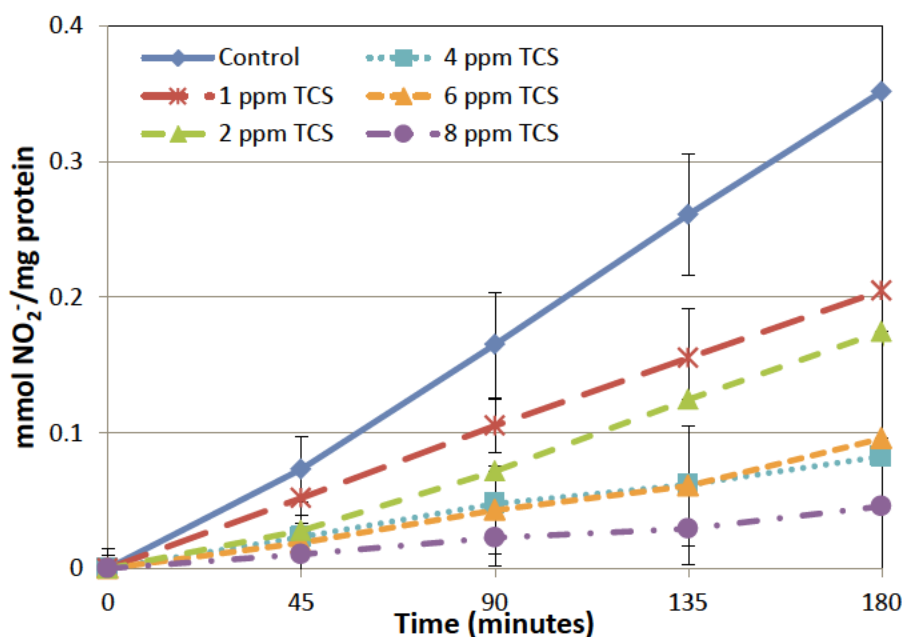
Figure 4. Nitrite production of *N. europaea* on a cell mass basis exposed to various concentrations of TCS. Error bars represent the standard deviation of triplicates to 95% confidence.

Results of three hour suspended cell batch inhibition tests showed that all tested concentrations of TCS inhibit the ability of *N. europaea* cells to oxidize ammonia to nitrite compared to the control cells. Nitrification inhibition was observed in the presence of TCS at concentrations below its solubility limit of 10 mg L^{-1}.

The percent inhibition of nitrification activity was determined by comparing the nitrite produced by inhibited cells verses that observed in control cells. The percent inhibition was calculated using the following equation:

$$\% \ Nitrification \ Inhibition = \left(1 - \frac{r_{inh}}{r_{cont}}\right) * 100\% \qquad (4.1)$$

where r_{inh} is the rate of nitrite produced by inhibited cells, and r_{cont} is the rate of nitrite produced by control cells.

Regression of percent inhibition fit a non-linear model with ammonia oxidation inhibition increasing with TCS concentration (Figure 5). *N. europaea* cells exposed to 1 ppm TCS experienced approximately 40% nitrification inhibition and cells subjected to 8 ppm TCS underwent approximately 90% inhibition. The inhibition response appears to fit a saturation type of model with respect to TCS concentration.
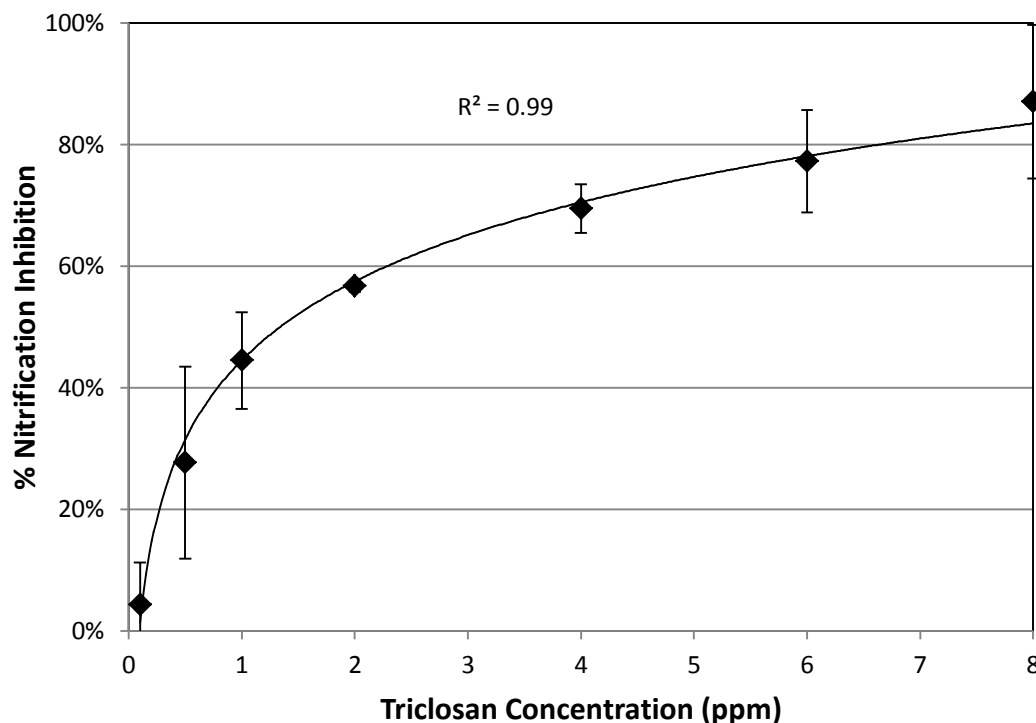
Figure 5. Triclosan inhibition of ammonia oxidation by *N. europaea* during three hour suspended cell batch tests. Error bars represent the standard deviation of tested concentrations to 95% confidence. Percent inhibition is given by $\% = \left(1 - \frac{r_{inh}}{r_{cont}}\right) * 100\%$, where r_{inh} is the rate of nitrite production by inhibited cells, and r_{cont} is the rate of NO_2^- production by uninhibited control cells.

4.2. Suspended Cell Inhibition and Recovery Tests

Recovery tests were conducted on previously inhibited *N. europaea* cells in order

to determine if exposure to TCS resulted in cell death or merely reversible inhibition at

the enzyme level. Recovery test results in which *N. europaea* cells that had demonstrated

nitrification inhibition when exposed to TCS for three hours, exhibited an increase in

nitrite production with time when washed of the inhibitor and placed in fresh media.

Figure 6 shows one recovery experiment in which *N. europaea* cells were inhibited by 1

and 4 ppm TCS (Figure 6A) and then subsequently washed of the inhibitor and allowed

to grow in fresh media for three hours (Figure 6B). Cells originally exposed to 1 and 4

ppm TCS showed approximately 70 and 30% of the nitrite produced by control cells,

respectively. When washed of the 1 and 4 ppm TCS inhibitor and allowed to recover, the

cells exhibited 94 and 77% of the activity observed in control cells, respectively.

Figure 6. **(A)** Nitrite production based on cell mass of *N. europaea* exposed to TCS during a three hour suspended cell batch inhibition test and **(B)** recovery of washed cells on a nitrite production per cell mass basis. Error bars represent the standard deviation of triplicates to 95% confidence.

Recovery experiments were conducted following longer inhibition tests in which *N. europaea* cells were exposed to TCS for 10 and 24 hours in order to determine if cell recovery was possible after exposure to TCS for longer periods of time.   These results also provided information on whether TCS fit a linear semi-log disinfection model with rates of microbial inactivation increasing with exposure time and TCS concentration.

N. europaea exposure to TCS over longer periods of time resulted in prolonged inhibition of ammonia oxidation. Figure 7 shows one recovery experiment in which *N. europaea* cells were inhibited by 6 and 8 ppm TCS for 10 hours (Figure 7A) and then subsequently washed of the inhibitor and allowed to recover in fresh media for three hours (Figure 7B). Cells treated with 6 and 8 ppm TCS for 10 hours produced approximately 22 and 6% of the nitrite produced by control cells, respectively (Figure 7A). Recovery experiments with cells previously exposed to 6 and 8 ppm TCS for 10 hours (600 minutes) resulted in approximately 50 and 22% of the nitrite produced by control cells, respectively (Figure 7B).

Figure 8 shows the results of a recovery experiment, in which *N. europaea* cells were inhibited by 6 and 8 ppm TCS for 24 hours (Figure 8A) and then subsequently washed of the inhibitor and allowed to recover in fresh media for three hours (Figure 8B). Nitrite production by *N. europaea* cells exposed to 6 and 8 ppm TCS over 24 hours was approximately 83 and 37%, respectively, when compared to the nitrite production observed in control cells (Figure 8A). It is interesting to note that despite exposure to 6 ppm TCS, nitrite production continued throughout 24 hours of exposure. Exposure to 8 ppm TCS caused a greater decrease in nitrite production.

Recovery experiments performed on cells previously inhibited by both 6 and 8 ppm TCS for 24 hours resulted in approximately 42 and 52% of the nitrification activity observed in control cells, respectively (Figure 8B).
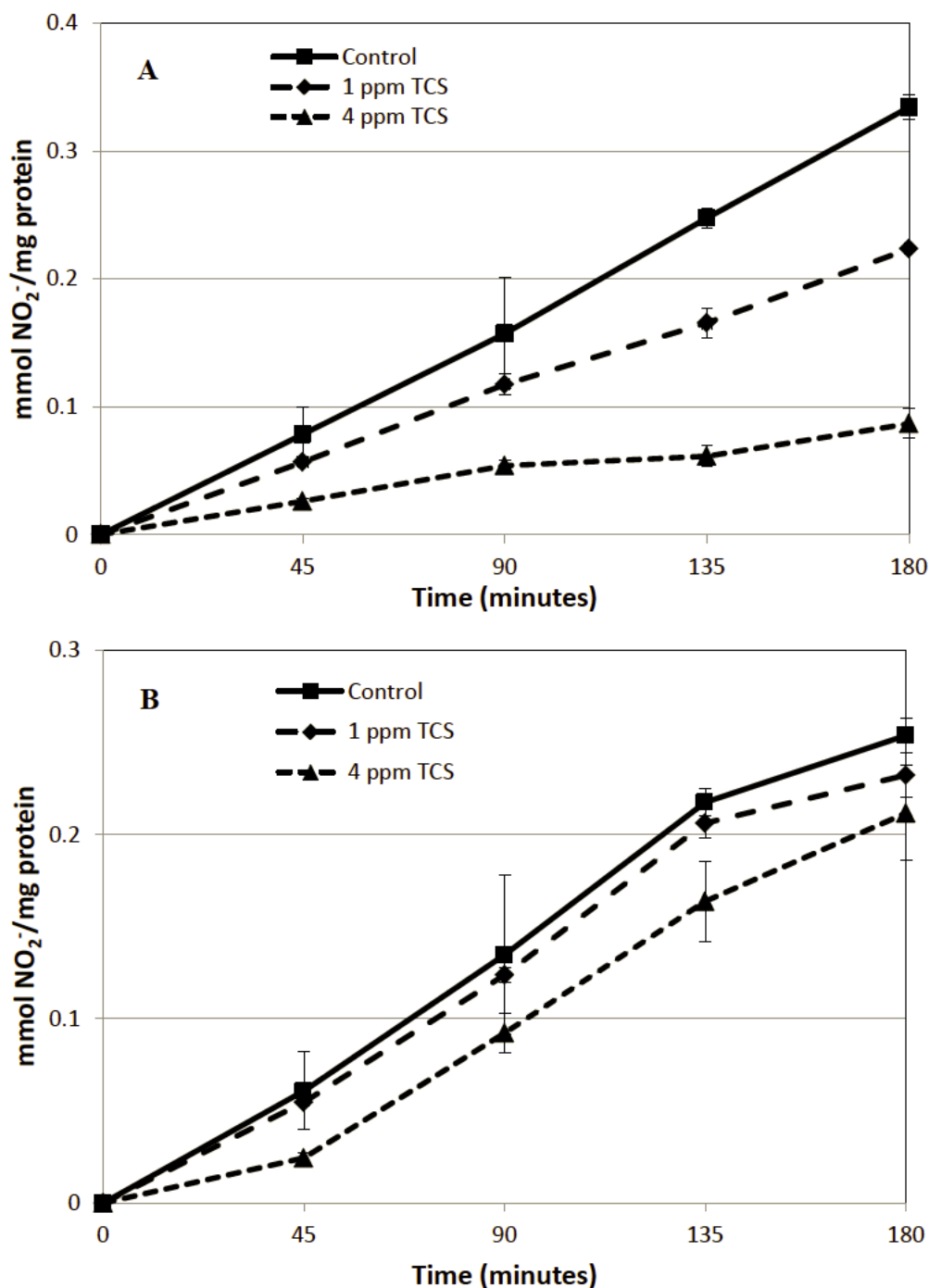

Figure 7. **(A)** Nitrite production based on cell mass of *N. europaea* exposed to TCS during a 10 hour suspended cell batch inhibition test and **(B)** recovery of washed cells on a nitrite production per cell mass basis. Error bars represent the standard deviation of triplicates to 95% confidence.
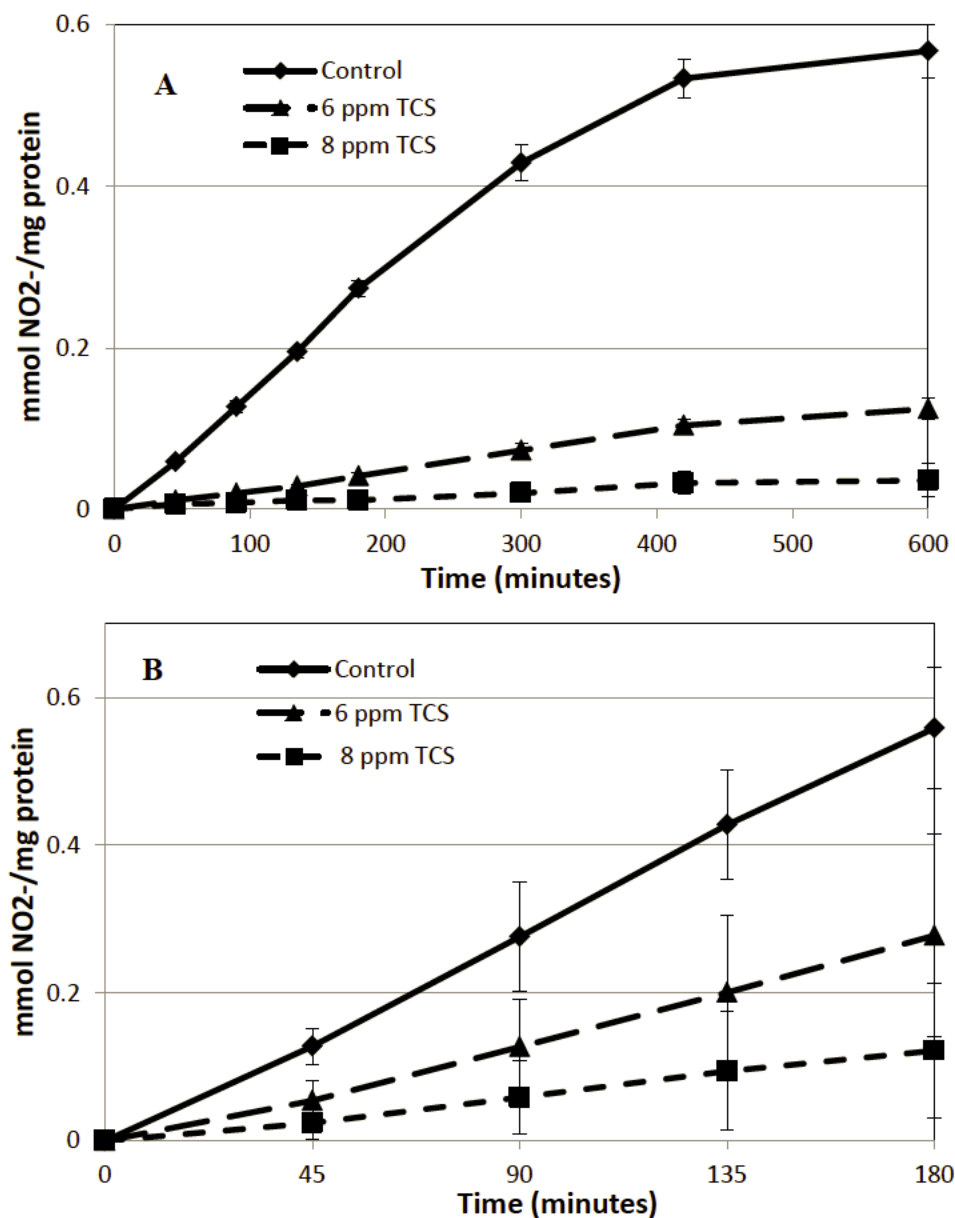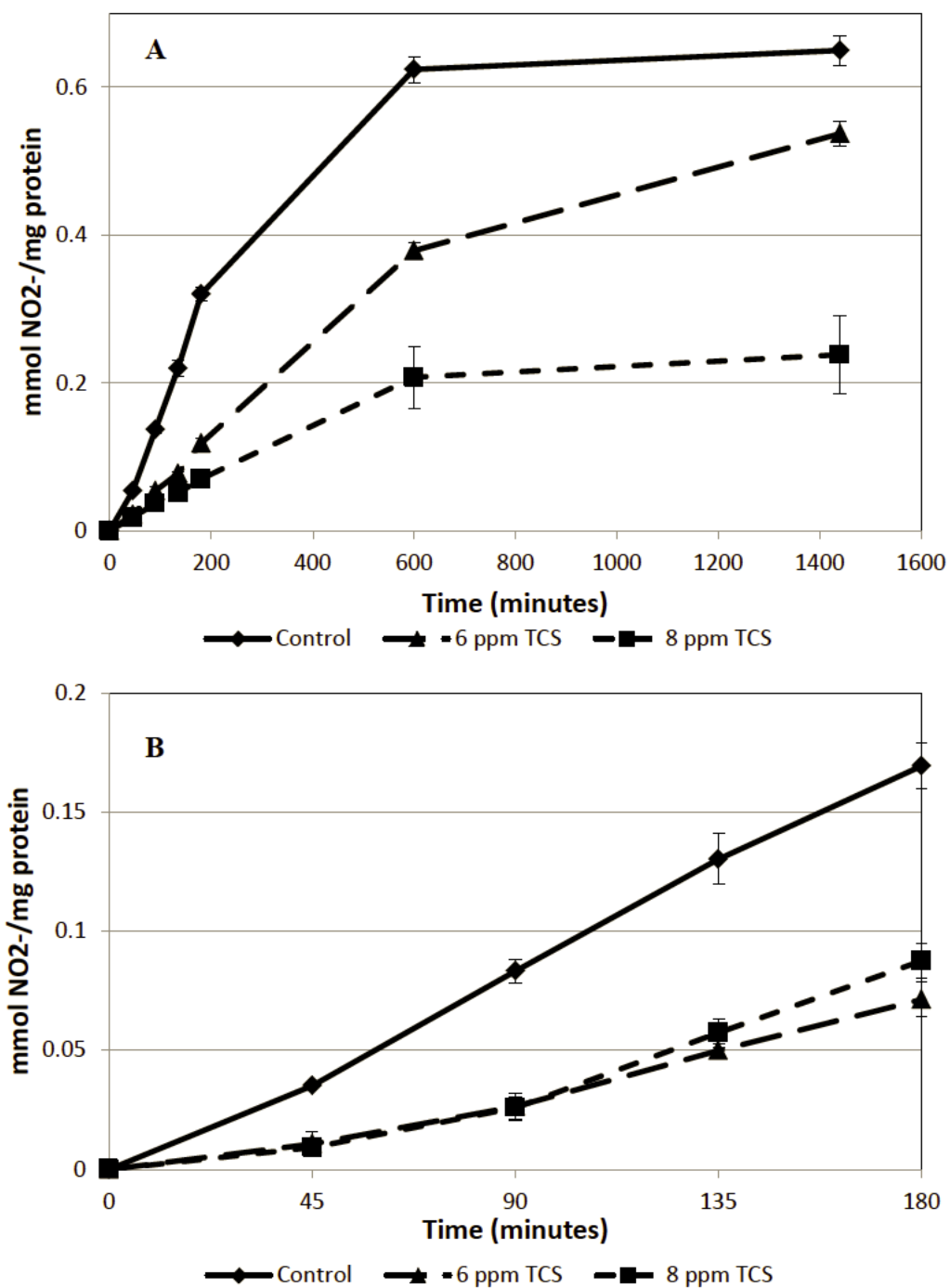
Figure 8. (A) Nitrite production based on cell mass of *N. europaea* exposed to TCS during a 24 hour suspended cell batch inhibition test and **(B)** recovery of washed cells on a nitrite production per cell mass basis. Error bars represent the standard deviation of triplicates to 95% confidence.

Cells exposed to TCS for 3 and 10 hour periods were inhibited to a greater extent than those exposed for 24 hours. This is only apparent due to the decreasing activity of the control cells over extended periods of time. In the 10 and 24 hour studies, the decrease in rates of nitrite production by control cells was due to a limited supply of free ammonia as a substrate as pH decreased. The decrease in pH over prolonged periods of time due to the limited supply of ammonia can be verified in Figure 15 C and F and Figure 16 C and F depicting *N. europaea* growth over a week without pH maintenance. When the control cells were permitted to consume ammonia for prolonged periods of time, the release of H^+ ions decreased the pH causing nitrite production to eventually cease. However, prior to a decrease in pH, cells exposed to TCS over 3, 10 and 24 hour periods exhibited similar inhibition of nitrite production when compared to control cells.

It is interesting to note that the rate of nitrite production by cells subjected to 8 ppm TCS for 24 hours slowed prior to producing enough nitrite to cause a major pH shift. For example, in Figure 8A the nitrite production rate of the control and 6 ppm TCS did not decrease with 0.35 and 0.38 mM NO_2^- mg protein^{-1} produced, respectively. The results indicate that prolonged exposure to TCS at high concentrations causes loss of cell viability.

While TCS was shown to be an inhibitor of ammonia oxidation, the partial recovery of previously exposed *N. europaea* cells indicates that it was not highly toxic to the cells over short periods of time.

At high concentrations of TCS some variability was observed in the extent of inhibition and recovery between tests with different batches of cells (Table 3).

Table 3. Comparison between percent inhibition and recovery of *N. europaea* cells inhibited by 6 and 8 ppm TCS.

Exposure Time (hours)	6 ppm TCS		8 ppm TCS	
	% Activity During Inhibition	% Activity During Recovery	% Activity During Inhibition	% Activity During Recovery
3	18	83	11	82
3	17	103	3	48
10	22	50	6	22
24	83	42	37	52
24	-	-	8	13

The greater variability in cell activity exhibited in the recovery tests compared to the inhibition tests is most likely due to the amount of cell mass lost during washing of the inhibitor prior to recovery experiments. A denser cell mass, although subjected to a higher concentration of TCS, might exhibit greater recovery than a less dense cell mass subjected to a lower concentration of TCS and vice versa. Sorption of TCS onto the cells could also potentially cause a greater variability in cell activity during recovery tests. However, TCS sorption to the cells was not measured in this study. It was assumed that TCS was completely removed by the three cycles of cell washing that was performed following inhibition tests.

In order to determine if loss of cell viability was occurring with time, a plot was constructed of the natural log of the ratio of the recovered cell activity compared to the recovered control activity versus the time of exposure (Figure 9). An approximate linear relationship between the normalized cell activity and the length of exposure was observed. Figure 9 shows that an increase in the TCS concentration results in a steeper slope. The linear relationship between the natural log of cell activity and exposure time suggests disinfection type kinetics where cell inactivation is proportional to the length of exposure and disinfectant concentration.

The rate of inactivation of microorganisms can be expressed by a first-order relationship referred to as Chick's Law:

$$-\frac{dN}{dt} = kN \qquad (4.2)$$

where - dN/dt is the rate of inactivation or microbial kill, k is the rate constant, N is the number of active organisms at any given time, t (68). Chick's Law states that the rate of bacterial kill is directly proportional to the number of active organisms remaining at any specific time. Integration of this equation between the limits of $t = (0, t)$ and $N = (N_0, N)$ results in the following relationship:

$$ln\frac{N}{N_0} = -kt \qquad (4.3)$$

Accordingly, a plot of log N/N_0 versus t should yield a linear relationship for disinfection data to match Chick's Law. In the case of *N. europaea* cell recovery following TCS inhibition, the activity of the recovering control cells was substituted for N_0 and the activity of recovering treated cells was substituted for N. The resulting plot matched the disinfection theory expressed by Chick's Law.

The rate of inactivation of cells exposed to 6 ppm TCS for various lengths of time was -0.03 hr^{-1} whereas for cells exposed to 8 ppm TCS the rate of inactivation was -0.05 hr^{-1}. The greater inactivation rate at the higher concentrations of TCS is consistent with disinfection theory, such as chlorination based on Chick's Law and the Chick-Watson Law. Cells subjected to 0.05 ppm TCS showed a less significant rate of inactivation of -0.003 hr^{-1}. This suggests that low concentrations of TCS that may be environmentally relevant do not inactivate *N. europaea* during short term exposures. Inhibition and

recovery test results for cells treated with 0.05 ppm TCS over 3, 10 and 24 hour periods can be seen in the Appendix.

The results indicate that high concentrations of TCS near the solubility limit are required to inactivate cells during short term exposure. They also suggest rapid disinfection with *N. europaea* during short term exposure requires a high aqueous concentration of TCS.
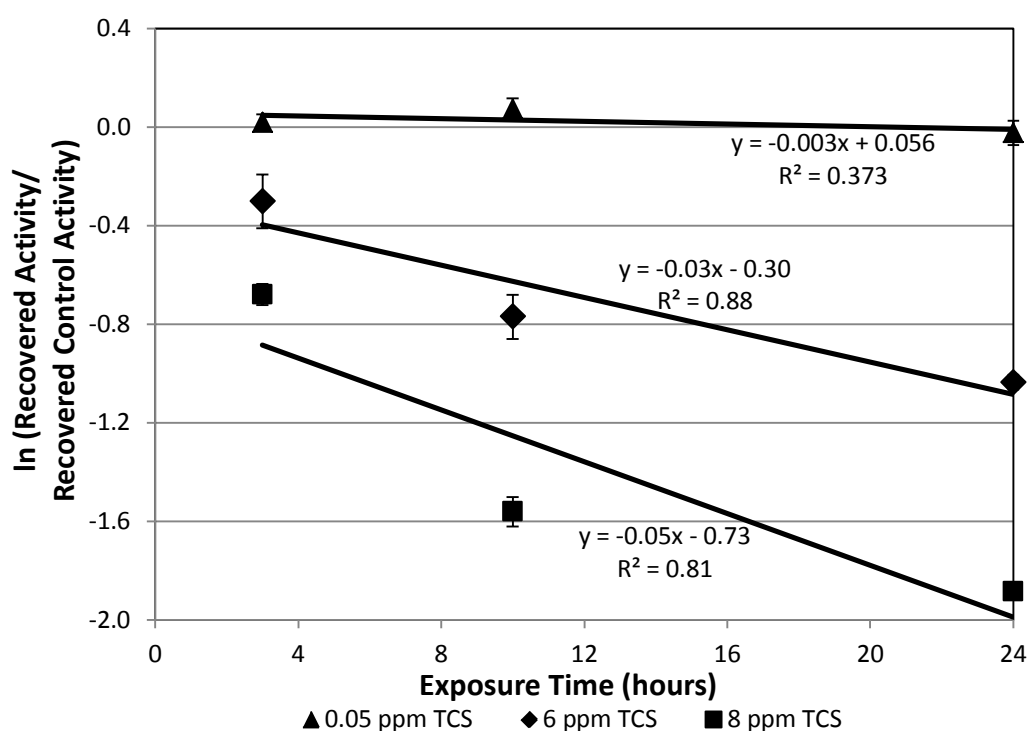
Figure 9. Nitrite production activity of recovering *N. europaea* cells normalized to recovering control cells that had previously been exposed to TCS for 3, 10, and 24 hour periods during suspended cell batch inhibition experiments.

4.3. Evaluation of Triclosan Transformation Potential During N. europaea Inhibition

The transformation of TCS was investigated by measuring TCS concentration using Ultra High Pressure Liquid Chromatography (UHPLC) coupled with mass spectrometry (MS). Aqueous samples obtained during three hour suspended cell batch inhibition experiments were analyzed. Active cells and controls that lacked cells are

shown in Figure 10. The absence of a decrease in TCS concentration in the presence of

N. europaea indicates that cometabolic transformation of TCS by *N. europaea* did not

occur. There was no distinguishable difference observed between the controls and the

reactors containing cells.   Some samples contained higher detected concentrations of

TCS than the target concentration that was supposed to be added to each bioreactor. This

is most likely attributable to dilution inaccuracies during the creation of stock solutions.

There are several missing sample points that resulted from problems encountered during

UHPLC-MS analysis. However, complete data sets for most cases showed no evidence

of TCS transformation over an 800-fold range in concentrations as shown in Figures 10

and 11. The confidence intervals cover a very small range in concentrations, and in some

cases are smaller than the size of the data symbols. This emphasizes the precision of the

measurement method for TCS using UHPLC-MS that was developed for this study.
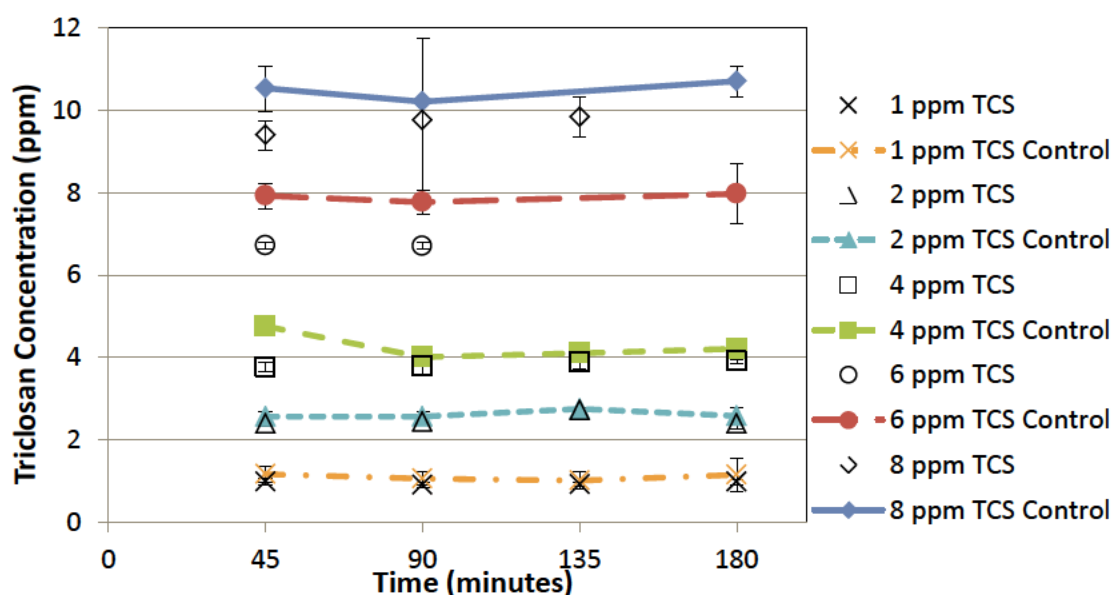
Figure 10. UHPLC-MS analysis of TCS concentration during three hour suspended cell batch tests inhibited with 1, 2, 4, 6, and 8 ppm TCS. Data not connected by dashed and solid lines represent concentrations of TCS during exposure to *N. europaea* cells. Data connected by dashed and solid lines represent control concentrations of TCS. Error bars represent the standard deviation of triplicates to 95% confidence. Error bars not depicted are smaller than the symbols.
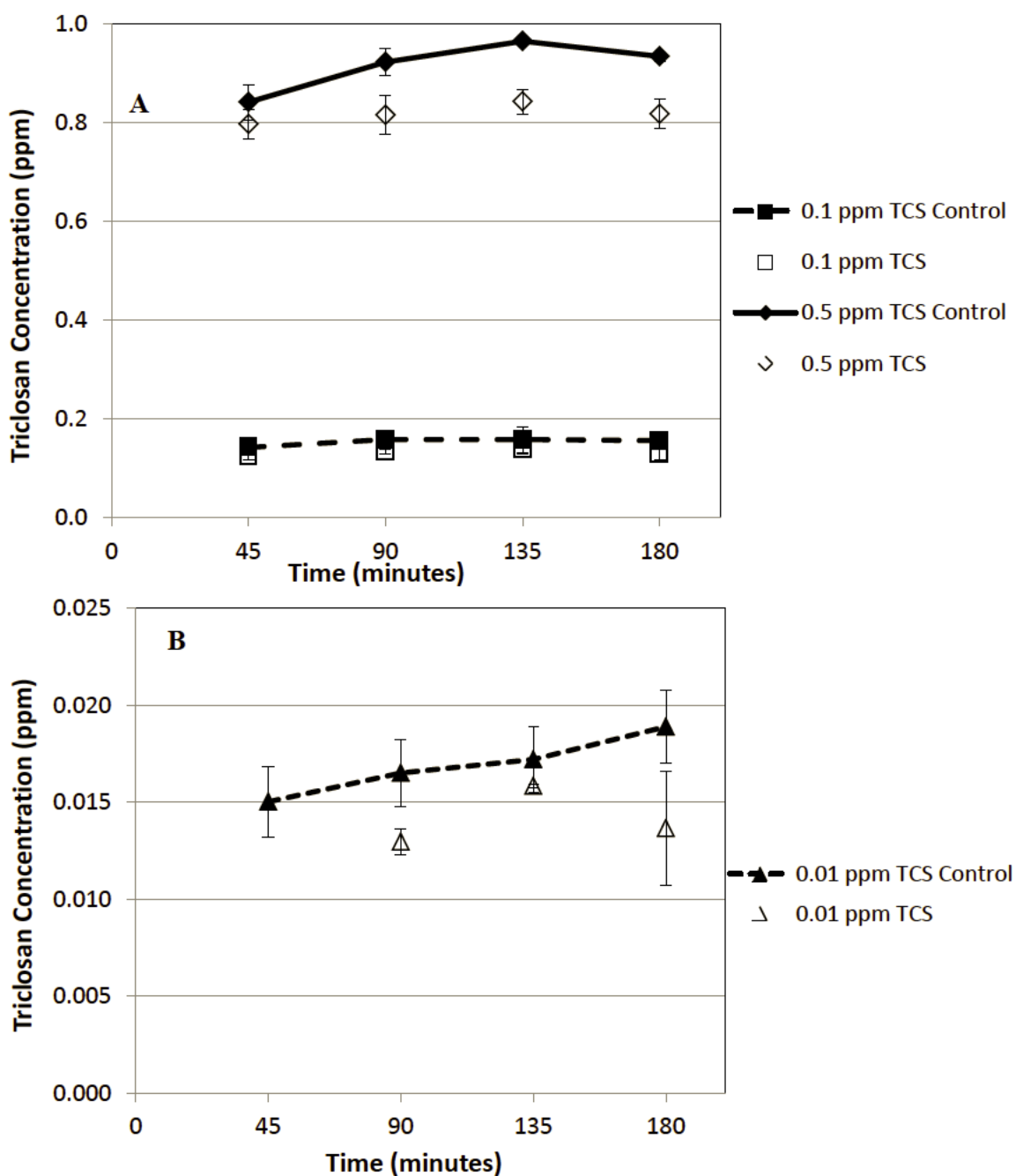
Figure 11. UHPLC-MS analysis of TCS concentration during three hour suspended cell batch tests inhibited with **(A)** 0.5 and 0.1 and **(B)** 0.01 ppm TCS. Data not connected by dashed and solid lines represent concentrations of TCS during exposure to *N. europaea* cells. Data connected by dashed and solid lines represent control concentrations of TCS. Error bars represent the standard deviation of triplicates to 95% confidence. Error bars not depicted are smaller than the symbols.

4.4.Specific Oxygen Uptake Rate Results

Specific oxygen uptake rate tests were performed during suspended cell batch inhibition studies in order to determine whether TCS inhibition is AMO and HAO specific. Specific oxygen uptake rate results for the AMO enzyme showed inhibition of AMO activity when exposed to TCS concentrations below 1 mg L^{-1} (Figure 12A). HAO SOURs results when exposed to TCS concentrations below 1 mg L^{-1} showed no decrease in HAO activity (Figure 12B). The decrease in AMO activity with time at tested TCS concentrations and the minimal effect of TCS on HAO indicates that direct TCS inhibition of the AMO enzyme is occurring, either through competition for the active site of AMO or through interactions of TCS with AMO. AMO can substitute a variety of chemicals as substrates and as a result non-growth substrates can compete with NH_3 and cause inhibition of *N. europaea*. Triclosan possesses a similar structure to phenol, a compound that has been shown to be a non-growth substrate for AMO and inhibit ammonia oxidation by *N. europaea* (10). This suggests that AMO is substituting TCS as a non-growth substrate for ammonia which is thereby inhibiting the nitrification process. However, transformation tests indicate TCS was not transformed by AMO.
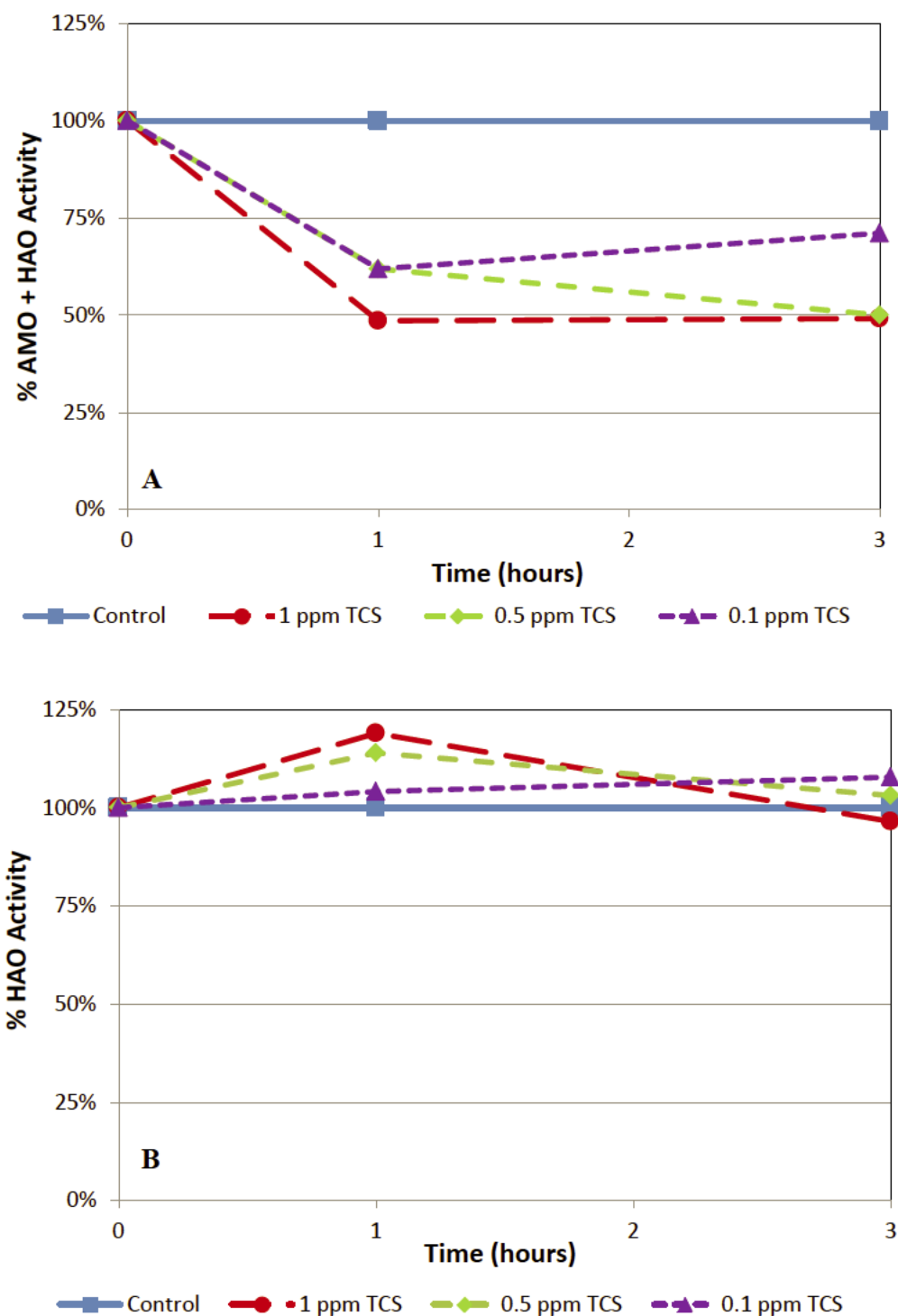
Figure 12. (A) Percent AMO + HAO enzyme activity and **(B)** percent HAO enzyme activity of *N. europaea* cells exposed to TCS during a three hour suspended cell batch experiment. Enzyme activity was determined using SOURs tests.

4.5.Triclosan Inhibition of Chemostat-Grown N. europaea

A three hour suspended cell batch TCS inhibition test was performed with *N. europaea* cells that had been grown in a chemostat in order to compare with the batch-grown cells. Studies of phenol inhibition showed chemostat-grown cells were less inhibited than batch-grown cells (20). The three hour suspended cell batch inhibition results also showed an increasing inhibition of ammonia oxidation with TCS concentration (Figure 13). Nitrite production by chemostat-grown cells treated with 1 ppm TCS was approximately 92% of that observed in control cells. In comparison batch-grown cells showed nitrification activity of approximately 60% when exposed to 1 ppm TCS. While 4 and 8 ppm TCS achieved 30 and 15% of the activity of the control cells, respectively. Batch-grown cells exposed to similar concentrations of TCS exhibited 30 and 13% nitrification activity of the control cells, respectively.

The comparison between non-linear regression of the inhibition of batch-grown cells and the inhibition of chemostat-grown cells as a function of increasing TCS concentrations is shown in Figure 14. The chemostat-grown cells exhibited a lower percent nitrification inhibition when exposed to 1 ppm TCS and similar nitrification inhibition when exposed to 4 and 8 ppm TCS. This could indicate that chemostat-grown cells are more resistant to nitrification inhibition when exposed to lower concentrations of TCS. Studies over a broader range of TCS concentrations are needed to determine the dependence of inhibition based on the growth state of the cells.
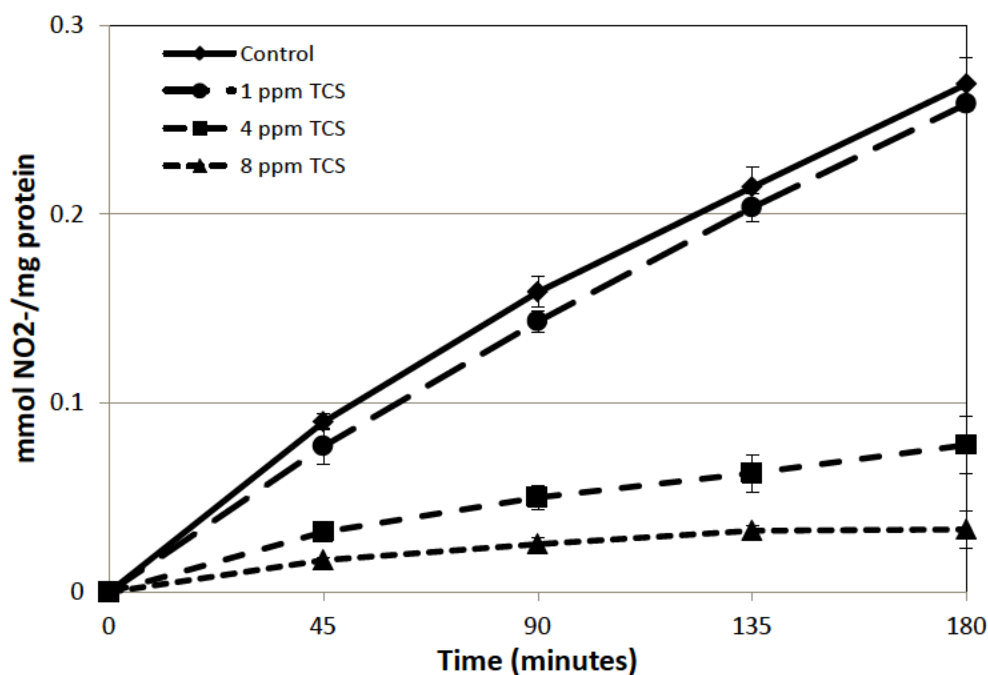
Figure 13. Nitrite production based on cell mass of chemostat-grown *N. europaea* cells exposed to TCS during a three hour suspended cell batch inhibition test.
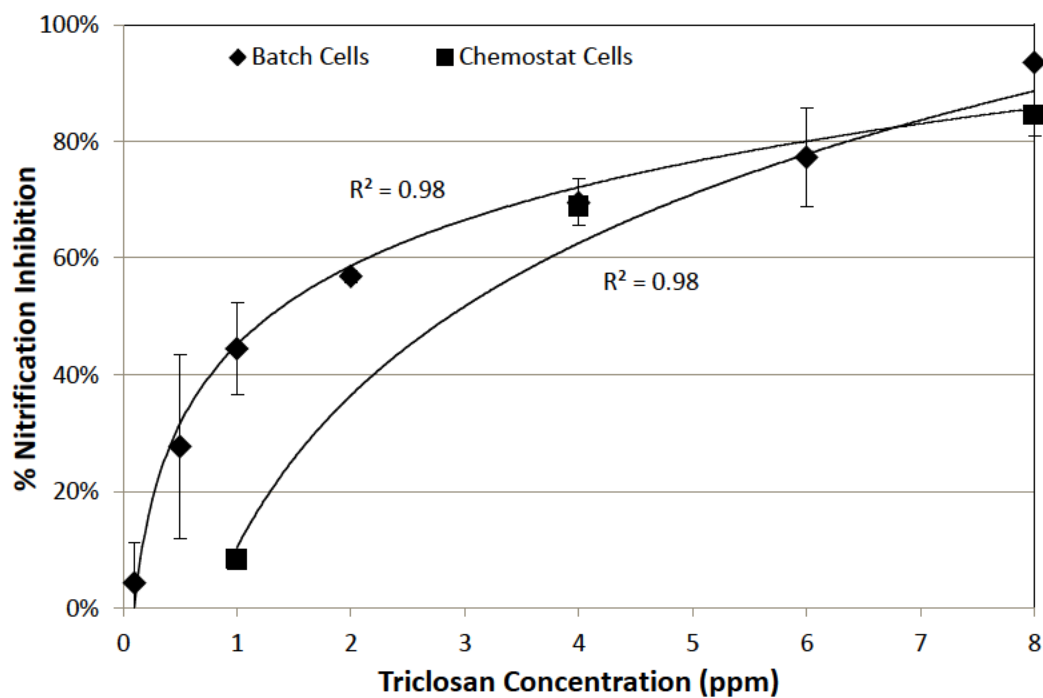
Figure 14. Comparison between TCS inhibition of ammonia oxidation by batch-grown and chemostat-grown *N. europaea* during three hour suspended cell batch tests. Error bars represent the standard deviation of tested concentrations to 95% confidence. Percent inhibition is given by $\% = \left(1 - \frac{r_{inh}}{r_{cont}}\right) * 100\%$, where r_{inh} is the rate of nitrite production by inhibited cells, and r_{cont} is the rate of nitrite production by uninhibited control cells.

4.6.Triclosan Inhibition of Long Term Growth

Long term growth inhibition experiments were conducted in order to determine if low concentrations of TCS inhibit the growth of *N. europaea*. To ensure the unhindered growth of *N. europaea* cells during long term growth inhibition experiments, inocula of *N. europaea* cells were created with 1 and 10% dilutions of the cell density (OD_{600} 0.072) used during suspended cell batch inhibition tests. These lower cell densities ensured that ammonia would not be a limiting factor for growth and provided the potential for an increase in cell numbers due to cell growth during the incubations. No attempt was made to maintain a constant pH during cell growth aside from the maintenance provided by the buffered media. Thus, the pH was expected to drop if effective cell growth occurred. A decrease in pH along with an increase in the optical cell density was used as an indicator of cell growth.

The optical density, nitrite production and pH shift during the growth of the 10% cell density inoculum are shown in Figures 15 A, B, and C, respectively. The growth of the 10% cell density inoculum was significantly inhibited by 0.1 and 1 ppm TCS (Figure 15A). When exposed to 0.1 ppm and 1 ppm TCS, the 10% cell density inoculum achieved approximately 80% and 50% of the nitrite produced by the control respectively (Figure 15B).

The pH of the control decreased from 8.0 to 6.0 over 50 hours while the pH of cells treated with 0.1 and 1 ppm TCS decreased much less.

The optical density, nitrite production and pH shift during the growth of the 1% cell density inoculum are shown in Figures 15 D, E, and F, respectively. The growth of the 1% cell density inoculum was also completely inhibited by 0.1 and 1 ppm TCS and

essentially no nitrite was produced (Figure 15D). Due to its lower cell density, the 1%

cell density inoculum was slower to grow and produce nitrite, A lag period of

approximately 20 hours is observed in Figures 15 D and E for the controls compared to

the 10 hour lag exhibited by the 10% cell density controls (Figures 15 A and B). The

complete inhibition of growth and nitrite production by the 1% cell density inoculum

treated with 0.1 and 1 ppm TCS is further verified by the lack of decrease in pH (Figure

15F).
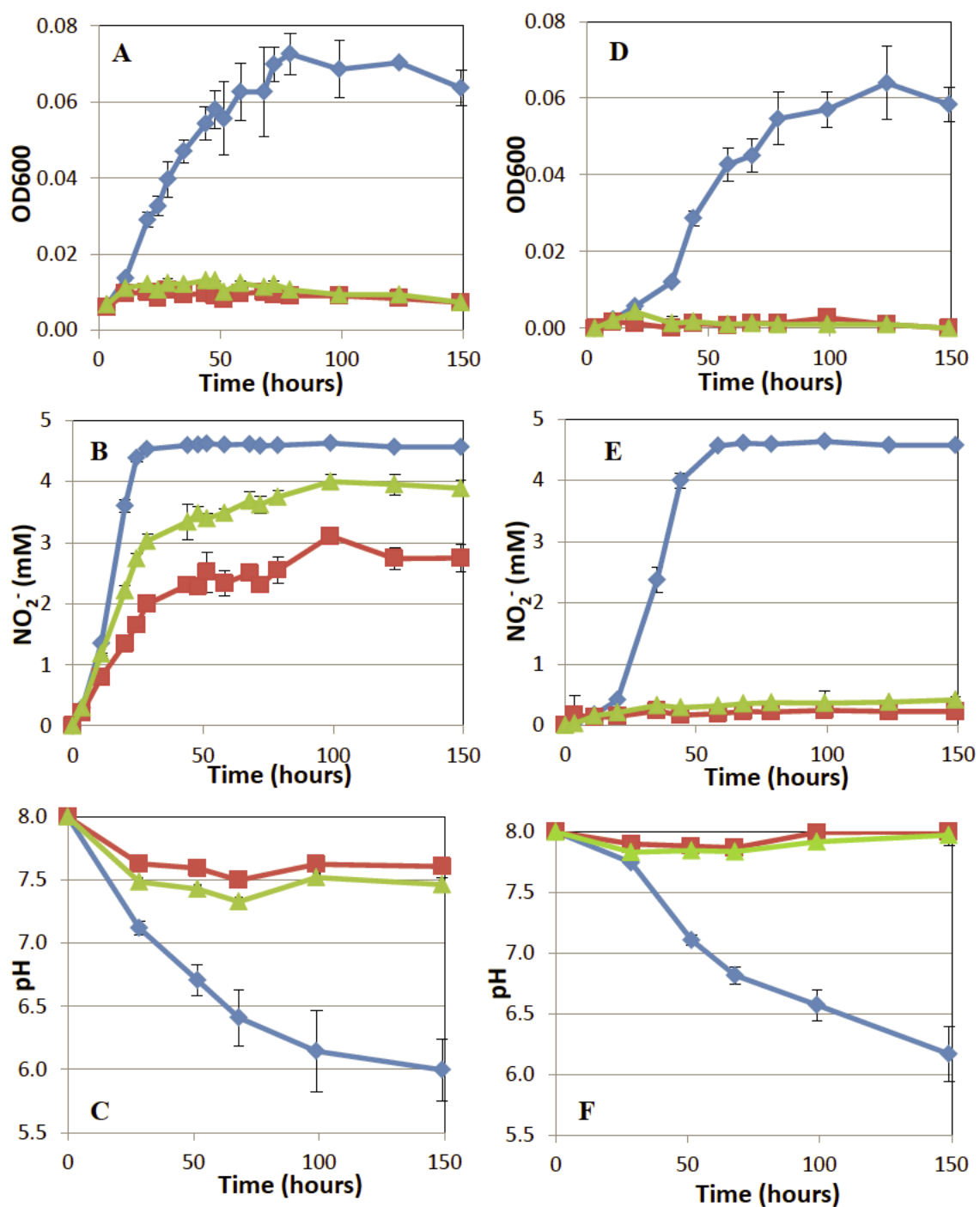
Figure 15. (A) Cell growth (OD$_{600}$ measurement), **(B)** nitrite production and **(C)** pH changes throughout the growth of a 10% cell density inoculum of *N. europaea* and **(D)** cell growth, **(E)** nitrite production and **(F)** pH changes throughout the growth of a 1% cell density inoculum of *N. europaea*. Changes in control (♦), 0.1 ppm (▲), and 1 ppm TCS (■) during growth. 10% cell densities of *N. europaea* were based on the amount of cells used during suspended cell inhibition tests. Error bars represent the standard deviation of triplicates to 95% confidence.

The inhibition of the growth of *N. europaea* at lower concentrations of TCS was investigated in order to pinpoint the magnitude of TCS toxicity towards cell synthesis. The optical cell density, nitrite production and pH shift during the growth of the 10% cell density controls and cells treated with 0.01 and 0.05 ppm TCS are shown in Figures 16 A, B, and C, respectively. The growth of the 10% cell density inoculum was strongly inhibited by 0.05 ppm TCS, but cells grew in the presence of 0.01 ppm TCS (Figure 16A). The cells treated with 0.01 ppm TCS achieved approximately 70% of the growth exhibited by control cells before reaching steady state. The nitrite production of the 10% cell density inoculum treated with 0.05 ppm TCS achieved approximately 80% of the control production (Figure 16B). The 0.01 ppm TCS treatment achieves the same nitrite production as the control.

During the 250 hour growth of the 10% cell density culture, the control and cells treated with 0.01 ppm TCS experienced a decrease in the pH from 8.0 to 6.0 (Figure 16C). The cells treated with 0.05 ppm TCS decreased from 8.0 to 7.25 over the first 50 hours and then maintained a relatively steady pH.

The optical cell density, nitrite production and pH shift during the growth of the 1% cell density controls and cells treated with 0.01 and 0.05 ppm TCS are shown in Figures 16 D, E, and F, respectively. The growth of the 1% cell density culture was strongly inhibited by 0.05 ppm TCS, but cells grew when treated with 0.01 ppm TCS to approximately 70% of that observed in the controls (Figure 16D). Cells treated with 0.01 ppm TCS exhibited a lag in nitrification production of approximately 70 hours compared to control activity before achieving equivalent nitrite production rates with the control cells (Figure 16D). Nitrite production was essentially completely inhibited for cells

treated with 0.05 ppm TCS. The partial inhibition in nitrite production exhibited by the 1% cell density inoculum prior to reaching steady state can be accounted for by its lower cell density and therefore slower growth and subsequent nitrite production.

During the growth of the 1% cell density culture, control cells and cells treated with 0.01 ppm exhibited a decrease in pH from 8.0 to 6.0. Cells treated with 0.05 ppm TCS decreased the pH from 8.0 to approximately 7.7 over the first 80 hours and then maintained a relatively steady pH.

The similar decrease in pH exhibited by the control and cells treated with 0.01 ppm TCS for both cell density inocula verifies the partial inhibition of growth exhibited by the cells treated with a lower concentration. The lack of a significant decrease in pH for cells treated with 0.05 ppm TCS verifies the complete inhibition of growth and nitrite production exhibited.

The incomplete inhibition of growth demonstrated by cells treated with 0.01 ppm TCS suggests that lower, potentially environmentally relevant, concentrations of TCS may only partially inhibit bacterial growth. However, as discussed earlier, lower concentrations of TCS do not inactivate *N. europaea* during short term exposure. This indicates that low concentrations of TCS directly target cell synthesis while they do not exert toxicity to mature cells during short term exposure. This result is evident by comparing the observations shown in Figure 9, where little inactivation is observed with a short term exposure of 0.05 ppm TCS, while in the long term growth test, 0.05 ppm TCS completely inhibited growth.
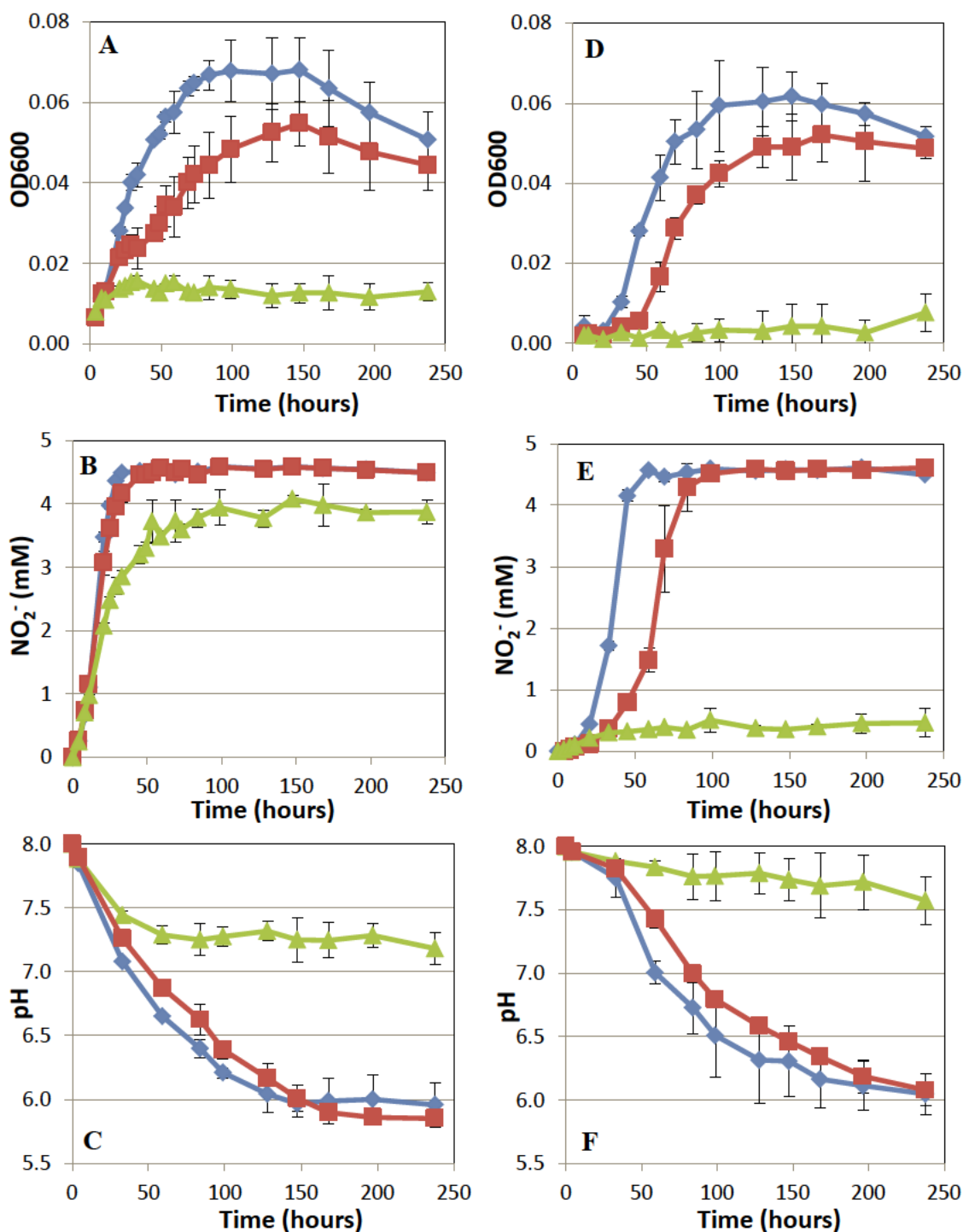
Figure 16. (A) Cell growth (OD_{600} measurement), **(B)** nitrite production and **(C)** pH changes throughout the growth of a 10% cell density inoculum of *N. europaea* and **(D)** cell growth, **(E)** nitrite production and **(F)** pH changes throughout the growth of a 1% cell density inoculum of *N. europaea*. Changes in control (♦), 0.01 ppm (■), and 0.05 ppm TCS (▲) during growth. 10% cell densities of *N. europaea* were based on the amount of cells used during suspended cell inhibition tests. Error bars represent the standard deviation of triplicates to 95% confidence.

Figures 15 A, B, and C and Figures 16 A, B, and C were combined to compare the inhibition of growth and nitrite production of the 10% cell density inoculum at all tested TCS concentrations (Figure 17). Growth and nitrite production of cells were shown to be inhibited at the tested TCS concentrations. Inhibition of nitrite production increased with increasing TCS concentration (Figure 17A). Cells exposed to 1 ppm TCS achieved approximately 50% nitrite production compared to control cells, while cells exposed to 0.05 and 0.1 ppm TCS achieved approximately 80% nitrite production compared to control cells. Cells subjected to TCS concentrations above 0.05 ppm were unable to synthesize cells for growth; whereas cells exposed to 0.01 ppm TCS exhibited 70% of the growth of the uninhibited control cells (Figure 17B).

Figure 17. **(A)** NO_2^- production and **(B)** growth (OD_{600} measurement) of *N. europaea* originally inoculated with a 10% cell density. Error bars represent the standard deviation of triplicates to 95% confidence.

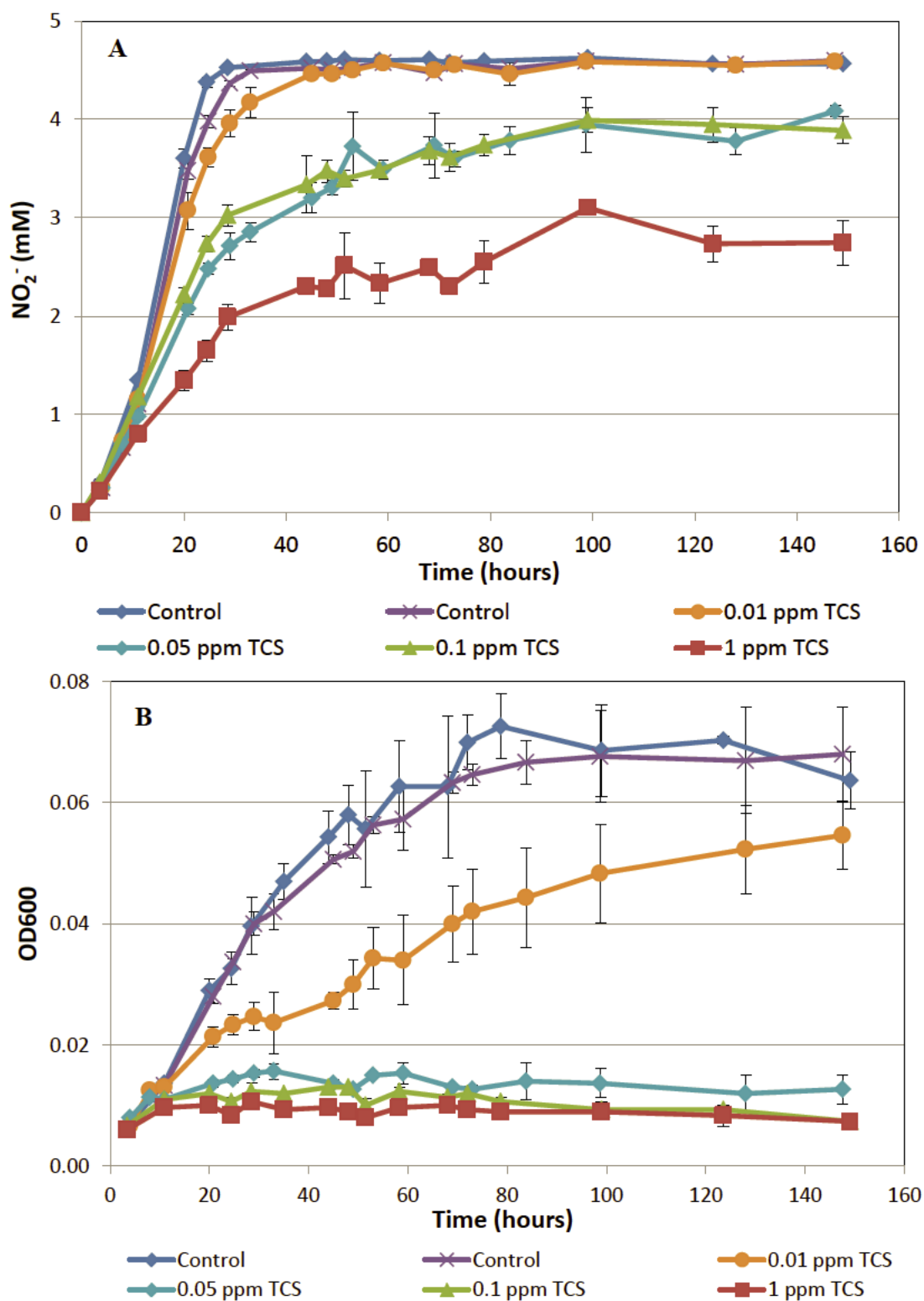Figures 15 D, E, and F and Figures 16 D, E, and F were combined to compare the inhibition of growth and nitrite production of the 1% cell density inoculum at all the tested TCS concentrations (Figure 18). Nitrite production was completely inhibited at TCS concentrations exceeding 0.05 ppm TCS, while exposure to 0.01 ppm TCS resulted in 80% of the control production. Once steady state was achieved, cells exposed to 0.01 ppm TCS achieved equivalent nitrite production as the control cells. Similar to the 10% cell density inoculum, the optical density of cells exposed to 0.01 ppm TCS achieved an estimated 70% nitrite production compared to control cells. Cells subjected to concentrations at or exceeding 0.05 ppm TCS were unable to grow.

The similarity in growth between the 1 and 10% original cell density suggests that the range in which the growth of *N. europaea* is either partially or completely inhibited falls between 0.01 and 0.05 mg L^{-1} TCS. TCS concentrations detected in natural waters are on the order of ng L^{-1} to µg L^{-1} (27, 32, 33, 40). The 0.01 ppm concentration tested at which growth of *N. europaea* was partially inhibited suggests that the growth of *N. europaea* could be inhibited at environmentally relevant concentrations. Concentrations of TCS detected in wastewater range from ng ml^{-1} up to 26.8 µg L^{-1} (27, 33, 35, 36, 37, 38). As *N. europaea* are utilized in wastewater treatment to perform the essential role of ammonia oxidation, these results suggest TCS concentrations in wastewater could either partially or significantly inhibit the growth of this functionally important microbe.

The TCS concentration of 0.05 ppm that is responsible for inhibiting cell growth is two orders of magnitude lower than the TCS concentration required for nitrification inhibition during short term exposure. Also, as shown in Figure 9, exposure to 0.05 ppm

TCS in short exposure tests did not result in cell inactivation. The results show that TCS acts as a biocide for *N. europaea* that mainly targets cells synthesis.
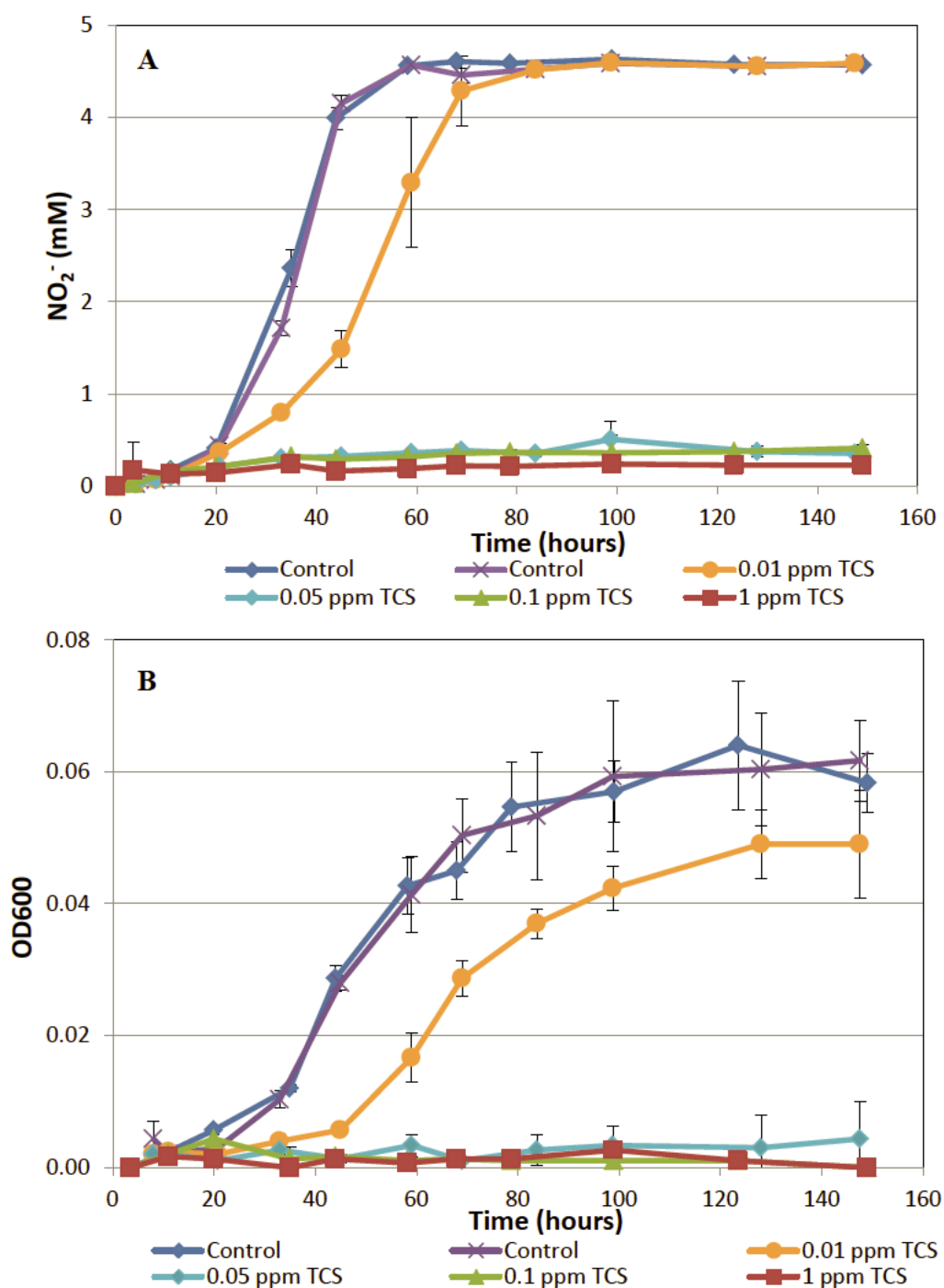
Figure 18. **(A)** NO_2^- production and **(B)** growth (OD_{600} measurement) of *N. europaea* originally inoculated with a 1% cell density. Error bars represent the standard deviation of triplicates to 95% confidence.

4.7.Evaluation of Triclosan Transformation Potential During N. europaea Growth

During *N. europaea* growth experiments, samples were extracted and analyzed using UHPLC-MS in order to investigate the cometabolic transformation of TCS by *N. europaea* during growth. A significant decrease in TCS concentration was not apparent (Figure 19). Samples containing TCS and media which were used as controls remained relatively constant over the course of the 6 day experiment. Samples containing 0.1 and 1 ppm TCS and media with *N. europaea* cells also remained relatively constant over the course of the 6 day experiment. The higher concentrations detected for the 0.1 and 1 ppm control samples could be due to some sorption of TCS to the cells. The lack of a decrease in TCS concentration over the 6 day experiment implies that the cometabolic transformation of TCS during the growth of *N. europaea* did not occur. The results are consistent with the short term assays conducted at higher cell concentrations.

Figure 19. (A) Changes in TCS concentration during long term growth of a 10% cell density inoculum and **(B)** a 1% cell density inoculum of *N. europaea*. Data not connected by solid lines represent concentrations of TCS exposed to cells. Data connected by dashed and solid lines represent control concentrations of TCS. Error bars represent the standard deviation of duplicates to 95% confidence.
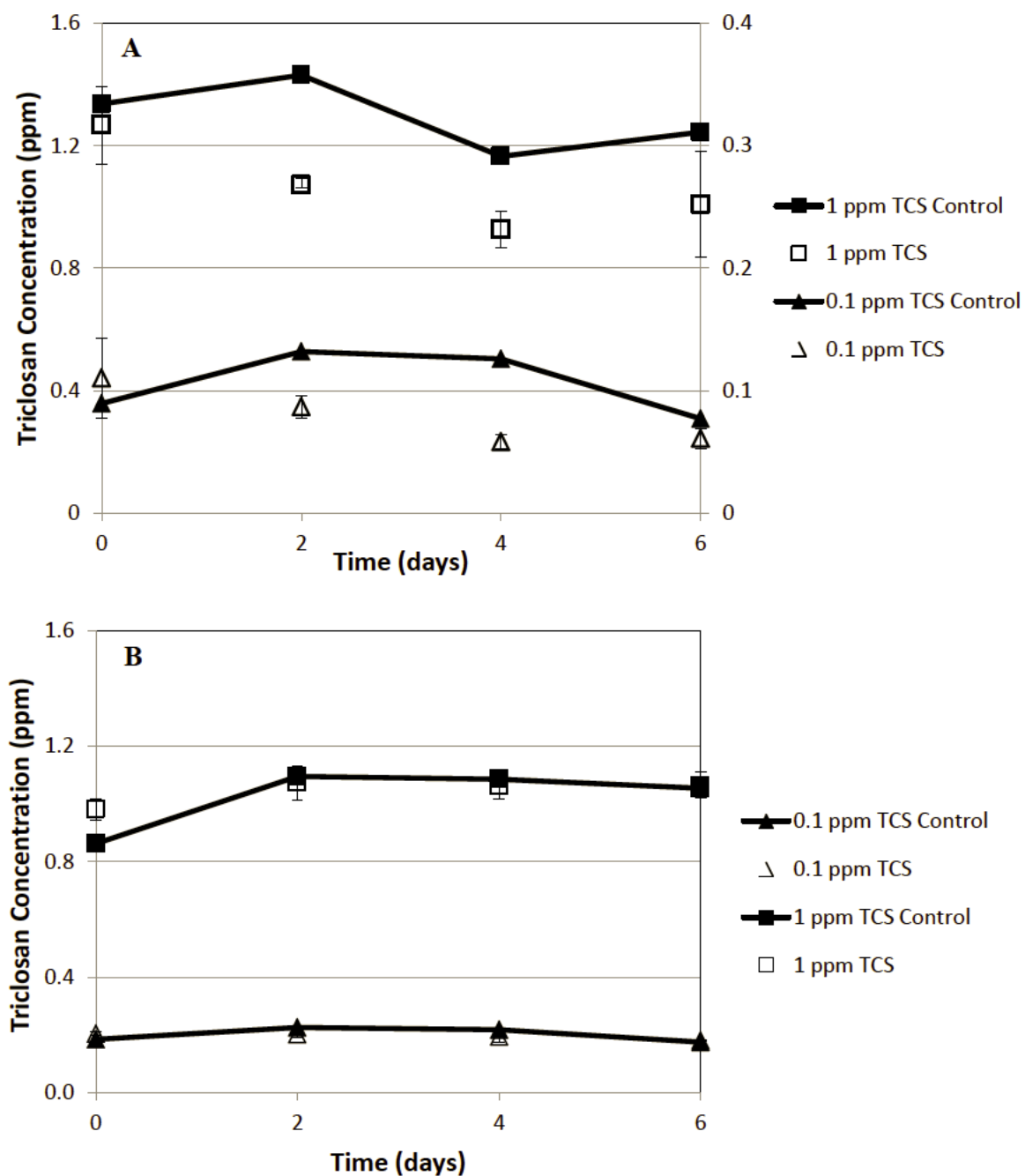
4.8.Triclosan Resistance

The development of resistance to TCS has been demonstrated in a variety of bacterial strains (13, 64, 65). To test if resistance developed in *N. europaea*, growth experiments were conducted with 1% cell density inoculum subjected to 0.01 ppm TCS. Once the cultures reached steady state growth (approximately 180 hours) they were washed and re-suspended in fresh media and the week-long growth cycle was repeated. Three growth cycles were conducted in order to determine if TCS resistant strains of bacteria were developing over several generations of growth.

Experimental growth tests of a 1% *N. europaea* cell density inoculum subjected to 0.01 ppm TCS resulted in a 70% increase in optical density compared to the complete inhibition of growth exhibited by cells treated with concentrations at or above 0.05 ppm. Control cells and cells treated with 0.01 ppm TCS that had reached steady-state growth were washed to remove TCS and a 1% cell density was re-suspended in fresh media to investigate the ability of *N. europaea* to develop antibiotic resistant strains over multiple generations of growth. Exposure to 0.01 ppm TCS was maintained in the cells with previous exposure. The washed cells were allowed to grow until steady state was reached at which time the cells were washed again and re-suspended in fresh media for a third growth cycle.

N. europaea cells were allowed to grow for 180 hours for each of three cycles which, based on a doubling time of 8 hours, would represent approximately 67 generations. The resulting three 180 hour growth cycles of *N. europaea* cells subjected to 0.01 ppm TCS were inhibited equally achieving approximately 70% of the optical density as the uninhibited control cells (Figure 20). The equivalent inhibition exhibited during all

three 180 hour growth cycles suggests that *N. europaea* did not develop resistance to TCS over the three growth cycles. Bacterial strains of *E. coli*, which have a doubling time of between 20 and 30 minutes, were found to develop resistance to TCS between 2 and 4 cycles of growth (51). *N. europaea* did not develop resistance to TCS over three growth cycles. However, longer term studies are needed to determine if strains with resistance would develop. The results do show the repeatable ability of *N. europaea* to grow when exposed concentrations of 0.01 ppm TCS. This is a positive observation with respect to wastewater treatment, where continuous exposure would occur.
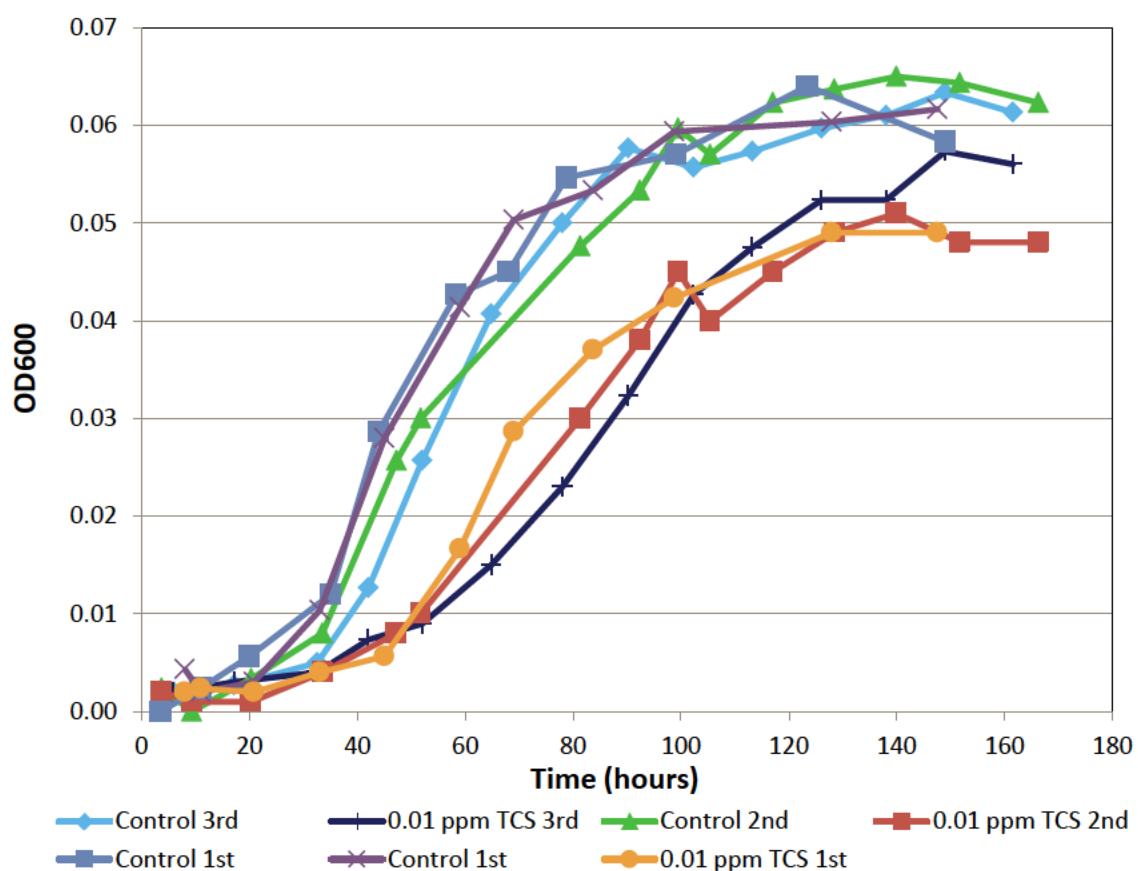

Figure 20. Growth (OD_{600} measurement) of three *N. europaea* growth cycles originally inoculated with a 1% cell density. The '1st', '2nd', and '3rd' in the series label represent the number of cycles of growth.

Chapter 5: Conclusion

5.1.Key Findings

The following conclusions may be drawn from this work:

- Inhibition of nitrite production in *N. europaea* cells does occur when exposed to concentrations of TCS below its solubility limit of 10 mg L^{-1}. Triclosan inhibition fit a non-linear model with nitrification inhibition increasing as TCS concentration increases with 40% inhibition occurring at 1 ppm TCS and 90% inhibition at 8 ppm TCS.

- Recovery test results indicate that *N. europaea* cells are inhibited by TCS and partially recover when washed and re-suspended in fresh media. Less recovery occurred when cells were exposed to higher concentrations and for long periods of time.

- Cell recovery fit a time and concentration dependent response associated with linear disinfection models such as Chick's Law and the Chick-Watson Law. The results fit a natural log versus TCS exposure time dependence, suggesting cell inactivation occurred as a function of time and concentration. Higher rates of cell inactivation were associated with higher concentrations and longer periods of exposure.

- AMO and HAO SOURs tests indicate direct inhibition of the AMO enzyme, either through competition for its active site or interactions between TCS and AMO, and that the HAO enzyme is unaffected by TCS inhibition during short term exposure.

- The inhibition of chemostat-grown cells exhibited an 18% increase in resistance to nitrification inhibition compared to batch-grown cells when exposed to 1 ppm TCS. Both chemostat and batch-grown cells exhibited similar nitrification inhibition when treated with 4 and 8 ppm TCS.

- Results of long term suspended cell growth experiments indicate that growth of *N. europaea* is approximately 70% at 0.01 ppm TCS compared to control cells; whereas concentrations at or exceeding 0.05 ppm TCS significantly inhibit cell growth.

- Growth resistance experiments did not show a development of resistance to 0.01 ppm TCS over three 180 hour growth cycles, or approximately 67 generations of growth.

- UHPLC-MS analysis results indicate that TCS is not transformed in the presence of *N. europaea* while it is metabolizing ammonia over a three hour direct exposure period or during long term growth.

5.2. Implications

The apparent inhibition of ammonia oxidation by *N. europaea* at concentrations of TCS below 10 mg L^{-1} indicates the existence of potential risks to these functionally important microbes when exposed to this antimicrobial in wastewater treatment plants (WWTP). Concentrations of TCS have been detected on the order of $\mu g\ L^{-1}$ in wastewater influent (27, 33, 35, 36, 37, 38). The TCS concentrations shown in this work that directly inhibit ammonia oxidation are much higher. Thus direct inhibition of ammonia oxidation is not expected to be a key process in the environment. TCS

inhibition of the growth of *N. europaea* is of much more concern since it occurred at concentrations of approximately two orders of magnitude lower than the direct inhibition of the AMO enzyme. A concentration of 0.01 ppm TCS inhibited growth by 30%, while 0.05 ppm TCS completely inhibited growth. These concentrations are of environmental relevance. No resistance to TCS by *N. europaea* over three repeated growth cycles was observed, which potentially represented 67 generations of growth.

The limited range between environmentally relevant concentrations of TCS that exhibit partial or complete inhibition of the growth of *N. europaea* emphasizes the importance in understanding the toxicity of TCS in biological treatment processes and the environment. The substantial overuse of this antimicrobial could result in its subsequent accumulation and increase in concentration to bactericidal levels in WWTP and the environment. The heightened sensitivity of AOB to environmental and chemical perturbations result in its inability to develop resistance to this widely used pharmaceutical. As a result of TCS accumulation in wastewater and the potential inability of AOB to develop resistance, the efficiency of AOB to remove ammonia during wastewater treatment could be impaired, with the resulting effluent supplying excess nutrients and promoting eutrophication of receiving water bodies.

Inhibition of AMO implies the occurrence of either competition of ammonia with TCS for its active site, or interactions between TCS and AMO that may result in the morphology of the AMO enzyme. The lack of inhibition of the HAO enzyme suggests only direct AMO inhibition occurs. The ability of AMO to utilize many non-growth substrates as a substitute for ammonia poses a risk to these functionally important

microbes as the accumulation of not only TCS, but other pharmaceutical compounds, amass in wastewater.

The lack of cometabolic transformation of TCS by *N. europaea* during three hour inhibition studies and long term growth suggests that TCS in the environment and in WWTP will not be significantly transformed by *N. europaea*. This is an important conclusion, as degradation products of TCS, such as methyl-TCS, are more stable and therefore potentially more toxic in the environment. Our results contradict the report by Roh et al. (2009), who concluded that TCS could be transformed by *N. europaea*.

The less nitrification inhibition exhibited by chemostat-grown cells compared to batch-grown cells when treated with 1 ppm TCS suggests that chemostat-grown cells may be more resistant to inhibition when exposed to lower concentrations of TCS. The continuous influx of nutrients to *N. europaea* during growth in a chemostat mimics the constant wastewater influent available as a nutrient source for AOB in WWTP. Lauchnor and Semprini (2013) indicate that the slower metabolic rate of cells grown under chemostat conditions compared to batch conditions was responsible for less inhibition by phenol. Our results with TCS are consistent with their observations.

5.3. Future Research

TCS inhibition was apparent during three hour suspended batch studies and during long term growth. Future studies directed at the inhibition of *N. europaea* by methyl-TCS might provide useful information about the toxicity of the more abundant, more stable by-product of TCS transformation. The transformation of TCS by *N. europaea* was not apparent during three hour suspended cell tests and during long term

growth. Similar studies with methyl-TCS should be performed as they may result in cometabolic transformation.

Growth experiments of 1 and 10% cell densities of *N. europaea* conducted with concentrations between 0.01 and 0.05 ppm TCS could be further studied to fill in and complete the curves for the range of TCS concentrations that exhibit partial to significant inhibition of growth. Long term resistance experiments with more than three growth cycles would also provide valuable information on whether or not more generations of growth are required for *N. europaea* to develop resistance to TCS.

Further examination of the inhibition of chemostat-grown cells by TCS could also provide more insight into its comparison to the inhibition of batch-grown cells. Direct TCS inhibition tests under chemostat growth conditions should also be investigated. TCS inhibition of the growth of biofilms could also provide interesting information. Comparison of TCS inhibition on the growth of batch, chemostat and biofilm cultures of *N. europaea* would provide interesting results on the inhibition of *N. europaea* under different growth states.

Numerical modeling of growth curves shown in Figures 17B and 18B simulating cell growth with TCS toxicity could also be further studied. A kinetic inhibition constant for the inactivation of cell growth by TCS could be incorporated into the biological growth model. Experimental data could be matched to growth inhibition models that have been proposed.

Future studies that include more in-depth genetic analysis could prove to be rewarding and might provide information about the inhibition mechanisms and specific

genes targeted by TCS in *N. europaea*. Further research should also be done to determine the mechanism by which TCS inhibits cell synthesis in *N. europaea.*

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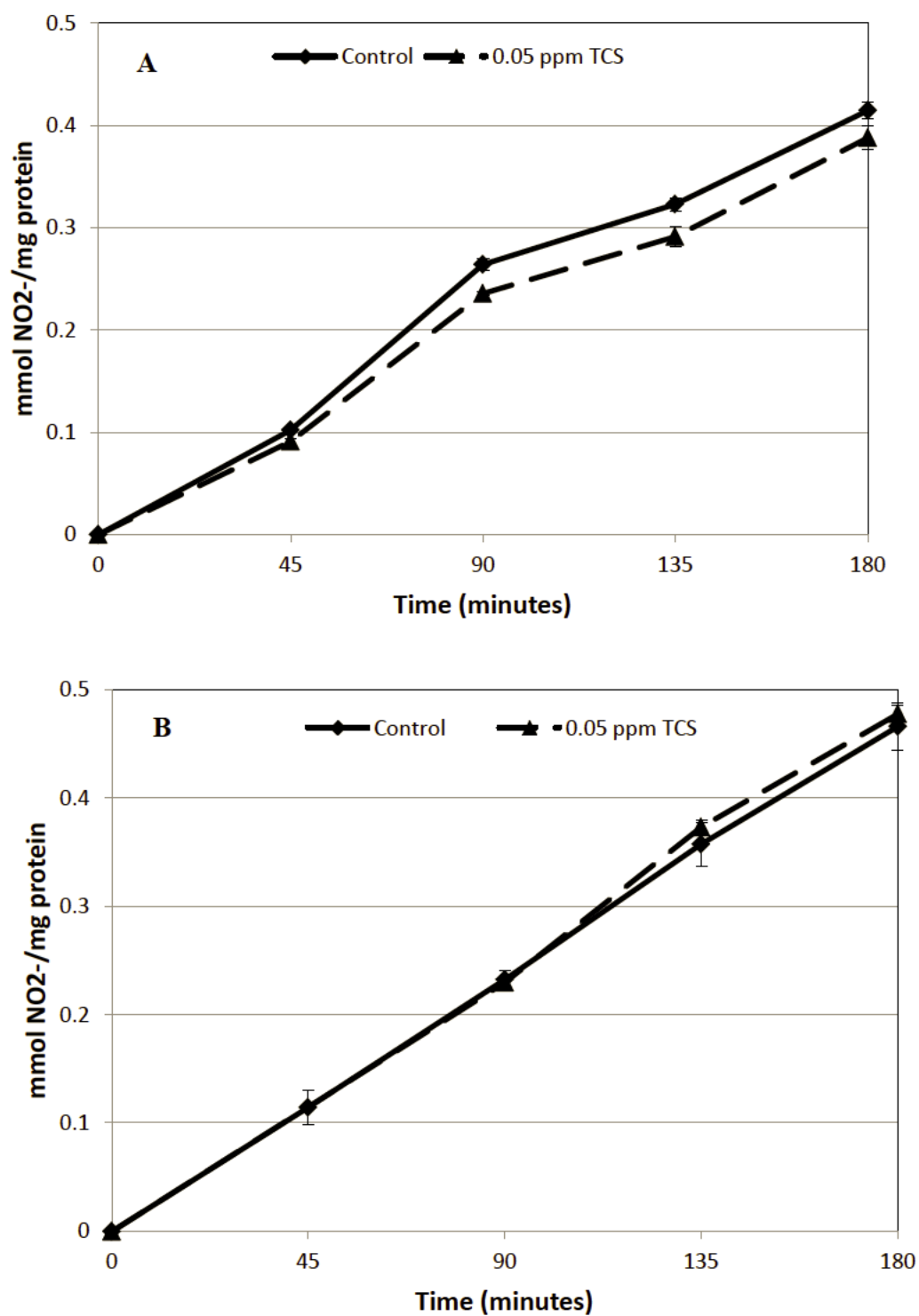
APPENDIX

Figure 1. **(A)** Nitrite production based on cell mass of *N. europaea* exposed to 0.05 ppm TCS during a three hour suspended cell batch inhibition test and **(B)** recovery of washed cells on a nitrite production per cell mass basis. Error bars represent the standard deviation of triplicates to 95% confidence.

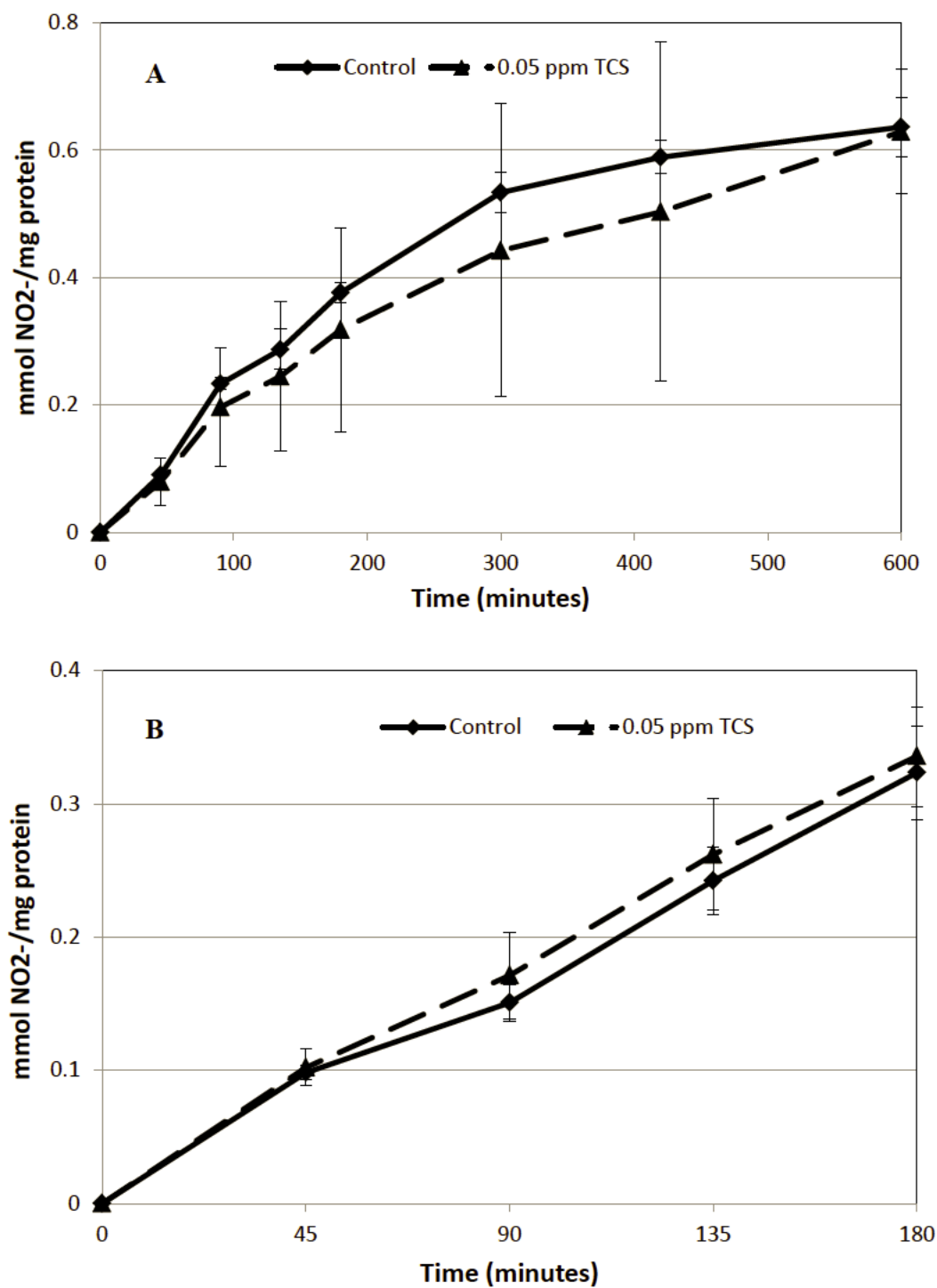**Figure 2.** **(A)** Nitrite production based on cell mass of *N. europaea* exposed to 0.05 ppm TCS during a 10 hour suspended cell batch inhibition test and **(B)** recovery of washed cells on a nitrite production per cell mass basis. Error bars represent the standard deviation of triplicates to 95% confidence.
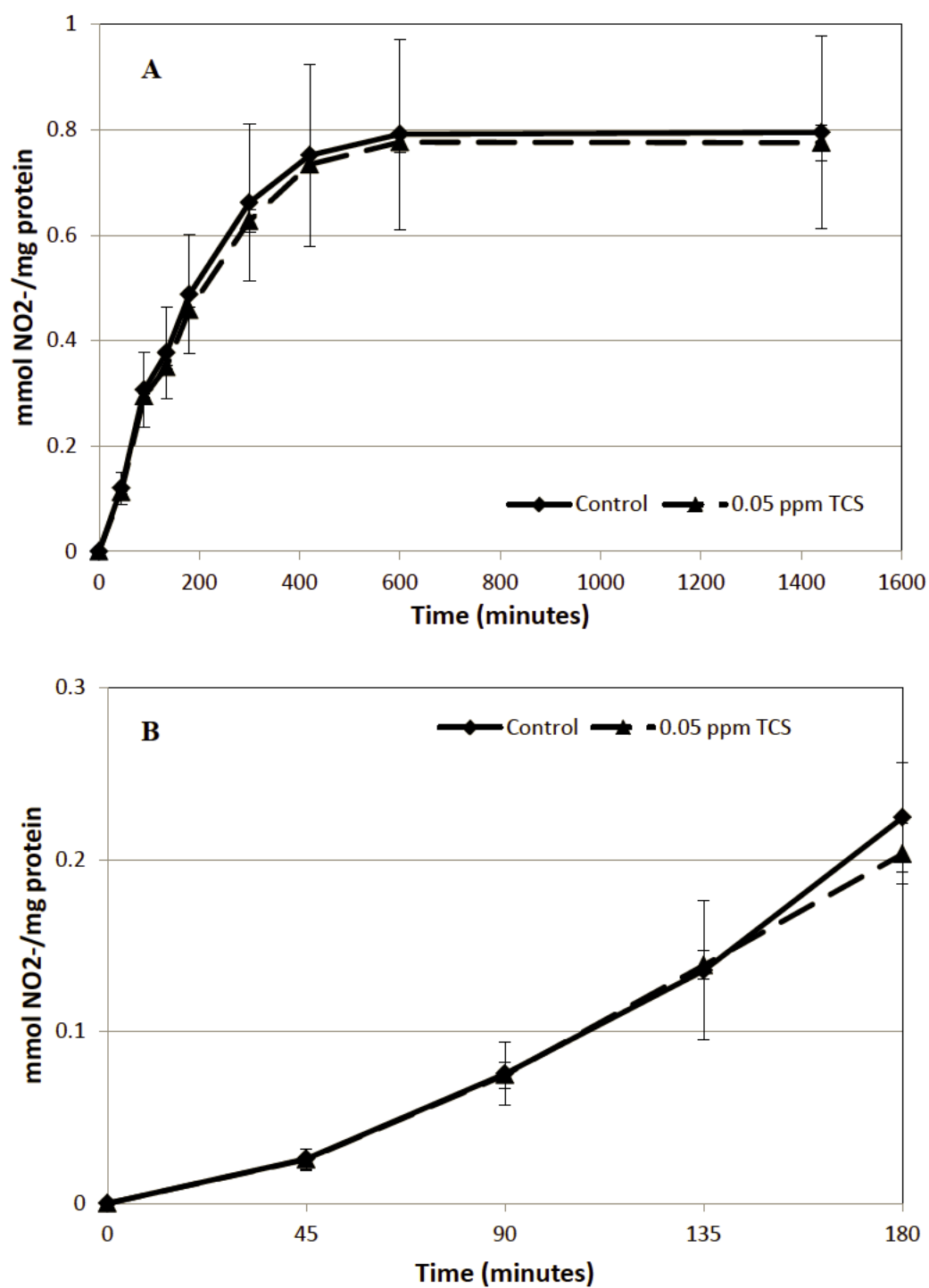
Figure 3. **(A)** Nitrite production based on cell mass of *N. europaea* exposed to 0.05 ppm TCS during a 24 hour suspended cell batch inhibition test and **(B)** recovery of washed cells on a nitrite production per cell mass basis. Error bars represent the standard deviation of triplicates to 95% confidence.