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IMPACT OF PROTOZOAN GRAZING ON NITRIFICATION AND THE AMMONIA- AND NITRITE-OXIDIZING BACTERIAL COMMUNITIES IN ACTIVATED SLUDGE

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A Thesis presented to Ryerson University

In partial fulfillment of the requirement for the degree of

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ABSTRACT

Impact of Protozoan Grazing on Nitrification and the Ammonia- and Nitrite-Oxidizing Bacterial Communities in Activated Sludge

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The effect of protozoan grazing on nitrification rates under different conditions was examined. The spatial distribution of ammonia- and nitrite-oxidizing bacteria (AOB and NOB) in activated sludge was also examined using FISH/CSLM. Batch reactors were monitored for ammonia, nitrite, nitrate, and total nitrogen concentrations and bacterial numbers in the presence and absence of cycloheximide, a protozoan inhibitor. In the absence of protozoan grazing, rates of nitrification were lower than in batches with protozoa. Spatially, both AOB and NOB were found clustered within the floc and neither inhibiting the protozoa or inhibiting ammonia oxidation appeared to lower the amount of AOB and NOB present or their position. These results suggest that a reduction in protozoan grazing pressure allowed the heterotrophic bacteria to proliferate which caused a corresponding decrease in the rate of nitrification. These results suggest that AOB and NOB are less active in the absence of protozoa and indicates the role of protozoa in the cycling of nitrogen.

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DEDICATION

This thesis is dedicated to each and every person who has ever spent time and energy on the pursuit of bettering the environment in which we live, in whichever way that may be.

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1.0 INTRODUCTION AND PURPOSE

The wastewater treatment process is used worldwide to treat both industrial and municipal sewage (Wagner and Loy, 2002). The efficient and effective treatment of wastewater is essential in order to ensure that our water resources are managed in the most environmentally and economically sustainable ways possible. In the past several decades, much research has focused on the role of nitrifying bacteria in the sewage treatment process. Nitrifying bacteria play a crucial role in the activated sludge phase of the sewage treatment process by removing nitrogen from the waste, thus preventing the discharge of ammonium salt into receiving waters (Viessman and Hammer, 1998). The discharge of ammonium salts into receiving waters leads to oxygen consumption, algal blooms, and fish kills via acute toxicity.

The accumulation and diversity of a nitrifying microbial community in the activated sludge plays a crucial role in the performance of nitrification in wastewater treatment. One important factor affecting the productivity of nitrifying bacteria is the presence or absence of protozoans. Protozoa are common predators of bacteria and thus have been shown to effect nitrogen transformation by increasing the mineralization of nitrogen immobilized in the bacterial biomass in soil and other systems such as suspended growth reactors (Clarholm, 1985; Verhagen and Laanbroek, 1992; Ratsak et. al., 1996; Strauss and Dodds, 1997). Grazing protozoa also ensure that there are ample nutrients available for slow growing nitrifying bacteria by grazing primarily on fast growing heterotrophic bacteria in activated sludge floc (Verhagen and Laanbroek, 1992). Thus, when a healthy population of protozoans are present, efficient nitrogen cycling occurs. In order to ensure that the efficiency of nitrification (and thus the removal of ammonium salts) from wastewater is being maximized, factors that affect the nitrifying community in activated sludge are being studied.

The overall goal of this study was to examine the effect of protozoan grazing on nitrification rates. The specific objectives were to monitor the concentrations of ammonia, nitrite, and nitrate under various conditions in the presence and in the absence of protozoa and to examine the spatial distribution of ammonia- and nitrite- oxidizing bacteria using fluorescent *In situ* hybridisation (FISH) and confocal scanning laser

microscopy (CSLM). To date, very few studies have been conducted that examine the link between the protozoan community and effective nitrogen cycling in activated sludge systems specifically. In order to achieve these objectives, bioreactors containing activated sludge from a municipal wastewater treatment plant (MWTP) were set up in the lab. In order to assure the batch reactors were operating within paramenter ranges approximating those of the activated sludge process, pH, temperature, dissolved oxygen, and chemical oxygen demand were measured. To assess nitrification efficiency, ammonia nitrogen, nitrite, nitrate, and total nitrogen concentrations were measured. To test for the effect of protozoan interactions cycloheximide, a protozoan inhibitor, was added to the reactors. To test for the effect of the inhibition of nitrification, allylthiourea, an inhibitor of ammonia oxidation, was added. To test for the effect of deflocculation, a problematic phenomenon in activated sludge treatment plants, ethylenediaminetetraacetic acid (EDTA) was added. Each set of conditions was established in the presence and absence of protozoans. The change in the position and quantity of nitrifying bacteria in the sludge was also established under each set of conditions by using a confocal scanning laser microscope (CSLM) combined with fluorescent *in-situ* hybridization (FISH), a technique used to identify specific bacterial species. By measuring the physical and chemical parameters described above and using the CSLM combined with FISH, we will be able to determine how each set of conditions affects the performance of nitrification, and the nitrifying bacteria community composition.

It is hoped that the outcome of this research will emphasize the importance of maintaining healthy protozoan-bacterial interactions in sustaining the effectiveness of bacterial nitrification. The relationship between protozoan grazing and bacterial nitrification i. ; ›ecially applicable in systems such as activated sludge where both protozoan and bacterial communities are present and the removal of ammonia nitrogen is of the upmost concern. Since nitrification is an essential step in the sewage treatment process, ensuring that it occurs with optimal effectiveness is in the best interest of the environment, industry, and society as a whole.

2.0 LITERATURE REVIEW

2.1 Wastewater Treatment

The treatment of wastewater is one of the most essential biotechnological processes and is used worldwide to treat both industrial and municipal sewage. The purpose of municipal wastewater treatment is to prevent pollution of the receiving waterways and the subsequent problems that arise from this pollution.

Conventional wastewater treatment consists of preliminary processes (pumping, screening, and grit removal), primary settling to remove heavy solids and floatable materials, and secondary biological aeration to metabolize and flocculate colloidal and dissolved organics. Combined preliminary and primary treatment processes remove approximately 30-50% of the suspended solids in raw municipial wastewater (Viessman and Hammer, 1998). The remaining organic matter is extracted in biological secondary treatment to the allowable effluent residual using activated sludge processes, trickling filters, or biological towers (Viessman and Hammer, 1998). Of these three biological treatment processes, the activated sludge process has proven to be the most effective and economical, and is thus the most frequently used biological treatment process in the western world (Viessman and Hammer, 1998).

The following sections will focus on wastewater treatment at Ashbridges Bay Waste Water Treatment Plant (WWTP) and the activated sludge process for the secondary biological treatment of wastewater.

2.1.1 Ashbridges Bay Treatment Plant: The Wastewater Treatment Process

The Ashbridges Bay Treatment Plant (ABTP), located in Toronto's East end at the foot of Leslie Street, is one of four sewage treatment facilities located within the borders of Metropolitan Toronto. The plant serves the City of Toronto, The Borough of East York, and parts of the cities of North York and Scarborough. It is operated by the Water Pollution Control subdivision of the Water and Wastewater Services department of the City of Toronto Works and Emergency Services (City of Toronto, 2004).

The ABTP is Canada's largest wastewater treatment facility and covers an area of 40.5 hectares (City of Toronto, 2004). The plant has a design capacity of 818, 000 cubic

metres of wastewater per day and has 12 primary and four secondary digesters to digest raw and waste activated sludge (City of Toronto, 2004). The plant processes wastewater for an estimated population of 1, 250,000 people.

The ABTP provides complete wastewater treatment including the removal of suspended and biological solids, phosphorous removal, and disinfection. Wastewater is carried to the ABTP by a network of pipes and sewers maintained by the City of Toronto Works and Emergency Services department (City of Toronto, 2004). Preliminary treatment occurs via a combination of an aerated grit tank, which slows down wastewater flow and allows the settling of sand, gravel, and other heavy materials to the bottom of the tank, and mechanical bar screens, which remove large solids contaminants such as sticks and rags (Viessman and Hammer, 1998). All materials removed by the grit tanks and the bar screens are washed and then transported to a sanitary landfill for disposal (City of Toronto, 2004).

After preliminary treatment, the screened wastewater flows into a primary settling tank where it is held for several hours to allow solid particles to settle to the bottom of the tank and oils and greases to float to the top. This stage of treatment is known as primary treatment and its main function is to allow for the physical separation of solids and greases from the wastewater. The oils and greases are skimmed off the top of the water and collected. This is known as primary or raw sludge, and is pumped to large digestion tanks for further treatment (City of Toronto, 2004).

Following primary treatment the partially treated wastewater from the settling tank flows by gravity to covered aeration tanks where it is mixed with solids that contain microorganisms (activated sludge) that use oxygen to consume the remaining organic matter in the wastewater as their food supply. The aeration tank uses air bubbles to provide the mixing and oxygen that are needed for the microorganisms to multiply (City of Toronto, 2004). The wastewater, air, and activated sludge are mixed in the aeration tanks for four to six hours. The activated sludge phase of the wastewater treatment process is known as secondary wastewater treatment. After secondary treatment the aerated mixture of wastewater and activated sludge is sent to the final clarifier. Here the solids settle out to the bottom where some of the material is sent to the solids handling

process and some is recycled back (returned sludge) to replenish the population of microorganisms in the aeration tank to treat incoming wastewater (City of Toronto, 2004).

The wastewater that remains is disinfected via chlorination to kill harmful microorganisms before being released into receiving waters.

The primary solids from the primary sedimentation tanks and solids collected from the final clarifier are sent to digestion tanks containing microorganisms. During this process, the microorganisms use the organic material present in the solids as a food source and convert it to by-products such as methane gas and water. Digestion results in a 90% reduction in pathogens and the production of a wet soil-like material called biosolids that contain 95-97% water. The remaining biosolids are de-watered using mechanical equipment such as filter presses or centrifuges to reduce the volume prior to being sent to landfill, incinerated or beneficially used as a fertilizer or soil amendment.

2.1.2 Biological Treatment: The Activated Sludge Process

Activated sludge processes are used for both secondary biological treatment and complete aerobic treatment without primary sedimentation (Viessman and Hammer, 1998). In activated sludge treatment, effluent from primary treatment is pumped into a tank and mixed with a bacteria-rich slurry known as activated sludge (Maier et al., 1999). The mixture of activated sludge, wastewater, and suspended solids present in the aeration tank is often referred to as "mixed liquor suspended solids", or simply "mixed liquor". Air or pure oxygen is pumped through the mixture and promotes bacterial growth and decomposition of the organic material (Maier et al. 1999). The mixture then goes to a secondary settling tank where water is siphoned off the top of the tank and sludge is removed from the bottom (Maier et al., 1999). Some of the sludge is used as an inoculum for the incoming primary effluent, while the remainder is removed. One important feature of activated sludge is the recycling of a large proportion of the biomass. This results in a large number of microorganisms that oxidize organic matter in a relatively short period of time (Maier et al., 1999). The primary feeders in activated sludge are bacteria and the secondary feeders are protozoans. Activated sludge removes the biodegradable organics as well as the unsettleable suspended solids from the wastewater.

The activated sludge process has been primarily aimed at the effective removal of carbon, nitrogen, and phosphorous from the wastewater. Since its conception early in the last century, the activated sludge process has undergone various modifications (Keller et al., 2002). Some of these modifications include changes to the size, number, and configuration of the reactors, changes to both the recycled and influent flow, and changes to the position and type of aeration devices (Viessman and Hammer, 1998). Despite these modifications, today's systems are still surprisingly similar to these early ones, and the three main functions of the activated sludge process remain the same: the removal of soluble biodegradable organic compounds from wastewater by a flocculant slurry of microorganisms through sedimentation, the recycling of a small amount of return sludge from the clarifier underflow to the reactor, and the dependence of the systems' high performance on the mean cell residence time of microorganisms (Grady and Lim, 1980).

The use of activated sludge for secondary wastewater treatment has proven to be both economical and an effective method for the removal of organic compounds from wastewater (Grady and Lim, 1980). The activated sludge process has been renowned for producing an effluent of high quality at a reasonable cost. The activated sludge process has been known to remove certain pollutants with an efficiency of 90% or more (Viessman and Hammer, 1998).

2.1.3 Parameters Affecting the Activated Sludge Process

The activated sludge process is a treatment process relying largely on microorganisms and as such is sensitive to all of the factors that affect the microbial community. Environmental parameters that adversely affect the desired microbial growth in an activated sludge aeration tank can cause production of sludge with poor settling characteristics (Viessman and Hammer, 1998). This can cause problems such as insufficient removal of organic material from wastewater, excessive presence of activated sludge floc in the final clarifier effluent, and the excessive growth of filamentous bacteria and algae (Viessman and Hammer, 1998). This section provides an overview of the most important factors affecting the activated sludge process in sewage treatment.

2.1.3.1 Oxidation, Temperature, and pH

Activated sludge is a completely aerobic treatment process since the biological flocs are suspended in a liquid medium containing dissolved oxygen. It is essential that aerobic conditions be maintained in the aeration tank; however, in the final clarifier the dissolved oxygen concentration can become extremely low. Dissolved oxygen (DO) extracted from the mixed liquor is replenished by air supplied to the aeration tank (Viessman and Hammer, 1998). The activated sludge process depends on the physiology of heterotrophic organisms that, in the presence of oxygen, utilize the organic substances present in the wastewater as a carbon source for cell synthesis and as a source of energy.

The biochemical oxygen demand (BOD) of domestic and industrial wastewaters is the amount of molecular oxygen consumed by microorganisms during the biochemical oxidation of organic (carbonaceous BOD) and inorganic (ammonia) matter (Maier et al., 1999). BOD is exerted by three classes of matter: carbonaceous material, oxidizable nitrogen, and certain chemical reducing compounds. The chemical oxygen demand is the amount of oxygen necessary to oxidize all of the organic carbon completely to CO_2 and H_2O. COD represents the concentration of both biodegradeable and nonbiodegradeable organics in wastewater (Maier et al., 1999). The chemical oxygen demand (COD) is a more commonly measured parameter in wastewater treatment plants because measuring COD is much easier and less time consuming than the traditional 5-day BOD measurement. In wastewater treatment, the general biological reaction that takes place is:

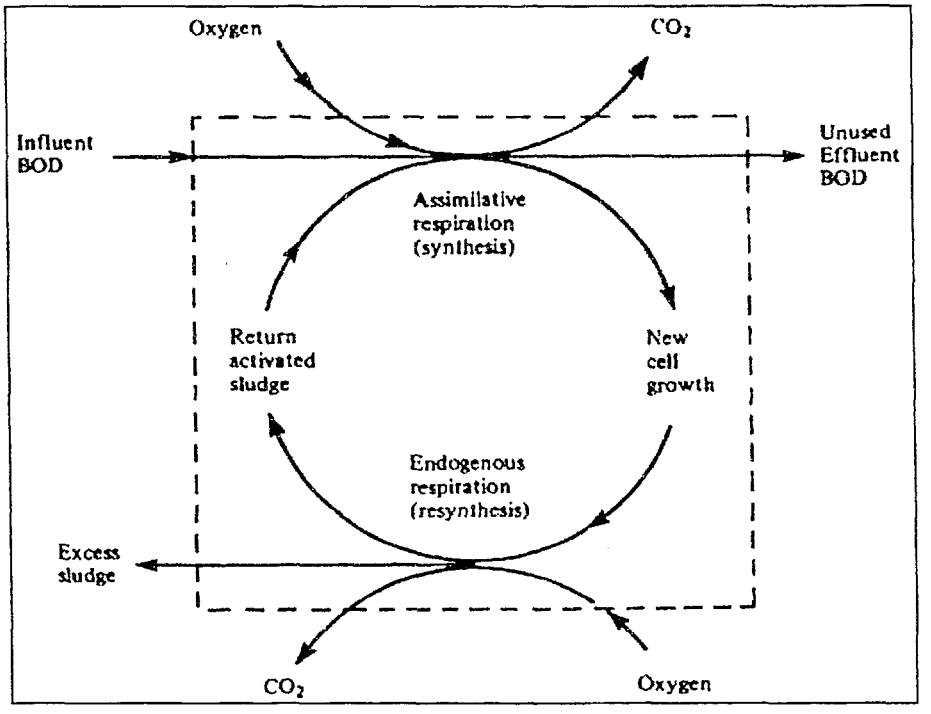
$$\text{waste organics} + \text{DO} \xrightarrow{\text{MICROORGANISMS}} CO_2 + \text{new cell growth} \quad \text{(Equation 2.1)}$$

(Viessman and Hammer, 1998)

In activated sludge, both BOD and COD are present in the form of waste organic material. When wastewater mixes with the organisms of activated sludge in the presence of dissolved oxygen, two phenomena responsible for the initial removal of BOD/COD take place. The suspended and colloidal solids and some of the soluble organic substances in the wastewater are absorbed onto the surface of the activated sludge floc. At the same time, waste organics are being converted to carbon dioxide and energy stored

within the microbial cells through intense biological activity. Further wastewater organic matter is subsequently removed by continued aeration. Figure 2.1 provides a summary of the generalized biological process reactions in the activated sludge process.

Figure 2.1 Generalized biological process reactions in the activated sludge process (Viessman and Hammer, 1998. pp 254).



Sufficient aeration and the maintenance of a completely aerobic environment is essential for the effective removal of BOD/COD from wastewater during the activated sludge treatment process.

Bacteria are classified at psychrophilic, thermophilic, or mesophilic depending on their optimum temperature range for growth. The vast majority of biological-treatment systems operate in the mesophilic temperature range of 20-40°C. The activated sludge process operates at the temperature of the wastewater as modified by that of the air. Generally, this falls within the range of 15-25°C. As a general rule, biological activity doubles for every 10-15°C temperature rise within the range 5-35°C (Viessman and Hammer, 1998). Temperature also has an effect on nitrification rates. At a pH of 8.5, a nitrification rate of 100% occurred at 30°C, with this rate decreasing by one-half for every 10-12°C temperature drop above 10°C.

Another important factor in ensuring that organic matter is being removed from wastewater at optimal rates is the maintenance of pH within acceptable limits. The hydrogen-ion concentration of the culture medium has a direct influence on microbial growth. Most biological treatment systems operate best in a pH neutral environment. The general range for operation of activated sludge systems is between pH 6.5 and 8.5 (Clark et al, 1977; Grady and Lim, 1980). At pH 9 and above, microbial activity is inhibited. Below pH 6.5 fungi are favoured over bacteria in competition for food. It is also important to note that the optimal pH for nitrification, an extremely important process for the removal of ammonia nitrogen in wastewater, is 8.2-8.6, with 90% of the maximum occurring between pH 7.8 and 8.9, and less than 50% of the optimum below 7.0 and above 9.8 (Viessman and Hammer, 1998).

2.1.3.2 Nutrient Levels

Carbon, nitrogen, and phosphorous are the most important nutrients in the activated sludge process. Since organic carbon is often present in abundance in wastewater and readily used by microorganisms, it rarely causes problems by being limiting or in excess. The levels of nitrogen and phosphorous are both very important parameters for two reasons.

1) An activated sludge system that is lacking in nitrogen and phosphorous has a significant effect on the growth of sludge microorganisms, and can also result in the proliferation of undesirable filamentous microorganisms.

2) Wastewater effluents containing high concentrations of nitrogen and phosphorous contribute to the eutrophication of receiving waters.

Phosphorous occurs in nature in the form of inorganic and organic phosphates. Phosphorous is required by the cell primarily for the synthesis of nucleic acids and phospholipids (Madigan et al., 2000).

Nitrogen is a major element in proteins, nucleic acids, and several other cell constituents (Madigan et al., 2000). Nitrogen is found in nature in both organic and inorganic forms, with the bulk being in inorganic form, either as ammonia (NH_3), nitrate (NO_3^-) or N_2. The primary sources of nitrogen in domestic waste are feces, urine, and food-processing discharges. About 40% of the nitrogen in wastewater is in the form of ammonia, and the remaining 60% is bound in organic matter (Viessman and Hammer, 1998).

Other mineral nutrients such as sulfur (S) potassium (K), magnesium (Mg), calcium (Ca), sodium (Na), iron (Fe), copper (Cu), and cobalt (Co), are needed in smaller amounts for various cellular functions (Madigan et al., 2000).

2.1.3.3 Flocculation

An important characteristic of the bacteria present in the activated sludge of the secondary wastewater treatment process is their tendency to aggregate into structures known as flocs. Activated sludge flocs are complex consortia of various microorganisms (Wagner et al., 1994). In addition to microorganisms, sludge flocs also consist of extra polymeric substances (EPS), organic particles (e.g. detritus, extracellular polymers, and cellular debris), inorganic particles (e.g. clays and silts), and interfloc spaces which allow for the retention or flow through of water (Davey & O'Toole, 2000; Droppo, 2001). Many factors, such as pH, temperature, nutrient availability, and oxygen levels, can effect the floc composition, structure, and function. Through their physical, chemical, and biological activities, activated sludge flocs not only regulate their own environment, but also have the ability to effect the surrounding water quality through their physical,

chemical, and biological activity (Mobarry et al., 1996; Droppo, 1996). The flocculating nature of bacteria in the activated sludge system is essential because the unique structure of the floc allows for the creation of "micro-ecosystems", with a species composition and interaction, and physical chemical, and biological activities that would not be possible without the aggregation of bacteria into flocs.

Good flocculant growth is necessary for the successful operation of the activated sludge process because suspended, colloidal, and ionic particles in the wastewater are removed by adsorption and agglomeration in the aeration tank. A flocculated particle is in continuous interaction with its surroundings, as the medium in which the floc is transported provides the floc with energy, nutrients and chemicals for biological growth, chemical reactions, and morphological development (Droppo, 1996). The formation of flocs is also extremely important in the final clarifier stage of wastewater treatment in order to ensure optimal settling and the efficient separation of the sludge biomass from the treated wastewater. The capability of a floc to adsorb material depends on the surface area of the floc and the number of available cell surfaces. Once all of the adsorption sites on a floc are occupied, the floc has only a very limited ability to adsorb further material until all of the currently adsorbed materials have been metabolized. The activated sludge process depends on the re-inoculation of the aeration tanks with recycled, settled sludge. This method ensures that sludge entering the tanks is composed only of floc-forming organisms that settle rapidly in the final clarifier. Thus, the process is microbially self-regulating with the required selected flocs re-inoculated into the system.

2.2 Microbial Populations in Activated Sludge System

The activated sludge process relies very much on the principle of "everything is everywhere, the environment selects" (Cloete and Theron, 2003). This has resulted in the selection of a complex and enriched culture comprising a mixture of generalists and specialist microorganisms. The microbial community of activated sludge consists of bacteria, protozoa, fungi, algae, and filamentous organisms. Of these organisms, it is the bacteria, protozoa, and filamentous organisms that the actively participate in the biological treatment of wastewater in the activated sludge system (Viessman and Hammer, 1998). Activated sludge microorganisms can be divided into two major groups:

decomposers consumers. Decomposers are responsible for the biochemical degradation of polluting substances and cycling of nutrients in wastewaters. This group is mainly composed of bacteria, fungi, and cyanophyta (Cloete and Muyima, 1997). Consumers are organisms that utilize bacterial and other microbial cells for energy. This group is known as activated sludge microfauna and consists of both phagotrophic protozoa and microscopic metazoa (Cloete and Muyima, 1997).

Although the macroenvironment of activated sludge has been well researched and described, an incomplete understanding of the activated sludge floc microenvironment exists. In order to continually improve on the activated sludge process and the accompanying technology, a complete understanding of the microorganisms and their physiology in the activated sludge habitat is necessary. In addition, it is impossible to adequately assess the function of microorganisms in the activated sludge habitat without a complete knowledge of all of the interacting populations in that habitat or in the microenvironments created within it (Cloete and Theron, 2003). A detailed knowledge of the bacterial and protozoan communities, and the interactions between these communities, can provide data on population changes over space and time. This knowledge could result in the construction of much needed accurate models to:

1) Predict population responses to environmental disturbances in the activated sludge system, such as the impact of higher than normal phosphorous or nitrogen loadings
2) More accurately define the food webs present in the activated sludge system and therefore better define energy input and utilization within the system
3) Have a much more thorough knowledge of biogeochemical cycling
4) Describe new organisms with unique physiological properties
5) Estimate microbial behaviour as defined by diffusion limitations, and, hence, the establishment of gradients and microhabitats within the activated sludge ecosystem.

(Cloete and Theron, 2003)

Currently, many techniques are being used in order to assess the complex bacterial community of the activated sludge system. Some of these techniques include culture-dependant techniques, nucleic acid hybridization techniques, fluorescent antibody

techniques, and whole cell *in situ* hybridization using oligonucleotide probes (Cloete and Theron, 2003). Techniques for assessing protozoan communities in activated sludge are less complex and rely on more traditional microscopic observation methods. The following sections provide an overview of the bacterial and protozoan communities present in activated sludge and the known interactions between these communities to date.

2.2.1 Bacterial Populations in Activated Sludge

Communities of prokaryotic organisms present in activated sludge reactors are responsible for most of the carbon and nutrient removal from sewage and thus represent the core component of biological wastewater treatment. Traditional culture-dependant and light microscope methods have revealed a variety of microbial isolates and indicated that activated sludge samples contain a whole array of different bacterial species (Cloete and Theron, 2003; Wagner and Loy, 2002). During the past decade, a variety of molecular approaches have been developed and used to study bacterial community diversity in activated sludge in a cultivation-independent manner (Wagner and Loy, 2002).

Based on 16S ribosomal ribonucleic acid (rRNA) gene library analyses, several general microbial diversity surveys of activated sludge systems have been performed since 1995. In these studies, sequences affiliated with the *Beta-*, *Alpha-*, and *Gammaproteobacteria*, as well as the *Bacteroidetes* and the *Actinobacteria* were consistently the most frequently retrieved (Wagner and Loy, 2002). These findings are consistent with previous fluorescent in situ hybridization (FISH) studies of activated sludge systems conducted using species specific oligonucleotide probes (Wagner et al., 1993; Snaidr et al., 1997). A summary of the 16S rRNA based diversity surveys for a full-scale WWTP is shown in table 2.1.

Table 2.1 Summary of 16S rRNA-based diversity surveys of a high-load aeration basin of a full-scale municipal waste water treatment plant

(modified from Wagner and Loy, 2002).

		High-load aeration tank of full-scale municipal WWTP
	No. Clones	62
	No. OTUs	25
	No eOTUs	32
	Coverage (%)	77
Relative frequency of bacterial divisions in % (No. OTUs within the respective division)	Proteobacteria α β γ δ ε	 3(1) 52(9) 18(7) 15(1)
	Firmicutes	10(5)
	Verrucomicrobia	2(1)
	Choroflexi	2(1)

** OTU denotes operational taxonomic unit. The number of total expected OTUs (eOTUs) was calculated according to the formula: eOTUs = (number of OTUs X 100%) X coverage.

Bacteria may be classified as heterotrophs or autotrophs depending on their nutrient requirements. Heterotrophic bacteria use organic compounds as an energy and carbon source for synthesis (Madigan et al., 2000). It is generally agreed that most of the bacteria present in activated sludge systems are aerobic respiring chemoheterotrophs (Seviour, 1999). Chemoheterotrophs are responsible for the degradation and utilization of the complex and diverse array of organic materials present in the activated sludge mixed liquor, which are in turn converted to carbon dioxide and cell biomass. Autotrophic bacteria use carbon dioxide as a carbon source and oxidize inorganic compounds for energy. The proper operation of the activated sludge bacterial community depends of the presence of both heterotrophic and autotrophic organisms.

The three groups of bacteria that are the most essential to the activated sludge process are the filamentous bacteria, the autotrophic bacteria responsible for nitrogen removal, and the bacteria catalyzing phosphorous removal. While many of the key species from each group have been identified using traditional culture-dependant and light microscope

methods, there are many uncultured bacterial species that have recently been identified as key players in the activated sludge process. As the development of molecular techniques for *in situ* analyses of the function of uncultured microorganisms continues, more will be discovered about the ecology and physiology of these important organisms. Increased research and development of a variety of molecular and environmental genomic techniques will allow for a more complete understanding of the relationship between microbial community composition, function, and process stability, and also allow access to the genome and transciptome of uncultured, but functionally important WWTP bacteria (Wagner and Loy, 2002).

2.2.2 Protozoan Populations in Activated Sludge

Protozoans are single-celled organisms that reproduce by binary fission. They have complex digestive systems, and use solid organic matter as an energy and carbon source (Viessman and Hammer, 1998). Protozoans are a vital link in the aquatic chain of the activated sludge treatment process because they ingest bacteria and algae, and also contribute to the flocculating characteristics of activated sludge. Protozoa are plentiful in activated sludge plants, and it is common to find populations of these organisms in the order of 50,000 cells/mL in the mixed liquor of activated sludge plants (Curds, 1982).

The protozoans of significance in activated sludge treatment systems are strict aerobic organisms (Viessman and Hammer, 1978). Protozoan species in activated sludge from WWTPs generally belong to three main groups, namely: ciliates, flagellates, and amoeba (Al-Shahwani and Horan, 1991). Other higher sludge organisms such as nematodes and rotifers may also be present, but to a much lesser degree (Curds, 1982). Of these groups, the ciliates are the most numerous, comprising approximately 70% of the protozoan community in activated sludge (Curds, 1975).

Ciliates may be categorized as free swimming and stalked. Free swimming forms move rapidly in the water, ingesting organic matter at a very high rate (Viessman and Hammer, 1998). The stalked forms attach by a stalk to particles of matter and use cilia to propel their head about and bring in food. Table 2.2 lists species of protozoa identified in biological sewage treatment programs especially activated sludge systems (Al-Shahwani and Horan, 1991). For a single reproduction a ciliated protozoan consumes thousands of

bacteria, with two major beneficial effects on the activated sludge ecosystem. Removal of the bacteria stimulates further bacterial growth, resulting in accelerated extraction of organic material from the mixed-liquor. Second, the flocculating properties of activated sludge are improved by reducing the number of free bacteria in solution, and this results in a biological floc with improved settling characteristics (Viessman and Hammer, 1998). Free swimming ciliates are present in higher numbers when the surrounding solution contains a high number of dispersed bacterial populations (Viessman and Hammer, 1998). When dispersed bacteria become scarce, stalked protozoans increase in numbers. Stalked protozoans do not require as much energy as free-swimming protozoans, and therefore they compete more effectively in a system with low bacterial concentrations (Viessman and Hammer, 1998). Thus, in well-flocculating activated sludge with limited numbers of dispersed bacteria, attached ciliates are the most biologically important protozoa (Curds, 1982; Viessman and Hammer, 1998). Attached ciliates can grow alone or in large colonies. Figure 2.2 shows several species of attached ciliates commonly present in activated sludge samples (UCLA, 2003).

In all of the publications on the role or protozoa in the purification of sewage, it is generally agreed that the primary role of protozoa in the activated sludge process is the clarification of the effluent (Curds, 1982). The exact extent and means by which protozoa clarify the effluent is still not completely understood. The significant drop in both the numbers of bacteria and the amount of nonsettleable suspended solids in effluents could be the result of two factors: predation or flocculation. A considerable amount of evidence in literature shows that protozoa in pure culture are able to flocculate suspended particulate matter and bacteria, which aids in both the clarification of effluent and in the formation of sludge (Curds, 1982; Curds and Hawkes, 1982; Viessman and Hammer, 1998). Some earlier studies postulated that protozoan-induced flocculation was brought about directly by the secretion of a mucous-like substance from the peristome region of the protozoa (Curds, 1982). However, it is now generally agreed upon that the major role of protozoa in activated sludge treatment processes is the removal of dispersed growths of bacteria by predation, and that increased flocculation is a result of this activity (Curds, 1982; Viessman and Hammer, 1998).

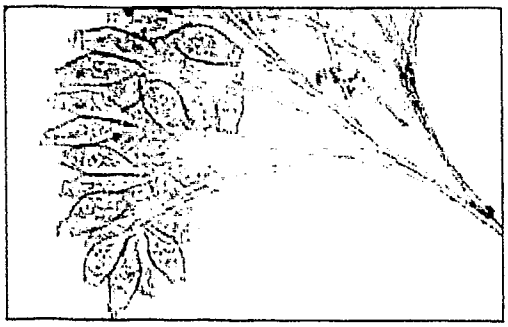
Table 2.2 The most predominant ciliated protozoa found at a domestic biological sewage treatment plant ranked on their percentage occurrence at > 1000/ml (Al-Shahwani and Horani, 1991).

Ciliated Protozoa	Occurrence (%)	Rank
Trachelophyllum pusillum	72	1
Aspidisca costata	70	2
Vorticella striata	62	3
Vorticella convallaria	58	4
Opercularia coarctata	56	5
Carchesium polypinum	52	6
Vorticella microstoma	50	7
Vorticella campanula	49	8
Vorticella fromentali	46	9
Litonutus fasciola	45	10
Tetrahymena pyriformis	37	11
Vorticella nebulifera	36	12
Vorticella alba	30	13
Epistylis rotans	28	14
Chilondonella cucullus	28	15
Vorticella communis	27	16
Vorticella aequilata	21	17
Epistylis plicatilis	21	18
Vorticella elongata	18	19
Litonutus caranatus	12	20
Podophyra fixa	8	21
Paramecium caudautum	8	22
Acineta grandis	7	23
Euplotes moebiusi	6	24
Colpidium colpoda	5	25
Aspidisca lynceus	2	26
Spirostomum teres	1	27
Blepharisma spp.	1	28
Hemiophrys fusidens	1	29

Figure 2.2 Three common ciliated protozoan species found in activated sludge tanks (a) Carchesium spp., (b) Opercularia spp., (c) Vorticella convalaria (UCLA, 2003).

(a)

(b)



(c)

In addition to the recognized role of protozoa as bacterial predators whose activities induce good effluent quality, there have also been attempts to relate the physico-chemical parameters of activated sludge (i.e. food/microorganism (F/M) ratio, the effluent biological oxygen demand (BOD), and the mixed liquor suspended solids (MLSS) content) to the species of ciliated protozoa present (Al-Shahwani and Horan, 1991; Esteban et al., 1991; Madony et al., 1993). This research is based on the general observation that the relative dominance of different protozoan groups changes with changes in plant performance, and that the numbers of morphological species drop as plant performance deteriorates. Madoni (1994) developed a concept of a sludge biotic index (SBI) based on this idea. The general idea behind the use of protozoa as 'indicator species' for the activated sludge process is that if the protozoan population is ideal, then the bacterial population will also be optimal, and the removal of waster products will occur in the most efficient way possible.

More recent research on the role of protozoa in the activated sludge system has focused on the role of protozoa in enhancing nutrient cycling and carbon mineralization. In literature there are three dominant hypotheses to explain why protozoa may enhance the mineralization of organic compounds. One such hypothesis is that as a by-product of their metabolic activities, protozoa excrete mineral nutrients (phosphorous as phosphate, nitrogen as ammonia or nitrate). This excretion results in increased substrate availability (i.e. C:N:P ratio), which in turn causes an accelerated use of carbon-sources by bacteria (Varma et al., 1975; Coleman et al., 1978; Clarholm, 1985). Another hypothesis is that protozoa excrete growth stimulating compounds such as vitamins, amino acids, and nucleotides which enhance bacterial activity (Ratsak et al., 1996). The impact of protozoan grazing on the selection of bacteria is another hypothesis used to explain the effect of protozoan grazing on nutrient mineralization. Grazing of bacteria leads to the selection of species that can grow fast and inefficiently and thus increase the use of carbon sources (Ratsak et al., 1996). Inefficient bacterial species must dissimilate more materials to form the same amount of biomass. Grazing of bacteria by protozoa in itself also results in a decrease in biomass concentration. All this amounts to a loss of energy in the resulting activated sludge food chain (Ratsak et al., 1996).

Understanding the effect that protozoa have on bacteria through predation, and also the effect that protozoa have through the cycling of nutrients in the ecosystem is an important factor in obtaining a better understanding of the activated sludge system and thus improving the existing biological treatment process.

2.2.3 Interactions Involving Bacterial and Protozoan Populations

Activated sludge consists of a complex and diverse consortium of bacterial, protozoan, and metazoan populations. Indeed, an activated sludge tank may be thought of as a complex ecosystem created by humans. Like in any ecosystem there is an interactive association between microorganisms called a consortium, that results in combined metabolic activities. These microbial populations interact within the community. The microbial community is structured in such a way that each population contributes to its maintenance. Some microbial populations in a community compete for resources in such a way that they may adversely influence each other, sometimes resulting in the exclusion of a species from the community. Other microbial populations have cooperative relationships where the interactions between populations are beneficial, allowing them to live within close proximity of each other. Yet another type of relationship is that exhibited between a predator and its prey.

A knowledge of the relationships, both cooperative and competitive, and predatory, between various microbial populations in mixed cultures is essential to understanding the activated sludge treatment process.

2.2.3.1 Microbial Competition in Activated Sludge

When organic matter is made available to a mixed population of microorganisms, competition arises for this food between various species. Theoretically, the competitive exclusion principle dictates that competition will eliminate all but one of the populations competing for the same substrates. In reality, microorganisms that compete for the same substrates frequently co-exist because of spatially separated micro-habitats within an ecosystem or because of differing affinities for substrates at varying concentrations that can lead to population fluctuations (Seviour, 1999). Competition for available substrates is the main driving force that determines community structure and the diversity of species that can coexist. Primary feeders that are most competitive become the dominant

microorganisms. Under normal operating conditions, bacteria are the dominant primary feeders in activated sludge (Viessman and Hammer, 1998).

The species of dominant primary bacteria present depends on the nature of the organic waste present and on the environmental conditions in the activated sludge aeration tanks. When environmental conditions are adverse to bacterial growth (i.e. low pH, low dissolved oxygen, nutrient shortages), the normal bacterial microflora may be outcompeted by filamentous fungal species who proliferate under these conditions (Seviour, 1999). This phenomenon is known as activated sludge bulking, and is commonly a problem in plants with low pH. When environmental conditions are stable and ideal for bacterial growth, a well-adapted bacterial population will often become dominant. At the initial stage of sludge formation, when the activated sludge is still suspended and the ratio of food (organic materials present in the waste water) to microorganisms (F/M ratio) is high, physically large and fast-growing r-strategist bacterium species dominate (Ratsak et al., 1996). This is true in sewage treatment facilities where an abundance of available organic substrates favours the growth of rapidly dividing bacteria. When the F/M ratio becomes lower due to decomposition of organic material by both bacteria and protozoa, or by a reduction in organic influent loading rates, the fast-growing r-strategists are easily replaced by a more diverse community of slow growing K-strategist bacterial species that have a high affinity for nutrients that are present in low concentrations (Maier et al., 1999).

The presence of protozoa can have a significant effect on competition between bacterial species. For instance, protozoan grazing is generally believed to stimulate the decomposition rate, thus releasing nutrients such as N and P into the surrounding environment. (Ekelund and Ronn, 1994). This in turn encourages the growth of fast growing, heterotrophic bacterial species. Another example of the influence of protozoan presence on bacterial competition can be found in a study by Verhagen and Laanbroek (1992) who examined the influence of grazing by the flagellate *Adriamonas peritocrescens* on competition between the nitrifying bacteria *Nitrosomonas europaea* and *Nitrobacter winogradski* and the heterotrophic species *Athrobacter globiformis.* The numbers of both *Nitrosomonas europaea* and *Nitrobacter winogradski* decreased in the presence of the protozoa, presumably due to selective grazing on the nitrifying bacteria.

The numbers of heterotrophic bacteria concurrently increased. Importantly, the rate of nitrate production did not did not decrease more in the presence of protozoa than in their absence. This result is important since it suggests that there was a higher nitrifying rate per cell in the presence of protozoa, thus protozoan activity influenced both the number and type of dominant bacterial species and bacterial activity.

Primary bacteria in an activated sludge system are maintained in the declining or endogenous growth phases. Under these conditions, the primary bacteria die and lyse, releasing their cell contents back into the solution. In this process, raw organic matter is synthesized and resynthesized by various bacterial groups (Viessman and Hammer, 1998).

Competition between bacterial species is a major factor dictating the primary bacterial community of activated sludge and ultimately the degradation of organic material in wastewater. Although the full extent of bacterial interactions is not well understood, it is generally agreed that the dominant species in treatment plants are largely a result of competitive interactions between species and this field will continue to be examined extensively in the future.

2.2.3.2 Microbial Predation in Activated Sludge

Microbial predation in activated sludge, and all of the interactions and influences involved in the predator-prey relationship, is one of the most complex and least understood topics in biological wastewater treatment. It is only within the past 25 years that protozoa have been recognized as an important force shaping the biological community and the ultimate goal of wastewater treatment: the maximum reduction of BOD with a minimal production of biological solids (Ratsak et al., 1996).

It has been discussed previously in this paper (refer to sections 2.2.2 and 2.2.3.1) the ways in which protozoa influence bacterial growth through the excretion of mineral nutrients (phosphorous as phosphate, nitrogen as ammonia or nitrate) and through the excretion of growth stimulating compounds. Protozoans also affect the bacterial population through predation. Protozoans grow in association with activated sludge bacteria in a prey-predator relationship. That is the bacteria synthesize organic matter and protozoans consume bacteria. Activated sludge consists of a diverse array of

bacterial populations including species of *Zoogloea*, members of the *Flexibacter-Cytophaga-Bacteroides* group, Pseudomonas, *Bacillus*, *Citrobacter*, *Sphaerotilus natans*, and *E. coli*. These bacteria become prey to activated sludge protozoa and other higher organisms such as rotifers and nematodes. Ciliate protozoa dominate the protozoan biomass of activated sludge and as such are the most common predators of activated sludge bacteria (Curds, 1982; Ratsak et al., 1996; Viessman and Hammer, 1998; Seviour, 1999). Ciliates are in-turn consumed by multicellular animals that act as top predators in activated sludge food webs, such as rotifers and nematodes. The basis of the detrital food webs present in activated sludge are the bacteria that degrade organic matter.

Grazing by protozoa has many direct and indirect effects on bacterial populations in activated sludge. Grazing by protozoa can induce several bacterial responses. Selection pressure brought on by competition and predation has been proven to cause the extinction of several specific bacterial species. In an early study by Curds and Fey (1969), the density of viable *E. coli* cells was reduced from 1.4×10^6 to 0.65×10^6 in activated sludge in the absence of protozoa. This was probably due to the ability of other organisms, such as *Flavobacterium* species, to lyse bacteria (Curds and Fey, 1969). In the presence of four ciliates, *Opercularia coarctata*, *Vorticella microstoma*, *Hypotrichidium conicum*, and *Tetrahymea pyriformis* a further reduction in *E. coli* density to 0.05×10^6 cells/ml was observed (a 95% reduction). This result indicates that although other factors are obviously involved in the removal of E. coli, that predation by protozoa plays a major role in the removal of E. coli. Such a dramatic reduction in density also suggests that perhaps besides selection by predation alone, grazing by protozoa was also causing the proliferation of other bacterial species thus increasing the density of these species and outcompeting *E. coli*. This finding was supported by a study by McCambridge and McMeekin (1980) who studied the effect of protozoan grazing on the removal of *E. coli* from estuarine water.

The direct reduction of in number of bacteria from sewage to effluent after aerobic treatment has been noted by many authors (Curds and Fey, 1969; Guede, 1979; McCambridge and McMeekin, 1980; Curds, 1982). It is also known from literature that the dominant type of protozoans in activated sludge (ciliates) feed on bacteria. Thus, it is likely that protozoan predation is responsible for the bacterial removal observed. This

conclusion was supported by the work of Curds and Vandyke (1966) who showed that ciliates in activated sludge feed upon a variety of bacterial species present. As further evidence, a later study by Curds et al., (1968) showed that nonflocculated bacteria do occur in large numbers in activated sludge, but only when protozoa are not present.

Besides directly reducing bacterial numbers through predation, there is evidence that protozoa are able to change the bacterial composition in an activated sludge tank. The was demonstrated with computer simulations (Curds, 1974) and in chemostat mixed cultures of the protozoa *T. pyriformis* grazing on *E. coli* and *Azotobacter vinelanddi* (Jost et al., 1973). Coexistence of both bacterial species was only possible in the presence of protozoan grazing. It was also observed that the purification rates of the wastewater were greater when both bacterial species and protozoa were present, likely because more types of substrate could be metabolized. Another possibility is that a synergistic relationship exists between the bacterial species that allows them to metabolize substrate more efficiently when both are present.

Researchers have also observed that bacterial cell size (Shikano et al., 1990) and the ability to form aggregrates (Goede, 1979) were selective criteria for grazing by protozoa.

Another prey-predator relation theory is that the predation by ciliates stimulates bacterial growth rates and hence substrate uptake rates. Grazing activities of ciliates prevent bacterial populations from reaching carrying capacity. Thus, bacteria populations are kept in the endogenous phase of growth, and their rate of substrate assimilation is greatly increased (Curds, 1982).

Thus, it is obvious that protozoan predation has an important role in the activated sludge process. Some of the better understood direct effects of predation are the removal of harmful bacteria such as *E. coli*, the increase of bacterial diversity in the presence of protozoa, and the direct removal of suspended bacteria by predation. The relevance of the prey-predator relationship to the activated sludge process may be summarized in two major beneficial effects:

1) Removal of bacteria stimulates further bacterial growth, resulting in accelerated extraction of organic material from solution (enhanced carbon mineralization).

2) The flocculation characteristics are improved by reducing the number of dispersed bacteria, and a floc with improved settling characteristics results.

Ultimately, protozoan predation results in a better quality effluent with high clarity (Curds, 1982).

Through grazing activities, protozoa also have an indirect but nonetheless essential role in the nutrient cycling that occurs within the activated sludge ecosystem. A review of the current knowledge of nutrient cycling in the activated sludge ecosystem will be undertaken in the next section.

2.3 Nutrient Cycling, Metabolism, and Energy Flow within an Ecosystem of Activated Sludge

Feeding of bacteria by protozoan and the metabolic activities of all activated sludge bacteria is an essential part of the nutrient cycling that occurs with the activated sludge ecosystem. Without these activities, the ultimate goal of wastewater treatment, a reduction in effluent BOD to levels that will not be harmful to the environment, would not occur.

It has already been discussed in the above sections the effect that protozoa have on enhancing the mineralization of organic compounds through the excretion of mineral nutrients and through grazing activities. It has also been discussed how heterotrophic and autotrophic bacteria greatly contribute to the stabilization of wastewater through the degradation and utilization or organic compounds as an energy and carbon source, and through the oxidation of inorganic compounds, respectively.

The process of metabolism is the basis of energy and nutrient cycling in any ecosystem. Metabolism is the biochemical process (a series of biochemical oxidation-reduction reactions) performed by living organisms to yield energy for synthesis, motility, and respiration to remain viable (Viessman and Hammer, 1998).

In heterotrophic metabolism, organic carbon is the substrate used as a source of energy. However the majority of organic matter in wastewater is in the form of large molecules that cannot penetrate the bacterial cell membrane. In order to metabolize high molecular weight substances, bacteria must be capable of hydrolyzing these large molecules into

diffusible fractions that are able to penetrate the cell membrane. Thus, the first biochemical reactions are hydrolysis reactions of complex carbohydrates into soluble sugar units, protein into amino acids, and insoluble fats into fatty acids. Under aerobic conditions such as those present in activated sludge aeration tanks, soluble organic compounds are oxidized to end products of carbon dioxide and water. A representation of this process is shown in equation 2.2.

$$\text{Organics} + \text{Oxygen} \rightarrow CO_2 + H_2O + \text{energy} \quad \text{(Equation 2.2)}$$

The growth and survival of heterotrophic microorganisms are dependant on their ability to obtain energy from the metabolism of substrates. Biochemical metabolic processes of heterotrophs are energy-yielding oxidation-reduction reactions in which reduced organic compounds serve as hydrogen donors and oxidized organic or inorganic compounds act as hydrogen acceptors. Oxidation is the loss of electrons or hydrogen or the addition of oxygen. Reduction is the gain of electrons or hydrogen, or the loss of oxygen (Viessman and Hammer, 1998). Energy stored in organic matter is released in the process of biological oxidation by dehydrogenation of substrate followed by transfer of hydrogen, or electrons, to an ultimate acceptor. Aerobic metabolism using oxygen as the ultimate acceptor yields the highest amount of energy (Viessman and Hammer, 1998).

The biochemical process of substrate utilization to form new protoplasm for growth and reproduction is called synthesis (anabolism). Microorganisms process organic matter to create new cells. The cellular protoplasm is formed is a combination of hundreds of complex organic compounds including proteins, nucleic acids, carbohydrates, and lipids. The major elements in biological cells are carbon, hydrogen, oxygen, nitrogen, and phosphourous. These elements are essential in the synthesis of organic matter and are known as nutrients. Relationships between metabolism, energy, and synthesis are essential to understanding biological treatment systems. The primary product of metabolism is energy, and the primary use of this energy is synthesis. Energy release and synthesis are coupled biochemical processes. The maximum rate of synthesis occurs simultaneously with the maximum rate of energy yield (maximum rate of metabolism). Thus, in heterotrophic metabolism of wastewater organics, the maximum rate of removal

of organic matter for a given population of microorganisms occurs during maximum biological growth.

Autotrophic bacteria also perform essential metabolic activities in activated sludge by oxidizing reduced inorganic compounds, yielding energy for the synthesis of carbon from carbon dioxide, producing organic cell tissue.

In order for metabolism and synthesis to occur at optimum rates, the presence of nutrients in the proper amounts is essential so that growth does not become limited. It was mentioned in section 2.2.3.2 that nitrogen is one of the most important nutrients in the activated sludge process, because the release of ammonia into receiving waters is toxic to fish and other aquatic organisms. In activated sludge, autotrophic nitrifying bacteria are essential because they perform the conversion of ammonia to nitrate.

The following sections will deal with nitrogen cycling in activated sludge.

2.4 The Microbial Cyclic Conversion of Nitrogen and Nitrogen Cycling in Activated Sludge

In domestic wastewater, faeces, urine, and food-processing discharges are the primary sources of nitrogen with a per-capita contribution in the range of 4-6 kg of N/year. About 60% of it is in the form of ammonia and 40% bound in organic matter. The nitrogen forms of interest are organic, inorganic, and gaseous nitrogen. The process of fixing atmospheric nitrogen into ammonia is called nitrogen fixation. Bacterial decomposition also releases ammonia by deamination of nitrogenous organic compounds (also known as ammonification). Continued aerobic oxidation results in nitrification. Biochemical denitrification occurs with heterotrophic bacteria in an anaerobic or low pH environment. Ammonium produced is also assimilated by cells into amino acids and cell component during the process of assimilation. These reactions define the biological nitrification-denitrification process known as the nitrogen cycle. A schematic representation of the nitrogen cycle is shown in Figure 2.3.

Nitrogen is fixed into ammonia by over 100 different free-living bacteria, both aerobic and anaerobic, as well as some actinomycetes and cyanobacteria (Maier et al., 1999). Some examples of species that can fix nitrogen include non-symbiotic bacteria such as

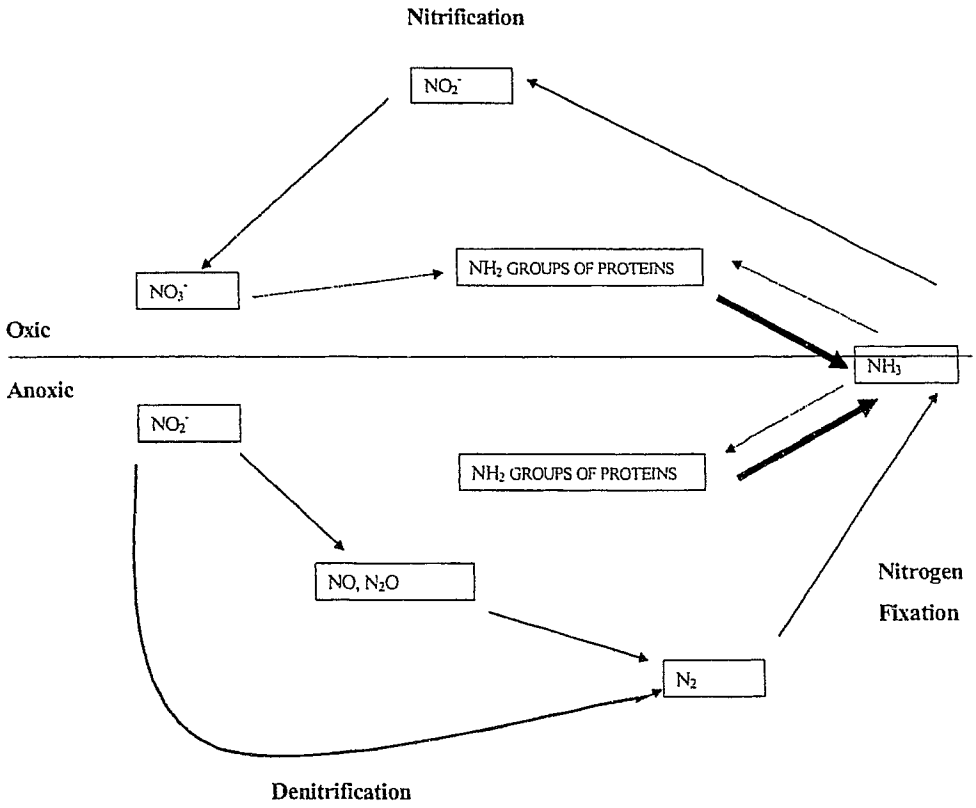
Azotobacter, *Mycobacterium*, and *Thiobacillus*; methane oxidizers as free-living aerobic bacteria such as *Clostridium* and *Klebsiella*; methanogenic bacteria as free-living anaerobes; and symbiotic bacteria such as *Rhizobium*, *Azospirillum*, *Frankia*, and *Citrobacter* (Wiebe, 1989). Ammonia can also be produced in the system through the action of bacterial decomposition of organic matter, also known as ammonification or ammonia mineralization.

In the activated sludge process, aeration of wastewater rich in ammonia induces nitrification, a key process in the removal of ammonia from wastewater. It is important to note that nitrification does not remove ammonia from wastewater, but simply converts it to the nitrate form, thereby eliminating problems to fish and reducing the nitrogen oxygen demand (NOD) of the effluent (Viessman and Hammer, 1998). During nitrification, ammonia is reduced first to nitrite, and then to nitrate by autotrophic nitrifying bacteria. These aerobic reactions yield energy for metabolic functions such as synthesis of carbon dioxide into new cell growth (Viessman and Hammer, 1998).

Many aerobic bacteria can reduce nitrite and nitrate to gaseous nitrogen in an anoxic environment. This process is termed denitrification. Denitrification results in the loss of nitrogen from the environment through the production of nitrogen gas. The following sections will focus on the processes of nitrification and denitrification in activated sludge.

Figure 2.3 The Nitrogen Cycle

The major processes of nitrification, denitrification and nitrogen fixation are labelled. Assimilation steps are marked with dotted arrows. Ammonification (ammonia mineralization) is marked with thick black arrows (modified from Madigan et. al, 2000).



2.4.1 Nitrification

Ammonium (and urea that is hydrolyzed to ammonium) is the major nitrogen compound of sewage and is removed in WWTPs by conversion first to nitrate and then to gaseous nitrogen. The conversion of ammonium to nitrate is known as nitrification and is one of the most important processes in the biological treatment of waste.

2.4.1.1 The Nitrification Process

Nitrification is catalyzed by two different groups of slow growing, autotrophic bacteria – the ammonia oxidizers and the nitrite oxidizers (Wagner and Loy, 2002). The conversions performed by these two bacterial groups can be described by equation 1.3 and equation 1.4 below:

$$NH_4+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ + 250\ kJ \text{ (Equation 2.3)}$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3- + 75\ kJ \text{ (Equation 2.4)}$$

Several conditions must be fulfilled before nitrification can occur in activated sludge systems. The most critical is the sludge retention time (SRT) in the aeration tank (Toerien et al., 1990). The sludge retention time is the inverse of the specific growth rate (μ) of the nitrifiers (McClintock et al., 1992). As such, the SRT or the mean cell residence time (MCRT) must be long enough that the nitrifying bacteria can multiply at a higher rate than they are removed from the system. By increasing sludge age or SRT, the washout of nitrifying bacteria can be avoided and the nitrite/nitrate removal efficiencies increase (McClintock et al., 1992; Viessman and Hammer, 1998; Blackall and Burrell, 1999). Extending the SRT likely aids in increasing the number of ammonia oxidizing bacteria in the system (McClintock et al., 1992).

Other conditions that are important in maintaining an optimal nitrification rate are an adequate hydraulic retention time (HRT) between the nitrifying bacterial biomass and the ammonia and nitrite in wastewater to ensure that the complete oxidation of these nutrients can occur (Blackall and Burrell, 1999). Successful nitrification also depends on providing a suitable pH, temperature, and dissolved oxygen level (refer to section 2.1.3.1 above).

Both the sludge age and the temperature will largely determine the kind of nutrients that will be removed in the activated sludge process. For nitrogen removal, a sludge age and temperature of between 3 days at 25°C, 6 days at 15°, or even up to 15 to 20 days at 7°C are needed for nitrogen removal (Viessman and Hammer, 1998).

Understanding the ecology of nitrifying bacteria in activated sludge is of considerable importance, because nitrification is often referred to as the "achilles heel" of the activated sludge process, and if nitrifiers are washed out of the system, the recovery of the nitrification process is usually a lengthy and expensive task (Wagner and Loy, 2002).

2.4.1.2 Nitrifying Microorganisms in Activated Sludge

In wastewater treatment plants, the oxidation of ammonia to nitrite is performed by a wide variety of different beta proteobacterial ammonium oxidizers (Wagner and Loy, 2002). A recent study by Juretschko et al. (1998) revealed that in addition to the widely known ammonia oxidizer *Nitrosomonas europaea*, ammonia oxidation is also performed by *Nitrosomonas eutropha, Nitrosococcus mobilis*, members of the *Nitrosomonas marina* cluster, and four other phylogenetic lineages for which no culture representative exists. These finding are consistent with other studies of the quantitative ammonia oxidizer community in WWTPs (Juretschko et al., 1998; Daims et al., 2001(b);Gieseke et al., 2001; Liebig et al., 2001).This study also concluded that, in contrast to other ecosystems such as soil, ammonia oxidizers of the genus *Nitrosospira* are not important in wastewater treatment processes.

Nitrite oxidation is performed by another group of bacteria, separate from the ammonia oxidizers discussed above. Recent *in situ* research found that yet uncultured *Nitrospira*-like microorganisms, and not *Nitrobacter* species, are the dominating nitrite oxidizers in activated sludge (Burrell et al., 1998; Juretschko et al., 1998; Okabe et al., 1999; Daims et al., 2000; Daims et al., 2001 (a); Gieseke et al., 2001). *Nitrospira*-like nitrite oxidizers are probably K-strategists (with high substrate affinities and low maximum activity or growth rate) for oxygen and nitrite and therefore outcompete *Nitrobacter* species under the substrate limiting conditions present in activated sludge (Wagner and Loy, 2002).

As discussed above, the activated sludge process relies on the biomass in flocs being recycled through the system as it actively removes nutrients from the wastewater. Floc

forming bacteria will therefore be selected for and retained in the system (Toerien et al., 1990). Microbes that are retained in the system, however, will only survive if they are able to tolerate the environmental conditions present in activated sludge plants, in particular the fluctuations in the dissolved oxygen levels. Fortunately, nitrifying bacteria possess the ability to flocculate and also can survive at various levels of dissolved oxygen (Toerien et al., 1990). Even with these abilities, the proportion of nitrifiers in the mixed liquor of an activated sludge tank is typically low (between 2-5%) (Blackall and Burrell, 1999). Their growth rate is also low compared with that of heterotrophs because their cell yield per unit of energy obtained is low (Blackall and Burrell, 1999). Also, the oxidation of organic compounds by heterotrophs releases far more energy that the oxidation of nitrogenous compounds by nitrifying bacteria (Blackall and Burrell, 1999).

Nitrifiers grow more slowly in sewage than in pure cultures or in other soil environments (Blackall and Burrell, 1999). This slower growth rate is probably due to the presence of inhibitors from the sewage and the other microbes, predation, and the presence of less than optimal and often fluctuating dissolved oxygen conditions, temperature, or pH conditions. Although the main role of heterotrophs in the activated sludge system is mainly the removal of biodegradeable carbonaceous compounds, their exact effect on the population of nitrifying bacteria is still unclear. There is evidence that they may be differential, either inhibiting or promoting nitrification activity (Blackall and Burrell, 1999).

It is well established in literature that growth rates of *Nitrosomonas* are much lower than those of *Nitrobacter* (Bock et. al, 1992). *Nitrosomonas* growth is limited by the ammonium concentration, while *Nitrobacter* growth is limited by the nitrite ion concentration. In the past, it was assumed that due to the slow growth of *Nitrosomonas*, the rate limiting step in nitrification is the conversion of ammonia to nitrite by *Nitrosomonas*. However, since recent *in situ* studies discussed above (Burrell et al., 1998; Juretschko et al., 1998; Okabe et al., 1999; Daims et al., 2000; Daims et al., 2001 (a); Gieseke et al., 2001) have indicated that *Nitrobacter* is not the most important nitrite oxidizer, this theory will have to examined in future studies.

In the future, more research is needed on the factors that influence the growth of nitrifying bacteria and factors that increase or decrease nitrification rates. Increased nitrification rates will lead to more complete removal of nitrogen from the system and a higher quality effluent.

2.4.2 Denitrification

Denitrification is the process where NO_3^- and or nitrite are reduced to NO, N_2O, or N_2, and represents one of the key processes in the nitrogen cycle. A simplified representation of the denitrification reaction is shown in equation 2.5.

$$NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H^2O \text{ (Equation 2.5)}$$

$$\Delta G = -212 \text{ kcal}/8e^- \text{ transfer}$$

Denitrification is actually a four-step process involving four separate enzymes: Nitrate reductase (reduction of nitrate to nitrite); Nitrite reductase (conversion of nitrite to nitric oxide); Nitric oxide reductase (conversion of nitric oxide to nitrous oxide); and Nitrous oxide reductase (conversion of nitrous oxide to dinitrogen gas) (Maier et al., 2000). The extent to which denitrification occurs depends on the conditions present in the system. For example, nitrous oxide reductase is inhibited by conditions of low pH and high oxygen levels. Thus, in conditions of low pH or high oxygen, nitrous oxide, rather than nitrogen gas, will be the final product of denitrification (Maier et al., 2000). The extent of denitrification is also determined by the initial nitrate levels in an environmental system. Low nitrate levels tend to favour the production of nitrous oxide, while higher nitrate levels favour the production of nitrogen gas (the favourable end product). Ideally, denitrification will occur when wastewater becomes anaerobic in the final clarifier step of the wastewater treatment process, and result in the release of nitrogen gas (Viessman and Hammer, 1998).

In contrast to ammonia and nitrite oxidation, the capability to anaerobically respire with nitrate (or nitrite) is widespread in the bacterial community. The dissimilatory reduction of nitrate is commonly found in bacteria, and more than 130 species of heterotrophic bacteria are now known that can denitrify (Zumft, 1992). As such, it is impossible to predict using modern methods such as environmentally retrieved 16S rRNA sequences,

whether a microorganism is actually performing denitrification (Wagner and Loy, 2002). This difficulty is reflected by the fact that the identity of important *in situ* denitrifiers in WWTPs is still not known (Wagner and Loy, 2002). Several studies have identified bacterial species capable of denitrification from WWTP samples (Gumaelius et al., 2001; Khan and Hiraishi, 2001), the mere detection of these bacteria does not prove that they are actually denitrifying (Wagner and Loy, 2002). Future attempts to identify the main denitrifiers in activated sludge may rely on the use of microautoradiography (MAR) combined with flourescent *in situ* hybridization (FISH). By combining MAR and FISH, metabolically active denitrifiers can be identified and enumerated (Wagner and Loy, 2002). Preliminary studies by Wagner (unpublished data) on activated sludge in an industrial wastewater treatment plant using MAR and FISH showed that Be*taproteobacteria* related to the *Azoarcus-Thauera* complex are likely abundant denitrifiers (Wagner and Loy, 2002).

2.5 The Role of Protozoan Predation in Nutrient Cycling and Energy Flow in Activated Sludge

Nutrient cycling and energy flow are centered on photosynthesis and plant growth, since plant tissue forms the greatest portion of the earth's biomass (Stout, 1980). However, all organisms participate in the flow of nutrients and energy through an ecosystem. The role of protozoa in nutrient cycling and energy flow is determined by their bionomics. The distinctive features of protozoa are their small size, their high rate of reproduction, their high conversion efficiency of nutrients to new cell tissue, and their potentially high metabolic rates (Stout, 1980). Protozoa generally live in association with a wide range of microorganisms that function together as a microcosm.

The role of protozoa in reducing bacterial numbers in the effluent and encouraging flocculation through predation has been discussed above. There is also evidence that prey-predator relations involving protozoa play a role in the cycling of nutrients and flow of energy within an ecosystem. Early studies by Johannes (1965) postulated that protozoan predation would stimulate bacterial growth rates and hence substrate uptake rates. This theory was supplemented by Hunt et al. (1977) who developed a simulation model for the effect of predation on bacteria in continuous culture. They concluded that

by lowering the bacterial biomass, predation increases the level of limiting nutrient, thereby increasing the growth rate of the bacteria and increasing the uptake of nonlimiting nutrient (Hunt et al., 1977). A more recent study by Simek et al., (1990) used microautoradiography (MAR) and found that there was a significant correlation between the total grazing rate of flagellates and the proportion of metabolically active bacteria.

Several more recent studies have focused specifically on the role of protozoan predation on nitrogen cycling in activated sludge. A study by Verhagen and Laanbroek (1992) observed that in suspended growth chemostat studies with nitrifying bacteria that the presence of protozoa increased per-cell nitrification rates. A very recent study by Petropoulos (2003) concluded that the efficiency of nitrification (as measured by nitrate, nitrite, and ammonia production rates) increased in the presence of protozoa, likely due to the ability of protozoa to regulate bacterial growth.

There is an abundance of research documenting how the presence of protozoa can change the composition of bacterial communities (Habte and Alexander, 1975; Hahn and Hofle, 2001; Ronn et al., 2002). There is also a strong interest in microbial ecology research to illuminate the relationship between structure and function of bacterial communities. An important question that must be researched further is whether predation as a structuring force for bacterial communities results in important consequences for the function of bacterial communities such as the rates of organic carbon decomposition, respiration, mineralization, and specific transformations of organic and inorganic matter (Jurgens and Matz, 2002). To date, studies which have examined the impact of protistan grazing on microbial processes have generally found an enhancement of bacterial activity and decomposition rates. However, more research involving MAR to prove increased metabolic rates in the presence of protozoan grazing needs to be undertaken. Also, the exact mechanism by which bacterial activity is enhanced by protozoan grazing is still not clear. Several studies have suggested that increased remineralization by protists and selection for fast growing bacteria may explain the enhanced nutrient cycling rates observed. Protists release inorganic and organic products into their surroundings. These are mainly recycled nutrients such as nitrogen, phosphorous, and organic carbon, but might also include stimulatory compounds which contribute to the dissolved organic

carbon pool and affect the physiological state and growth of bacteria (Jurgens and Matz, 2002). Protozoan grazing also influences elemental cycling mediated by bacteria, such as nitrification, either positively by stimulating the specific activity (Verhagen et al., 1993) or negatively by reducing bacterial abundance and promoting cell aggregation (Lavrentyev, 1997). Despite an abundance of research on the effect of protozoan grazing on cycling of nutrients, we still know very little about the biogeochemical consequences of grazing. Indeed, it is still debateable whether the availability of nutrients (bottom-up control) or predation (top-down control) is more important in bacterial community regulation (Jurgens and Matz, 2002). Future studies need to focus on linking decomposition rates with new techniques, such as FISH and MAR, to assess the bacterial community composition and metabolic activity.

2.6 Use of Experimental Laboratory Microcosms

Much of the difficulty in evaluating the role of protozoa in nutrient cycling in natural systems lies in monitoring the small and rapid changes in nutrient balance in which they play their role. Due to this, it has been necessary to develop laboratory scale microcosms which enable a closer and more complete monitoring of nutrient cycling than is possible in the field. Laboratory microcosms also allow for the manipulation of certain parameters on a small scale, and the more thorough monitoring of the effects of these manipulations.

The use of laboratory microcosms to examine a field system (i.e. activated sludge) is much more useful and convincing to examine a close relationship between the composition and activity of bacterial and protozoan populations (Stout, 1980). The use of laboratory scale microcosms to mimic activated sludge aeration tanks is more applicable than to completely natural environments since the activated sludge aeration tank is a man-made ecosystem and parameters are controlled to a certain extent (Stout, 1980). Several experiments by Curds (1974; 1972) have attempted the mathematical or computer simulated modelling of the activated sludge environment and the relationship between protozoan and bacterial populations with some success. Laboratory microcosms are often used as the first step in establishing observable relationships between protozoan

grazing and bacterial community structure and function that would not be possible to observe in full-scale plants due to the interference of countless other factors.

2.7 Potential Problems in the Activated Sludge Process

The ability of the activated sludge process to effectively reduce wastewater BOD/COD is dependant on the maintenance of optimal conditions for bacterial growth. Since the activated sludge process is a living system, it is sensitive to any change in conditions that fall outside of the range for optimal biological growth, or cause a change in the flocculating properties of the sludge. Some of the major problems that may be encountered in the activated sludge process are the inhibition of biological growth and/or metabolism and deflocculation of the sludge. These problems may be caused by a sudden and significant change in activated sludge parameters, or by the presence of certain toxic chemicals in the influent wastewater. Toxic chemicals may make their way into the influent wastewater by several pathways such as residues from manure and sludge used as fertilizer on fields, the improper disposal of chemicals from industrial or household sources, and spills (Halling-Sorensen, 2001).

Protozoan species are particularly sensitive to changes in activated sludge parameters such as pH, temperature, and dissolved oxygen (Viessman and Hammer, 1998). They are also very sensitive to the presence of toxic chemicals that may periodically be present in influent wastewater (Viessman and Hammer, 1998). Thus, when conditions in wastewater treatment plants are not optimum, protozoan species are often one of the first groups to decline in population. Cycloheximide is a known inhibitor of eukaryotic metabolism that is often used in experimental systems in order to inhibit protozoans (Tremaine and Mills, 1987; Kota et al., 1999; DeLorenzo et al., 2001). It is often used in the laboratory to mimic the effects that unfavourable system parameters or the presence of toxic chemicals would have on the protozoan population.

Bacterial species are also sensitive to changes in system parameters and the presence of chemicals. Many chemicals that may make their way into activated sludge systems act as antibacterial agents, inbibiting aerobic growth and nitrification of bacteria (Halling-Sorenson, 2001). One way such antibacterial agents may make their way into the environment is through the faeces and urine of humans treated against infectious diseases

(Halling-Sorensen, 2001). Some antibacterial agents known to inhibit nitrification are tetracylines, sulfonamides, and quinolones (Halling-Sorensen, 2001). The chemical allylthiourea is a known inhibitor of ammonia oxidation that functions by inhibiting the active site on the ammonia monooxygenase (AMO) enzyme in *Nitrosomonas* spp. ammonia oxidizing bacteria (Surmacz-Gorska et al., 1995). It is often used in laboratory studies as a selective inhibitor of ammonia oxidation in order to mimic the effects of certain antibacterial agents that may be present in influent wastewaters.

Deflocculation of activated sludge may be induced by certain conditions such as very low or high pH, temperature extremes, or the presence of certain deflocculating agents such as cadmium and benzene (Viessman and Hammer, 1999; Liao et al., 2002). Ethylenediamenetetraaceticacid (EDTA) is a known deflocculating agent that functions by breaking the salt bridges linking flocs in activated sludge (Liao et al., 2002). Although EDTA is not likely to be present in wastewater, it is often added in experimental situations in order to study the effects of deflocculation on the activated sludge process.

2.8 *In situ* characterization of nitrifying bacteria

Traditionally, microbial communities in WWTPs were analysed either by observation with a light microscope or by cultivation-dependant techniques (Wagner and Loy, 2002). More than 15 years ago, the introduction of rRNA-targeted oligonucleotide probes by Stahl et al. (1988), changed the field of applied microbiology forever as it allowed for the detection, identification, and quantification of microorganisms without the tedium of light microscopy or the limitations of culture-dependant methods (Lipski et al., 2000). The *in situ* characterization of nitrifying bacteria is a field that has expanded rapidly within the last decade. Fluorescent *in situ* hybridization (FISH) techniques using fluorescently labelled rRNA-targeted oligonucleotide probes in combination with confocal scanning laser microscopy (CSLM) have been used to characterize the community structures of samples taken from activated sludge systems (Wagner et al., 1994). The use of CSLM improved the capability of researchers to directly visualize the spatial distribution of defined bacterial populations inside the sludge flocs. Since its development in the late 1980's, the use of FISH combined with oligonucleotide probes and observation with

CSLM has been used extensively for the *in situ* identification and spatial analysis of nitrifying bacteria in activated sludge. The following section will review the technique of FISH using fluorescent labelled oligonucleotide probes combined with CLSM, and the application of this technique to nitrifying bacteria in activated sludge.

2.8.1 Whole Cell Fluorescent In situ hybridization

In contrast to hybridization techniques that are based on extracted nucleic acids, whole cell hybridization is applied to morphologically intact cells (Lipski et al., 2001). This allows the researcher to obtain information about the cell concentration and, when applied *in situ*, the spatial distribution of microorganisms in their environment can be studied (Lipski et al., 2001). The detection limit of FISH has been reported to be approximately 10^4cells/mL (Amann et al., 1995). FISH with rRNA oligonucleotide probes is a relatively new tool that enables researchers to rapidly detail information on cell morphology, detect the abundance of an uncultured microbe, and identify the in situ spatial distribution of bacteria in environmental samples such as those from activated sludge (Wagner et al., 1994; Wagner et al., 1996). Ribosomal rRNAs are excellent target molecules for FISH due to several beneficial properties. These are: present in all organisms, high natural concentration, and an information content that is high enough to provide signature nucleotide stretches for most phylogenetic taxa at and above the species level (Lipski et al., 2001).

Prior to whole cell hybridization, cells need to be permeabilized so that flourescently labelled probes are able to penetrate the cell membrane and hybridize to ribosomal RNA. Generally, this can be performed by fixation of the sample with paraformaldehyde or ethanol (Wilkinson, 1994).

A variety of different 16S rRNA oligonucleotide probes are currently being used to analyze bacterial communities present in activated sludge. This technique is useful for the rapid quantitative analysis of activated sludge to identify nitrifiers, and also to determine their spatial distribution relative to each other and heterotrophic bacteria within the activated sludge floc (Wagner et al., 1996). Understanding the nitrifying community and how their structure is affected by various fluctuating conditions in activated sludge is the key to obtaining optimal nitrifying populations and rates (Wagner et al., 1996).

Simultaneous hybridization (Manz et al., 1993), that is hybridization using more than one probe concurrently, may be used to characterize the ratio of different bacterial types and their spatial distributions in relation to each other in the flocs (Kim and Ivanov, 2000). To date, FISH results have indicated that some WWTPs are dominated by a single ammonia oxidizer while others have at least five or more ammonia oxidizers co-existing (Wagner and Loy, 2002). The composition of nitrite oxidizers in WWTPs as determined by FISH also varies, with *Nitrospira*-like bacteria dominating in substrate limiting conditions (Schramm et al., 1999) and *Nitrobacter* and *Nitrospira* co-existing in reactors with temporarily high nitrite concentrations (Daims et al., 2001(b)).

2.8.2 Oligonucleotide Probes

When discussing FISH, the term 'probes' is often utilized. This term refers to DNA oligonucleotide probes that target the highly conserved 16S rRNA sequences of the bacteria being studied. The primary structures of the ribosomal RNAs are composed of regions showing different degrees of conservation, some being highly conserved and some highly variable (Lipski et al., 2001). Highly conserved stretches of rRNA are used for the design of domain-specific probes such as the bacterial probe EUB 338 that targets most species in the bacterial domain (Lipski et al., 2001). The specificity of probes can also be adjusted to the different phylogenetic levels, and many such probes have been developed for the nitrifying bacterial community in activated sludge, as well as for other specific bacterial species (Loy et al., 1998).

In general, oligonucleotide probes used for hybridization techniques usually contain 15-25 nucleotides (Lipski et al., 2001). Probes to be used in fluroescent hybridization are covalently linked at the 5′ end to a single fluorescent molecule (Lipski et al., 2001). Commonly used fluorescent dyes include Cy3, Cy5, and carboxyrhodamine.

The oligonucleotide itself and hybridization parameters have an impact on the sensitivity and specificity of oligonucleotide probes. For example, the number of mismatches of non-target organisms should be at least two (Lipski et al., 2001). Hybridization conditions for each probe are dependant on the melting temperature (T_m) of the probe. Through the addition of formamide in the hybridization buffers, the incubation temperatures for hybridization may be reduced (Mobarry et al., 1996). The stringency of

the hybridization process may be adjusted by lowering the sodium chloride concentration in the washing buffer until the optimum specificity is reached (Mobarry et al., 1996). The relationship between the melting temperature, the sodium chloride concentration, the % formamide, the length of bases in the hybrid, and the %GC content of the probe can be described using Lathe's equation (Lathe, 1985):

$$T_m = 81.5 + 16.6\log[Na^+] + 0.41(\%GC) - 0.63(\%\text{formamide}) - [300+200(Na^+)]/N$$

(Equation 2.6)

Where:

T_m = melting temperature of the probe in degrees centigrade

$[Na^+]$ = molar concentration of sodium ions in the solvent

%GC = percentage of GC base pairs in the hybridized molecules

% formamide = percentage formamide (v/v) in the solvent

N = length in bases of the hybrid

Fluorescently labelled probes can be hybridized to microbial nucleic acid extracts or to whole cell preparations for use in whole cell *in situ* hybridization techniques.

2.8.3 Confocal Scanning Laser Microscopy

In many systems bacteria have a tendency to congregate into flocs or attach themselves to surfaces, forming biofilms (Wagner and Loy, 2002). To resolve spatial resolution at the cellular level, epifluorescence microscopy or confocal scanning laser microscopy (CSLM) is a valuable tool. Epifluorescent microscopy uses high pressure mercury lamps as a light source which emit light in a broad range from UV to far-red. Up to four fluorescent stains can be detected simultaneously with multibandpass filters. Fluorochromes that are out of the range of spectral sensitivity detected by the human eye (400-700nm) are recorded using cameras or other detectors (Lipski et al., 2001). A major problem with conventional epifluorescence microscopy is its poor applicability to thick specimens such as biofilms (Lipski et al., 2001). Fluorescence emitted above and below the focal plane causes a blurred image (Lipski et al., 2001). To exclude this fluorescence

from the image, confocal scanning laser microscopes contain a pinhole at the confocal plane of the microscope (Lipski et al., 2001). Thus, only fluorescence emitted from a small volume around the focal plane is detected, resulting in sharp images (Lipski et al., 2001). CLSM also allows the user to make image stacks of 3-dimensional objects, thus viewing the object on any number of different z-planes. It is only through the use of FISH combined with CLSM that researchers are able to determine both the composition, and spatial distribution, of bacteria within flocs.

2.8.4 The Combined use of FISH using rRNA Targeted Oligonucleotide Probes and CLSM for the Identification and Spatial Visualization of Nitrifying Bacteria

One of the first *In situ* analyses performed on activated sludge was performed by Wagner et al. (1994). In this study, several general bacterial probes were used to determine the spatial distribution of bacteria in activated sludge flocs. It was found that fluorescently labelled oligonucleotide probes penetrated even to the deeper regions of thick sludge flocs, and bacteria were not just located on the outside of the floc. This study also found sludge floc diameter to be between 5 and 50 μm after cell fixation and hybridization. This floc diameter corresponded well with other studies of floc size in untreated sludge samples (Andreadakis, 1993), indicating that the hybridization procedure does not affect the floc size (Wagner et al., 1994).

A later study by Wagner et al. (1995) was one of the first studies using FISH and CLSM attempting to identify ammonia oxidizing bacteria in activated sludge samples. In this study, Wagner used probes targeting ammonia oxidizers and found that ammonia oxidizing bacteria are clustered into microcolonies and generally located within the sludge flocs rather than on the outside (Wagner et al., 1995). This finding corresponds with subsequent findings that also found AOBs positioned near the interior of the floc (Mobarry et al., 1996; Wagner et al., 1996; Wagner et al., 1998).

Many early studies on the presence and position of nitrite oxidizing bacteria within activated sludge flocs focused on the *Nitrobacter* species. These studies found that ammonia and nitrite oxidizers often grow near the interior of activated sludge flocs in closely associated aggregates (Mobarry et al., 1996; Wagner et al., 1996). However, as indicated in section 2.4.1.2 above, recent studies have indicated that *Nitrospira*-like

bacteria, and not *Nitrobacter*, are the dominant nitrite oxidizers in activated sludge plants (Burrell et al., 1998; Juretschko et al., 1998; Okabe et al., 1999; Daims et al., 2000; Daims et al., 2001 (a); Gieseke et al., 2001,). It was found that two phylogenetically different groups of nitrospira-like nitrite oxidizers frequently form tight microcolonies with water permeable channels (Daims et al., 2001 (a)). It was also found that they are located near the interior of the floc, adjacent to ammonia oxidizers (Daims et al., 2001(a)).

It is important to note that physiologically inactive AOBs and NOBs will also be detected using FISH as these bacteria maintain high cellular ribosome contents even under unfavourable conditions (Wagner et al., 1995). The number of physiologically active ammonia and nitrite oxidizers can be determined using FISH combined with MAR with ^{14}C-labelled bicarbonate as substrate (Lee et al., 1999). Recently, Adamczyk et al. (2003) introduced the isotope array approach. This new technique can be used to identify microorganisms that consume a ^{14}C-labelled substrate and has been used successfully to quantify the CO_2 fixation activities of AOBs in activated sludge and is much less tedious and time consuming than the FISH-MAR method (Adamczyk et al., 2003).

2.9 Experimental Objectives

Although several studies have been conducted that relate grazing of protozoa to increased nitrification rates (Clarholm, 1984; Verhagen and Laanbroek, 1992; Strauss and Dodds, 1997; Petropoulos, 2003), very few of these (with exception to Petropoulos') have been examined in activated sludge systems, and none have attempted to establish the effect of protozoan grazing on bacterial nitrification in activated sludge under various conditions. There have also been many studies performed that have examined the position of nitrifying bacteria (both AOB and NOB) in activated sludge floc (Wagner et al., 1994; Wagner et al., 1995; Mobarry et al., 1996; Wagner et al., 1996; Burrell et al., 1998; Juretschko et al., 1998; Wagner et al., 1998; Okabe et al., 1999; Daims et al., 2000; Daims et al., 2001(a); Gieseke et al., 2001). However, none of these studies attempted to use FISH/CSLM to determine the position and relative abundance of nitrifying bacteria under various conditions and with and without the presence of protozoan grazing.

The objectives of this study were two-fold. First, I wished to examine the effect of protozoan grazing on nitrification rates under various conditions that may occur during the wastewater treatment process, such as deflocculation and partial inhibition of nitrification. I also wished to study the spatial distribution of ammonia- and nitrite-oxidizing bacteria in activated sludge under various conditions in the presence and absence of protozoan grazing. It was hypothesized that an increase in nitrification would be observed in the presence of protozoan grazing due to the ability of protozoa to control populations of heterotrophic bacteria that otherwise compete with nitrifiers for nutrients such as ammonia. It was also hypothesized that a change in the relative abundance and position of both nitrifying bacteria and heterotrophic bacteria would be observed under the different conditions tested and in the presence and absence of protozoan grazing. In the presence of protozoan grazing it was postulated that more nitrifying bacteria may be observed through FISH/CSLM, and in the absence of protozoan grazing it was postulated that more heterotrophic bacteria may be observed and that nitrifiers would be located deeper within activated sludge flocs.

It is hoped that the outcome of this study will provide further insight into the relationship between protozoa grazing and bacterial nitrification as applied to the activated sludge phase of municipal wastewater treatment.

3.0 MATERIALS AND METHODS

3.1 Sample Collection at Ashbridges Bay Municipal Water Treatment Plant

The sample was collected at the beginning of each trial at the Ashbridges Bay Treatment Plant (ABTP). It consisted of a mixed liquor sample obtained from aeration tank 2 of the activated sludge system at ABTP. The sample was collected and transported to the lab for immediate use. A time frame of the sample collection is outlined in table 3.1.

Table 3.1 The batch reactor sample collection time frame

Time of Sample Collection	Trials Run with Sample
February/2004	1 (Reference)
February/2004	2 (Cycloheximide)
May/2004	3 (Allylthiourea)
May/2004	4 (Allylthiourea+Cyloheximide)
June/2004	5 (EDTA)
June/2004	6 (EDTA+Cycloheximide)

3.2 Experimental Design

Bench-scale batch reactors were used to simulate the oxic and mixing conditions of the activated sludge process. The bench scale batch reactors consisted of 250 mL Erlenmeyer flasks containing 150 mL of the mixed liquor sample from ABTP and placed on a bench scale shaker (VWR-Canlab, Toronto, ON, Canada) (Figure 3.1). The flasks were sealed with a sterile porous sponge and continuously shaken at a speed of 150 rpm throughout the trial period to ensure adequate aeration and to prevent settling.

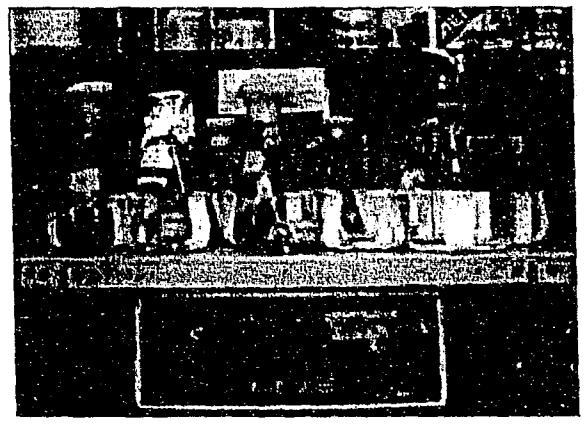


Figure 3.1 Picture of the laboratory batch microcosm system

Similar bench scale reactor microcosm systems have been used in other areas of microbial ecology (Christian et. al, 1982; Pis'man et al., 1995; Landry et al., 1982 Strauss et al., 1997; Taylor, 1978). The use of the batch reactor system provides several advantages including smaller volume requirement than a conventional bioreactor, no feed requirements, easy withdrawal of sample volumes, and the ability to run multiple reactors at the same time. In order to minimize the amount of manipulation and to observe the natural variation of nutrient levels over the trial period, no volume replacement liquid was used.

Each trial consisted of three replicate reactors, a total of six 14-day trials were run

3.2.1 The Addition of Cycloheximide to Batch Reactors

Chemical inhibition of protozoan bacterivores was achieved through the addition of the eukaryote inhibitor cycloheximide to trials 2, 4, and 6. A stock solution of cycloheximide (Sigma-Aldrich Chemical Co.; molecular weight 281.3) was made by dissolving cycloheximide powder in distilled, deionized water to a final concentration of 15 g/L. The solution was filter sterilized upon dispensation with a syringe filter (pore size 0.2 μm). Cycloheximide was used at a final concentration of 100 mg/L by adding 1 mL of the prepared stock solution to each reactor containing the 150 mL mixed liquor sample in trials 2,4, and 6 .

3.2.2 The Addition of Allylthiourea to Batch Reactors

The inhibition of ammonium oxidation by activated sludge bacteria was achieved through the addition of allylthiourea (Sigma-Aldrich Chemical Co.; molecular weight 116.19), a selective inhibitor of ammonium oxidation by *Nitrosomonas* (Surmacz-Gorska et al., 1995). A stock allylthiourea solution was prepared by dissolving allylthiourea in dissolved, deionized water to a final concentration of 2 g/L. The solution was filter sterilized upon dispensation with a syringe filter (pore size 0.2 μm). Allylthiourea was used at a final concentration of 5 mg/L in the reactors by adding 0.375 mL of stock solution to each reactor containing 150 mL of the mixed liquor sample in trials 3 and 4.

3.2.3 The Addition of EDTA to Batch Reactors

In order to observe the effects of deflocculation on nitrification and on the presence and position of nitrifying bacteria, ethylenediaminetetraacetate (EDTA, Na-form, B.D.H. Laboratory Chemicals Inc.; molecular weight 373.24) was added to mixed liquor samples. EDTA is a strong chelating agent which breaks flocs by removing divalent cations from the floc matrix, thus breaking the salt bridges linking flocs (Liao et al., 2002). EDTA was used at a final concentration of 150 mg/L in the mixed liqour samples in trials 5 and 6.

A summary of each trial is shown in Table 3.2.

Table 3.2 Summary of each trial and the modifications to reference conditions made

Trial	Modifications	Protozoan Inhibition
1	None	No
2	None	Yes
3	Allylthiourea added	No
4	Allylthiourea added	Yes
5	EDTA added	No
6	EDTA added	Yes

3.3 Parameter Measurements

Throughout each trial, samples were collected at intervals throughout the 14 day period for determination of chemical oxygen demand (COD), determination of nitrogen in the form of ammonia, nitrate, nitrite, and total nitrogen, enumeration of bacteria, and examination of bacterial flocs for the presence and location of both ammonia-oxidizing and nitrite-oxidizing bacteria. Physical parameters such as pH, temperature, and dissolved oxygen levels were measured daily.

Sample collection from each individual flask occurred by removing the porous sponge and withdrawing a sample using individually wrapped sterile pipettes. Temperature, dissolved oxygen, and pH were measured using probes which were rinsed with ethanol and distilled, deionized water between each measurement.

3.3.1 Electrometric pH Measurement Method

Measurement of pH was done on a daily basis throughout each 14-day trial with a hand held, battery operated pH meter (Corning™ pH-30 portable pH meter). . The meter was calibrated prior to each use based on the manufacturers instructions.

3.3.2 Temperature Measurements

Temperature measurements were taken using a mercury-filled Celsius thermometer (VWR-CanLab, Toronto, ON). The thermometer had a scale marked for every 0.1°C , with markings etched on the capillary glass (-10°C to 100°C).

3.3.3 Dissolved Oxygen Measurement

Dissolved oxygen measurements were performed at 4-day intervals (5 times through the 14-day trial period) using a dissolved oxygen testing kit (Salifert O_2 profi-test, NL Duiven, Holland). According to the manufacturers instructions, five mL of the mixed liquor sample was added to a test tube. Five drops of O_2-1 reagent was added to the sample and the sample was swirled gently for 20 seconds. Six drops of O_2-2 reagent was then added, the sample was swirled for 15 seconds, and then allowed to sit for a period of one minute. Finally, 6 drops of O_2-3 reagent was added, the sample was swirled for 20 seconds, and then 30 seconds was allowed for the colour to develop. The dissolved

oxygen concentration (mgO_2/L) was determined by comparing the developed colour to a colour chart provided by the manufacturer.

3.3.4 Chemical Oxygen Demand

The closed reflux, colorimetric method was used to measure the chemical oxygen demand (COD). This procedure was adapted from the Standard Methods for the Examination of Water and Wastewater (AHPA, 1998).

The acclimatized mix liquor sample was filtered through 0.45 μm pore size filter paper (Whatman, VWR-Canlab, Toronto, ON). The 2.5 mL of the filtered sample was placed in culture tubes with Teflon-coated caps (Hach Co., Loveland, CO, USA). The 1.5 mL of digestion solution (0.02 M $K_2Cr_2O_7$, 0.1 M H_2SO_4, 0.001 M $HgSO_4$ in reagent grade water) and 3.5 mL of sulfuric acid reagent (0.02 M Ag_2SO_4 in 0.5M H_2SO_4; VWR-Canlab, Toronto, ON) was then added. The culture tubes were then placed in a COD block heater (Hach COD reactor, model # 45600-00, Hach Co., Loveland, CO, USA), and refluxed at 150°C for two hours. The cooled samples were measured spectrophotometrically (Spec 20^+ Spectrometer, Spectronic Instruments Inc., Rochester, NY, USA) at a wavelength of 600 nm along with potassium hydrogen phthalate (KHP) standards ranging in concentration from 0 to 500 mgO_2/L. A standard curve was prepared by plotting absorbance readings of KHP standards against the known concentrations of the standards. The sample concentration was determined the comparing the sample absorbance with the standard curve. A filtered reagent grade water blank was prepared along with the samples.

3.3.5 Determination of Ammonia Levels

The phenate method was used to measure ammonia concentrations in the reactors. In this method, alkaline phenol and sodium hypochlorite react with ammonia to form indophenol blue which is proportional to the ammonia concentration in the sample (AHPA, 1998). The blue colour formed is intensified with the use of sodium nitroprusside (AHPA, 1998). The method was modified from the Standard Methods for the Examination of Water and Wastewater (AHPA, 1998).

The acclimatized mix liquor sample was filtered through 0.45 μm pore size filter paper and a one-hundredth dilution was prepared. In a clean, dry 5 mL glass cuvette, 2.5 mL of sample was added. Then, with thorough mixing after each addition, 100 μL phenol solution (11.1% v/v in 95% ethanol), 100 μL sodium nitroprusside solution (0.5 % w/v in reagent grade water), and 250 μL oxidizing solution (10 mL alkaline citrate; 2.5 mL sodium hypochlorite) were added to the sample in the cuvette. The samples were covered with paraffin wrapper film and placed in subdued light at room temperature where the colour was allowed to develop for a minimum of one hour. The colour was stable for 24-hours.

The samples were measured spectrophotometrically (Perkin-Elmer Lambda 20 Spectrophotometer, Perkin Elmer Inc., Wellesley, MA, USA) at 640 nm along with ammonium chloride standards. A standard curve was prepared by plotting absorbance readings of standards against ammonia concentrations of standards (0 to 25 mgNH_3-N/L). This calibration was repeated for every 14-day experimental run. The sample concentration was determined by comparing sample absorbance with the standard curve. Ammonia blanks and filtered reagent grade water blanks were prepared in addition to the samples.

3.3.6 Measurement of Total Nitrate and Nitrite Levels

The semi-micro method for determination of nitrite and of nitrate was employed using a kit purchased from Roche Co. (Nitrate/Nitite Colorimetric Method Kit, Roche Co., Montreal, Canada).

The main principle is that nitrate is reduced to nitrite by reduced nicotinimide adenine dinucleotide phosphate (NADPH) in the presence of the enzyme nitrate reductase (NR). This reaction can be represented as follows:

$$\text{Nitrate} + \text{NADPH} + H^+ \xrightarrow{\text{NR}} \text{Nitrite} + NADP^+ + H_2O \quad \text{(Equation 3.1)}$$

The nitrite formed reacts with sulphanilamide and N-(1-naphthyl)-ethylene-diamine dihydrochloride to give a red-violet diazo dye. This reaction is shown below:

$$\text{Nitrite} + \text{sulfanilimide} + \text{N-(1-naphthyl)-ethylenediamine} \rightarrow \text{diazo dye} \quad \text{(Equation 3.2)}$$

According to the manufacture procedure, 0.5 mL of filtered sample (0.45 μm filter paper) was pipetted into disposable microcuvettes for nitrate and nitrite determination. In some cases, it was necessary to dilute the sample into the 0.05 mg – 5 mg nitrite or nitrate/L concentration range. For the determination of nitrite, the samples were mixed with 770 μL of reagent grade water and allowed to incubate at room temperature for 30 minutes. The initial absorbance was measured at 540 nm (Perkin Elmer Lambda 20 Spectrophotometer). After the initial absorbance was measured, 250 μL of sulfanilimide (colour reagent I) and 250 μL of N-(1-naphthyl)-ethylenediamine (colour reagent II) were added to the mixture. The mixture was then allowed to stand in the dark for 10 to 15 minutes. The final absorbance reading was measured at 540nm.

For the determination of nitrate, 0.5 mL of sample was mixed, in the cuvettes, with 20 μL of lyophilised nitrate reductase solution (4 U nitrate reductase dissolved in 700 μL distilled water) and 250 μL NADPH buffer solution (0.5 mg NADPH dissolved in 3 mL of 1M K_2PO_4 buffer, pH 7.5). After the initial mixing and 30 minute incubation period, the initial absorbance was measured at 540nm. Then, 250 μL of sulfanilimide and N-(1-naphthyl)-ethylenediamine, the two colour reagents, were added. After a 10 to 15 minute incubation period in the dark, the final absorbance reading was taken. Nitrite and nitrate blanks and a filtered reagent grade water blank were prepared in addition to the samples.

The samples were measured spectrophotometrically at 540 nm against standards of sodium nitrite (0.05 mg –5 mg nitrite/L) and potassium nitrate (0.05 mg –5 mg nitrate/L) for nitrite and nitrate determination. The standard curves were constructed by plotting the change in absorbance obtained for the sodium nitrite and potassium nitrate standard solutions on the y-axis against the corresponding nitrite or nitrate concentrations in mg/L on the x-axis. The concentrations of nitrite and nitrate in the sample were then determined from the calibration curves using the change in absorbance measured.

3.3.7 Determination of Total Nitrogen Levels

The determination of total nitrogen levels was achieved analytically through the use of a total nitrogen testing kit purchased from Hach Co. (Nitrogen, Total, Test n'Tube™, Hach Co., Loveland, CO, USA). This test measures total nitrogen in the range of 0 to 25 mg N/L.

A 2mL filtered, one-tenth diluted sample was pipetted into a reagent vial containing a mixture of 1 M nitrogen hydroxide and 1 M nitrogen persulfate. One reagent blank and one filtered reagent water blank was also prepared along with samples. The reagent vials were capped and shaken vigorously for approximately 30 seconds. The vials were placed in a COD block heater pre-heated to a temperature of 105°C for 30 minutes. The hot vials were then removed from the COD heater and allowed to cool to room temperature.

The caps were removed from the cooled, digested vials and 2 mg of sodium metabisulfite was added to each vial. The vials were then shaken for 15 seconds, and then left to stand for a three-minute reaction period. After the reaction period, 2mg of chromotropic acid was added, vials were shaken for 15 seconds, and a 2-minute reaction period was allowed.

After the second reaction period, 2 mL of digested, treated sample was removed from each vial and added individually to new vials containing acid reagent. The vials were capped and inverted 10 times to ensure proper mixing. The vials were incubated at room temperature for five minutes, and then measured spectrophotometrically at a wavelength of 410 nm (Spec 20^+ Spectrometer, Spectronic Instruments Inc., Rochester, NY, USA). A standard curve was prepared by plotting absorbance readings against total nitrogen concentrations of standards (0 – 25 mg N/L). The total nitrogen concentration of samples was determined by comparing absorbance readings of samples to the standard curve.

3.4 Enumeration of Bacterial Abundance

3.4.1 Preparation of sample

The microscopic enumeration of total bacterial abundance was performed for each trial on day 1, day 7, and day 14. To aid in the enumeration of total bacterial cells, computational software (Northern Eclipse Manual Count (section 3.7.2) was used.

To perform bacterial counts, 1 mL of sample was removed from each reactor and placed in a microcentrifuge tube. Samples were then vortexed on a bench scale vortexer (VWR-Canlab, Toronto, ON, Canada) for 4-minutes to break up flocs. One-tenth successive dilutions of the sample were then prepared.

3.4.2 Enumeration of viable bacteria using the LIVE/DEAD® Method

For enumeration of bacterial abundance, a LIVE/DEAD® Baclight™ Bacterial Viability Kit purchased from Molecular Probes Inc. was used (Molecular Probes Inc., Eugene, OR, USA).

This kit uses a mixture of SYTO 9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain propidium iodide. These stains differ in both their spectral characteristics and in their ability to penetrate healthy bacterial cells.

Equal volumes of SYTO 9 and propidium iodide (containing 0.3 % v/v DMSO) were combined in a microcentrifuge tube and mixed thoroughly. For each 1 mL of bacterial suspension, 3 µl of the dye mixture was added in a microcentrifuge tube. It was mixed thoroughly and incubated in the dark at room temperature for 15-minutes. After this incubation period, 5 µl of the stained bacterial suspension was placed on a slide (VWR-Canlab, Toronto, ON, Canada) and an 18 mm square coverslip (VWR-Canlab, Toronto, ON, Canada) was placed over the suspension.

The computational enumeration of total bacterial cells (viable, green, and non-viable, red) was observed using a fluorescent microscope (Inverted Microscope, Axiovert 200, Carl Zeiss, Gottingen, Germany). The equipped filters being used were excitation 485 nm and emission 500 nm. Total bacterial cells (both live and dead) were counted.

3.4.3 Computational Enumeration of Microbes using the Northern Eclipse Manual Count Software Function

The computational enumeration of bacteria used the manual count function of the Northern Eclipse software part of the microscope apparatus. The manual count function was used as a simple counting function, which was useful for automatic counting. This function was used to capture an image of the stained bacterial cells. After selection of the image was completed, an automatic count of the image was taken per field of view.

After the computational count per field of view, the number of cells per mL was calculated using the following equation:

$$\text{cell/mL} = \frac{\text{cells per field of view X dilution factor}}{\text{Volume used (mL)}} \quad \text{(Equation 3.3)}$$

3.5 Fluorescent *In situ* hybridization

3.5.1 Oligonucleotide Probes

Six oligonucleotide probes were used *in situ* to observe the presence and the position of nitrifying bacteria within flocs in each trial. All probes were purchased from Sigma-Genosys, TX, USA. Of these six probes, three probes were targeted at the nitrite-oxidizing bacteria, two probes were targeted specifically at ammonia-oxidizing bacteria, and one probe was used as a general probe targeted at most bacterial species. A summary of the probe name, sequence, target species, and fluorescent label, is found in table 3.2.

Table 3.3 The sequence, target species, and fluorescent label of the six probes used in this study

Probe Name	Sequence	Target Species	Fluorescent Label	Reference
EUB338	5′-GCTGCCTCCCGTAGGAGT-3′	Most eubacteria	Carboxo-rhodamine 6G (CR6G)	Amann et al., 1990
NSM156	5′-TATTAGCACATCTTTCGAT-3′	AOB:*Nitrosomonas* group: *Nitrosomonas europea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis*	Cy3	Mobarry et al., 1996
NSO190	5′-CGATCCCCTGCTTTTCTCC-3′	*betaproteobacterial* AOB	Cy5	Mobarry et al., 1996
NSV443	5′-CCGTGACCGTTTCGTTCCG-3′	NOB: *Nitrosospira* cluster	Cy3	Mobarry et al., 1996
Nb1000	5′-TGCGACCGGTCATGG-3	NOB: *Nitrobacter* genus	Cy3	Mobarry et al., 1996
S-*-Ntspa-0712-a-A21	5′-CGCCTTCGCCACCGGCCTTCC -3′	NOB: *Nitrospira*-like bacteria	Cy5	Daims et al., 2001

Prior to use, probes were suspended in sterile distilled, deionized water at a final stock concentration of 500ng/μL and stored at –20°C. Immediately prior to use, probe stocks were diluted 1/10 and used at a final concentration of 50ng/μL.

3.5.2 Gelatin Coated Slides

This procedure was adapted from the Methods in Gene Biotechnology manual (Wu et al., 1997). Slides were soaked in a 10% KOH in ethanol solution for one hour then rinsed with distilled water and air dried. The dry slides were then immersed in a gelatin solution (0.1% gelatin, 0.01% $KCr(SO_4)_2$ at 70°C) for ten minutes. Slides were dried on a slant overnight to obtain a thin gelatin coating. The purpose of the gelatin coating is to ensure that the sample adheres to the slide with minimal loss of sample.

3.5.3 Cell Fixation

One mL of sample was collected and centrifuged in an Eppendorf Microcentrifuge at 14000 rpm for two minutes. The supernatant was discarded and the pellet resuspended in one mL of fresh, cold paraformaldehyde (4% in PBS) solution (Wu et al., 1997). The suspension was then incubated at 4°C for at least 3 hours. After this incubation period, the suspension was centrifuged at 14000 rpm for 2 minutes and the pellet was resuspended in 1mL of PBS solution (1L PBS: 8g NaCl, 0.2g KCL, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 dissolved in distilled , deionized water, pH adjusted to 7.2, autoclaved at 121°C for 20 minutes) (Wu et al., 1997). If the suspension was to be used at a later date it was centrifuged again at 14000 rpm for 2 minutes and the pellet was resuspended in 500 μl PBS and 500 μl 95% (v/v) ethanol and stored at -20°C for up to one month (Amann et al., 1990). If the suspension was used immediately, 300 μl PBS and 1 drop of Tween 20 was added to the sample. After centrifuging for 2 minutes at 14000 rpm the pellet was resuspended in 400 μl of 1% Tween 20. Ten μl of the fixed sample was spotted onto the gelatin slides and dried at 46°C for 15 minutes. This step was repeated twice to ensure a thick layer of sample on the slide. Samples were then dehydrated in 50, 80, and 95% (v/v) ethanol for three minutes each (Wu et al., 1997).

3.5.4 Whole Cell Hybridization

After dehydration with ethanol, samples were air-dried for approximately 20 minutes at room temperature. In a microcentrifuge tube, 8µl of the appropriate hybridization buffer (formamide in concentrations shown in table 3.3, 0.9M NaCl, 20mM tris/HCL pH 7.2, and 0.01% SDS) and 2 µl (50ng/l) of probe. 10 µl of this hybridization mixture was added to each spot of sample on the gelatin coated slide. The slides were then placed in an isotonically equilibrated humidity chamber and placed in a hybridization oven at 46° C for a minimum of two hours. The humidity chamber was prepared by placing paper towels soaked with 1M NaCl solution (58.44 g NaCl, filled to mark with distilled deionized water in a 1L volumetric flask) on the bottom of a plastic container. The slides were then placed face-up on top of the paper towel and the container was sealed before being placed into the hybridization oven.

Hybridization was followed by an equally stringent 20-min post hybridization wash at 48°C. The probe was removed with 5mL of the appropriate washing buffer (NaCl in concentrations shown in table 3.3, 20mM Tris HCl buffer, pH 7.2, 0.01% SDS, and 5mM EDTA). The immersed slides were then placed back into the hybridization oven set to a temperature of 48°C for 20 minutes. The washing buffer was removed by rinsing the slides for 30 seconds to one minute with milli Q water in suddued light. The slides were then air dried in subdued light before being examined under the confocal microscope.

3.5.5 Optimizing the Probe Specificity

In order to minimize non-specific binding of probes and over-stringent hybridization conditions, the stringency of hybridization was optimized for each probe using a method modified from one previously described by Manz et al. (1992). Holding the hybridization temperature constant at 46°C, the formamide concentration in the hybridization buffer was gradually increased. In the washing buffer, the sodium chloride concentration was adjusted according to the Lathe equation (Lathe, 1985). The stringency of the hybridization step and the washing step was determined by comparing the different combinations of hybridization and washing conditions using confocal scanning laser microscopy. By examining the pictures obtained on the CLSM, optimal stringency conditions defined by the percent formamide in the hybridization buffer and

the sodium chloride content of the washing buffer were determined for each probe. The conditions for each probe used in this study are shown in Table 3.3.

Table 3.4 Hybridization conditions used with the oligonucleotide probes.

Probe	Formamide Concentration (%)	NaCl Concentration (mM)
EUB338 (most eubacteria)	39	135
NSO190 (AOB bacteria of β-subclass Proteobacteria)	40	135
NSM156 (AOB, *Nitrosomonas* spp., *Nitrosococcus mobilis*)	5	350
NSV443 (NOB, *Nitrosospira* spp.)	38	135
Ntspa-0712-a-A-21 (NOB, *Nitrospira*-like bacteria)	50	28
Nb1000 (NOB, *Nitrobacter* spp.)	39	130

(Source: Loy et al., 2003)

3.5.6 Simultaneous Hybridization

Simultaneous hybridization with more than one probe requiring different stringency conditions were performed using successive hybridization procedures similiar to that described by Manz et al. (1993). The first hybridization was done with the probe requiring the higher stringency (or higher % formamide) followed by hybridization with the probe requiring lower stringency.

In this study, simultaneous hybridization was performed with a maximum of two probes. Four probe combinations were used in this study. The different probe combinations used in this study, the trials in which each combination was used, and the species targeted by each combination is presented in Table 3.4.

Table 3.5 The probe combinations used in this study.

Probe Combination	Trials Used	Target Species
EUB 338 + Ntspa-A21	1,2,3,4,5,6	Most eubacteria + NOB (*Nitrospira*-like bacteria)
Ntspa-A21 + Nb1000	1,2,3,4,5,6	NOB: *Nitrospira*-like bacteria + *Nitrobacter* spp.
Ntspa-A21 + NSV443	1,2,3,4,5,6	NOB: *Nitrospira*-like bacteria + *Nitrosospira* spp.
NSO 190 + NSM 156	1,2,3,4,5,6	AOB: AOB bacteria of β-subclass *Proteobacteria* + *Nitrosomonas* spp., *Nitrosococcus mobilis*

3.5.7 Confocal Microscopy

A Carl Zeiss (Oberkochen, Germany) LSM 510 scanning confocal microscope equipped with an Argon (Ar) ion laser (488 and 514 nm) and two Helium Neon (HeNe) lasers (543 and 633 nm) was used to observe fluorescent emission of probes. The configuration of the lasers and the various filter and pinhole settings available on the LSM 510 software is shown in Table 3.5. The Achrochrome 63X water immersion objective lens was used for all microscopic analysis. Z-stacking and image analysis was performed using the LSM 510 software package.

Table 3.6 Confocal scanning laser microscopy configuration settings for viewing probes labelled with each of three fluorescent labels

Fluorescent Label	Cy3	Cy5	Carboxyrhodamine
Peak Absorption wavelength(s) (nm)	552 (source: Sigma-Genosys 2004)	643 (source: Sigma-Genosys 2004)	502 (source: Sigma-Genosys 2004)
Primary Emission wavelength (nm)	570 (source: Sigma-Genosys 2004)	667 (source: Sigma-Genosys 2004)	524 (source: Sigma-Genosys 2004)
Laser	HeNe1(543nm)	HeNe2 (633nm)	Ar (488nm)
MBS	HFT 488/543	HFTUV488/543/633	HFT488
DBS1	Mirror	None	Mirror
DBS2	BG39	NFT545	NFT545
DBS3	Plate	None	None
Filter	LP560	LP650	LP560
Pinhole (airy units)	1.00	1.00	1.00

3.6 Laboratory Experiment Statistical Analysis

Statistical analysis compared reference batch reactors to all treatment reactors. Comparisons were also made between each set of reactors containing the same treatment in the presence and absence of protozoa (i.e. allylthiourea to allylthiourea+cycloheximide). Comparisons were made on the basis of the rate of COD production, rate of ammonia production, rate of nitrite production, and the rate of total nitrogen production using the unpaired t-test with a significance level of 0.05. Floc size and floc depth were also compared. The assumptions of the unpaired t-test are as follows:

1) Samples are randomly selected, or at least representative of, the larger population.
2) The two samples were obtained independently.
3) The observations within each sample were obtained independently.
4) The data are sampled from populations that approximate a normal distribution (must think about the biology of the situation when using this assumption for small samples).
5) The population standard deviations (σ) are identical.

All statistical calculations were done using the Analyze-it™ statistical add-in package on Microsoft excel (Microsoft Office 2000 for Windows 98, 1999 Microsoft Corp., Redmond, Washington).

Production rates of COD, ammonia, nitrite, nitrate, and total nitrogen, were calculated using the equation:

$$\Delta C/t=(C_f-C_i)/t \quad \text{(Equation 3.4)}$$

Where :

C_f = Final concentration of parameter

C_i = Initial concentration of parameter

t = number of days over which concentration was measured.

4.0 RESULTS

4.1 Performance of the Bench Scale Batch Microcosms

Bench scale batch microcosms were monitored over a 14-day period. Temperature and pH were monitored on a daily basis throughout the 14-day trials. Dissolved oxygen, COD, ammonia, total nitrate and nitrite, and total nitrogen levels were measured on day 1, 4, 7, 10, and 14. Cell counts to measure microbial abundance were performed on day 1, 7, and 14. For tables containing all data collected throughout the 14-day trial the reader is referred to Appendix A of this report.

4.1.1 Temperature, pH, and Dissolved Oxygen

The temperature (Figure 4.1) was relatively stable for the duration of each trial. Since the temperature varies with the ambient temperature in the laboratory setting, some variation in temperature is expected. Variation between trials run at different times occurred, and was most likely a result in variation the ambient laboratory temperature. For all trials, the daily temperature remained between 23.2°C and 26.4°C

The pH was also monitored on a daily basis for each trial (Figure 4.2). In the reference trial, the cycloheximide trial, the EDTA trial, and the EDTA + cycloheximide trial, the pH decreased from approximately 7.5 on day 1 of the trial to 4.9, 4.79, 4.71 and 4.73 respectively, by day 14. Between day 1 and day 10, the pH for the reference, cycloheximide, EDTA, and EDTA + cycloheximide trials did not drop below 6.4. At below 6.4, nitrification is known to occur at less than 50% of the maximum rate of nitrification (Seviour, 1999; Viessman and Hammer, 1998). For every milligram of nitrogen nitrified, 7.2 mg of alkalinity is consumed. Thus, there is a net production of acid during nitrification of wastewater, and a drop in pH as nitrification proceeds is expected.

Interestingly, in the allylthiourea and the allylthiourea + cycloheximide trials, the pH actually increased throughout the 14-day trial. In the allylthiourea trial the pH increased from a value of 7.49 at day one to a value of 8.87 on day 14 and in the allylthiourea + cycloheximide trial the pH increased from 7.49 on day 1 to 8.60 on day 14

Dissolved oxygen measurements were performed in the reference, cycloheximide, EDTA, and EDTA + cycloheximide trials (Figure 4.3). In the reference and cycloheximide trials, the dissolved oxygen levels remained constant at 8 mg O_2/L throughout the entire 14-day period. In the EDTA and EDTA + cycloheximide trials, the dissolved oxygen content was slightly higher during the first two days of measurement (day one and day four) but then dropped to 8 mg O_2/L and remained at this level for the duration of the trial. Dissolved oxygen levels below 0.3 mg/L are known to inhibit nitrification, while at 1.0 mg/L the nitrification rate is 90% of that observed in oxygen saturated water (Blackall and Burrell, 1999). It is important to note that the dissolved oxygen measured in this study is the dissolved oxygen content of the mixed liquor. The dissolved oxygen content in the interior of the biological flocs depends on floc characteristics such as size and depth. Thus, the nitrification rates will largely be affected by the dissolved oxygen available to nitrifying bacteria within the flocs. A mixed liquor dissolved oxygen content of approximately 8.0 mg/L is indicative of adequate aeration and it is probable that floc bacteria will have sufficient dissolved oxygen available to them.

Figure 4.1 Temperature Profile for all Treatments of the 14-day Batch Reactors.

Error Bars = +/- 1SD

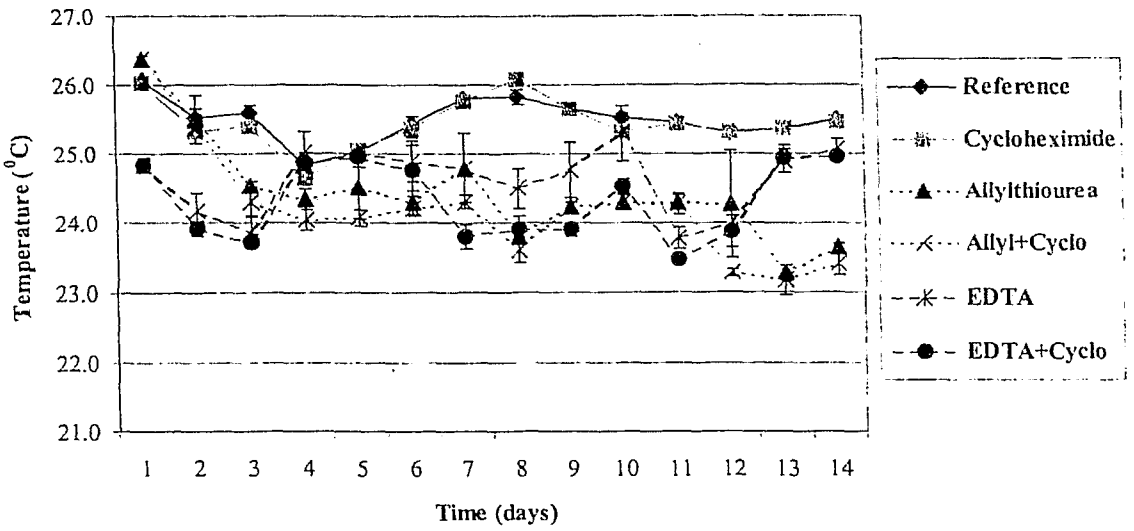


Figure 4.2 pH profile for all Treatments of the 14-day Batch Reactors.

Error Bars = +/- 1SD

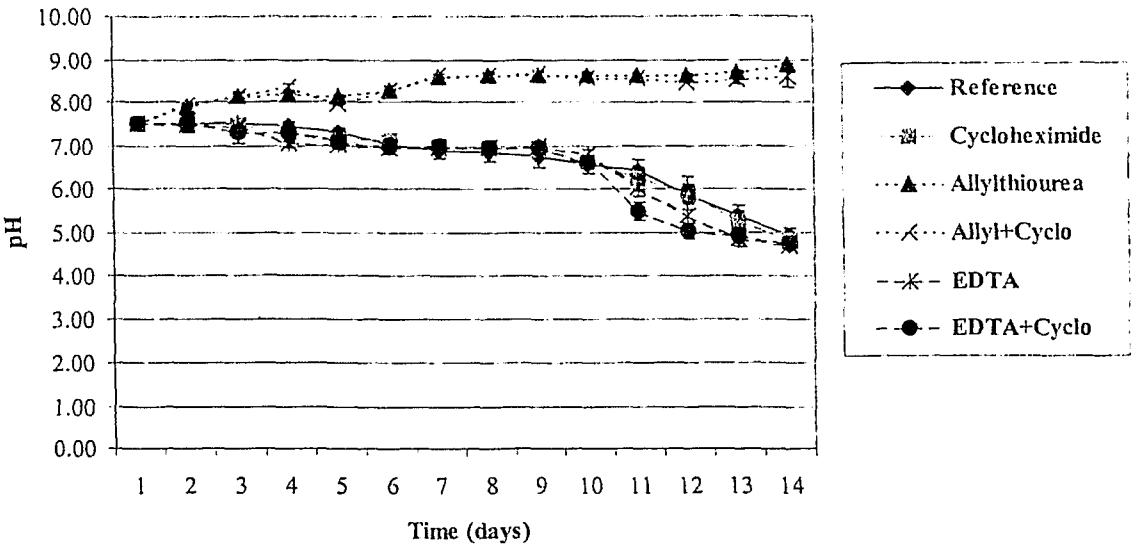
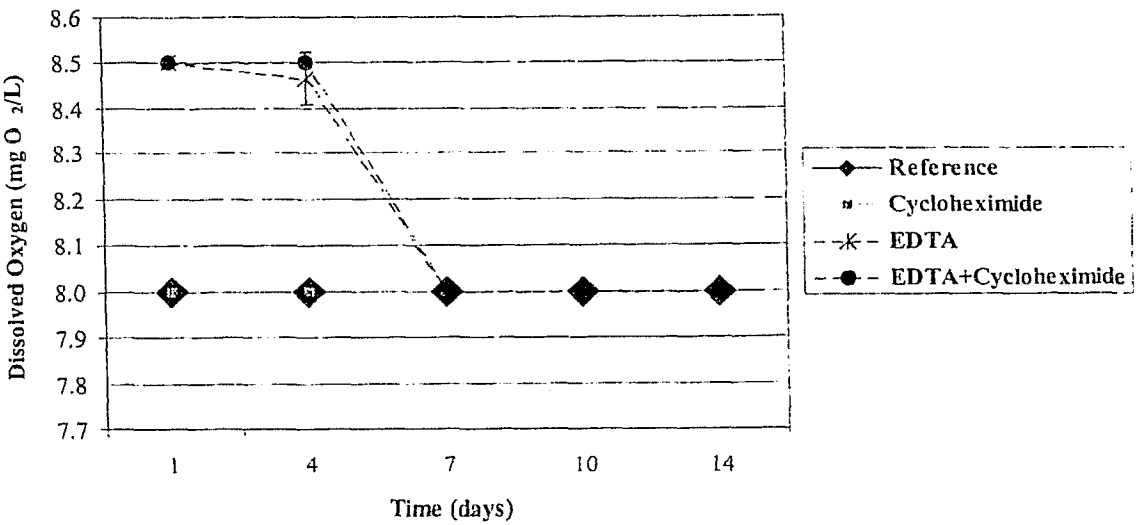


Figure 4.3 Dissolved Oxygen Profiles for all Treatments of the 14-day Batch Reactors.

Error Bars = +/- 1SD



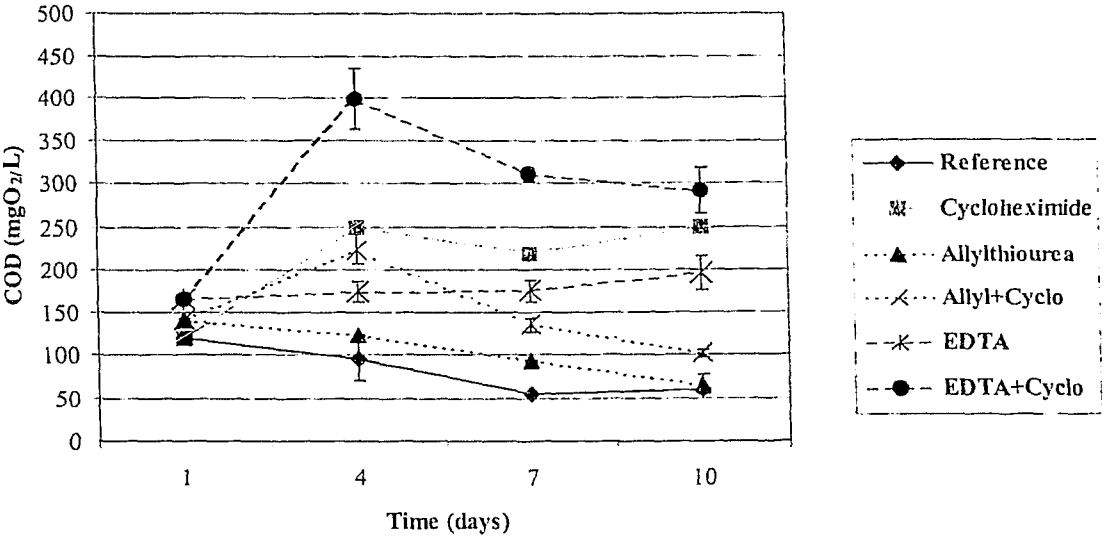
4.1.2 10-day Profiles of COD, Ammonia, Nitrite, Nitrate, and Total Nitrogen

The parameters COD, ammonia, nitrite, nitrate, and total nitrogen were monitored on day 1, 4, 7, 10, and 14 of each trial. Between day 10 and Day 14 of the reference, cycloheximide, EDTA, and EDTA + cycloheximide trials, the pH dropped rapidly from a value of above 6.5 on day 10 (in all trials) to a value of below 5.0 on day 14. As already mentioned in section 1.1.3.1, pH is an important factor in the maintenance of optimal conditions for waste removal from influent water. Nitrification has an optimum pH of between 7.0 and 8.2 and 7.5 and 8.5, and no nitrification activity has been observed at a pH below 6.5 or above 10.0 (Blackall and Burrell, 1999). In addition, at pH values of 6.5 and below, fungi are favoured over bacteria in competition for food (Blackall and Burrell, 1999; Viessman and Hammer, 1998). Therefore since this study was interested in the effect of protozoan grazing on nitrification and it is very unlikely that any nitrification activity occurred between day 10 and day 14 due to the rapidly decreasing pH the results for all parameters are limited to the measurements taken from day 1 to 10. Complete readings can be seen in appendix A. The chemical oxygen demand is a measure of the

oxygen equivalent or the organic matter susceptible to oxidation by a strong chemical oxidant. In the test, the organic matter is destroyed by a mixture of strong acids and subsequently converted to carbon dioxide and water. For a wastewater composed mainly of biodegradeable organic substances the COD concentration approximates the ultimate carbonaceous BOD value. The chemical oxygen demand was monitored in order to ensure that resources were not completely depleted throughout the monitoring period. In the reference batch treatments, the COD decreased 50% from a value of 120 mgO_2/L on day 1 to a value of 60 mg O_2/L on day 10. A decrease in COD over the 10-day monitoring period was also observed in both of the treatments containing allylthiourea. In the cycloheximide trials, the COD increased over the 10-day period from a value of 120 mgO_2/L on day 1 to 249 mg O_2/L on day 10. In the reactors treated with EDTA, the COD increased very slightly over the 10-day period, from 165 mg O_2/L on day 1 to 196 mg O_2/L on day 10. In the reactors treated with both EDTA and cycloheximide the COD increased at the greatest rate from165 mgO_2/L on day one to 290 mgO_2/L on day 10. It is interesting to note that in all reactors treated with cycloheximide, the COD peaked on day 4. The observed increases in COD in the cycloheximide and EDTA + cycloheximide trials could be due to the death and eventual decay of the metabolically inhibited protozoan species, which would increase the chemical oxygen demand. In the trial containing allylthiourea and cycloheximide, the effect of cycloheximide may have been offset by the high numbers of heterotrophic bacteria due to the combined effect of cycloheximide and no competition from nitrifying bacteria. It is also important to mention that the COD measured only takes into account the dissolved organic matter and does not measure organic matter in the flocculated material or that which is removed upon filtration. Due to the highly flocculant nature of activated sludge, the measured COD values may be artificially low because a large portion of the COD is removed upon filtration of the sample (as part of the sample preparation for the COD method). Significantly, the COD was not completely depleted in any of the trials, indicating that some organic matter was still present and the system was still active.

Figure 4.4 Chemical Oxygen Demand Profile for all Treatments of 10-day Batch Reactors.

Error Bars = +/- 1SD

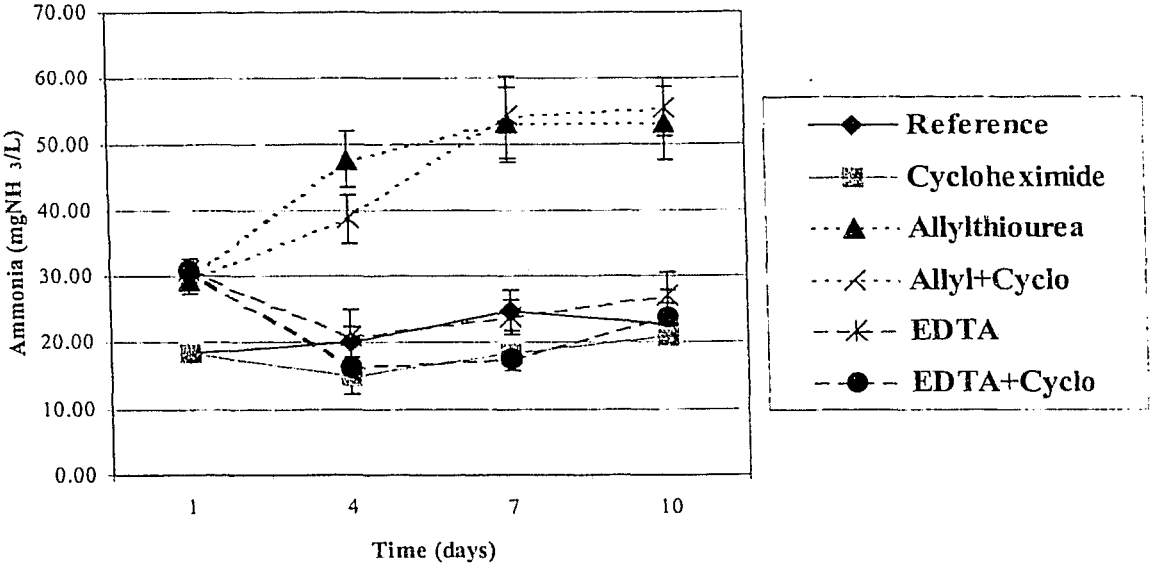


The parameters of ammonia, nitrite, nitrate, and total nitrogen were measured in order to monitor the nitrification activity of batch reactors. The measurement of ammonia, nitrite, and most importantly nitrate is a method commonly used to monitor nitrification in batch reactor systems (Garrido et al., 1998; Gernaey et al., 1998; Muller et al., 1995; Verhagen and Laanbroek, 1992; Verhagen and Laanbroek, 1991).

Ammonia is present in influent wastewater in abundance (usually in excess of 10 mg/L) and is also excreted by some organisms, such as protozoa, as a by-product of metabolism. When ammonia dissolves in water a portion reacts with water to form ammonium ions ($NH4^+$) and the remainder exists as un-ionized ammonia. Since un-ionized ammonia cannot be measured, its concentration in water is based on the measured concentration of total ammonia ($NH_3 + NH_4^+$). The change in ammonia concentrations in batch reactors over the 10-day period is shown in figure 4.5.

Figure 4.5 Ammonia Profile for all Treatments of 10-day Batch Reactors.

Error Bars = +/- 1SD



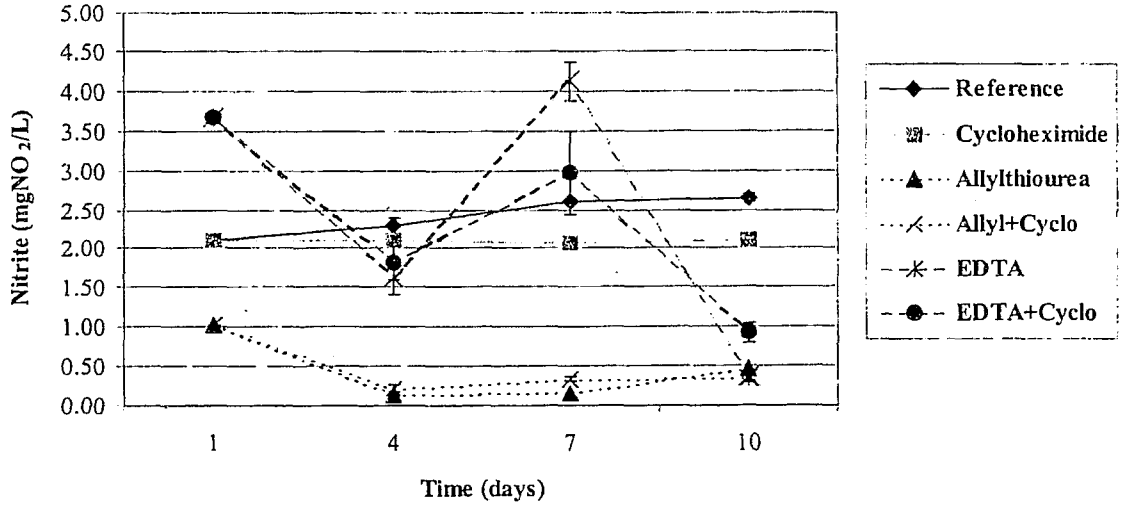
In the reference and cycloheximide reactors, the ammonia increased slightly from an initial value of 18.38 mg NH_3/L on day 1 to values of 22.82 mg NH_3/L and 20.97 mgNH_3/L in the reference and cycloheximide reactors respectively. In the reactors treated with EDTA, ammonia concentrations started out at 30.98 mg NH_3/L on day 1. Throughout the 10-day period in the reactors treated with EDTA, ammonia levels decreased to 27.26 mg NH_3/L and 23.92 mg NH_3/L in the EDTA and EDTA + cycoheximide reactors, respectively. In both the EDTA and EDTA + cycloheximide reactors, ammonia levels reached a minimum on day 4, and then increased again between day 4 and day 10. The greatest increase in ammonia levels was measured in the allylthiourea and allylthiourea + cycloheximide trials, where the ammonia level increased from 29.52 mg NH_3/L on day 1 to 53.19 and 55.56 mg NH_3/L on day 10, respectively. In the both the allylthiourea and the EDTA trials, the day 1 ammonia levels were much higher than in the reference and cycloheximide trials. However, because sample was collected at different times from Ashbridges Bay WWTP, ammonia levels in the sample

vary with the ammonia loading rates in the influent wastewater at the time of sample collection.

Nitrite levels were monitored over the 10-day period using a colourimetric method (Figure 4.6). Nitrite is an intermediate in the nitrification process, and is produced during the ammonia oxidation step of the nitrification process.

Figure 4.6 Nitrite Profile for all Treatments of 10-day Batch Reactors.

Error Bars = +/- 1SD



In the reference reactors, the nitrite levels increased slightly over the 10-day period from 2.10 mg NO_2^-/L on day 1 to 2.65 mg NO_2^-/L on day 10. In the cycloheximide reactors the nitrite levels remained fairly constant throughout the trial, but overall there was a slight increase in nitrite levels, from 2.10 mg NO_2^-/L on day 1 to 2.11 mg NO_2^-/L on day 10. In both the allylthiourea and allylthiourea + cycloheximide trials, the amount of nitrite decreased from 1.02 mg NO_2^-/L on day 1 to 0.45 mg NO_2^-/L and 0.32 mg NO_2^-/L in the allylthiourea and allylthiourea + cycloheximide, respectively. The nitrite levels in the reactors treated with EDTA and EDTA + cycloheximide were extremely varied. On day 1, the nitrite levels were 3.67 mg NO_2^-/L, and on day 10 nitrite levels had decreased to 0.36 and 0.92 mg NO_2^-/L in the EDTA and EDTA + cycloheximide reactors, respectively. Both the trials containing EDTA were characterized by a sharp decrease in nitrite on day 4, followed by a sharp increase on day 7, and a subsequent decrease by day

10. Again, variation in the day 1 nitrite levels is to be expected as initial nitrite levels in collected activated sludge samples will vary with the incoming effluent nitrite concentration and plant operating parameters at the time of collection.

Nitrate is the final product in the nitrification process and is the most commonly measured parameter in the tracking of nitrification in environmental samples. Nitrate was monitored throughout each trial using a colourimetric method (Figure 4.7A and Figure 4.7B).

Figure 4.7A Nitrate Profile for Reference, Cycloheximide, Allylthiourea, and Allythiourea + Cycloheximide Treatments of 10-day Batch Reactors.

Error Bars = +/- 1SD

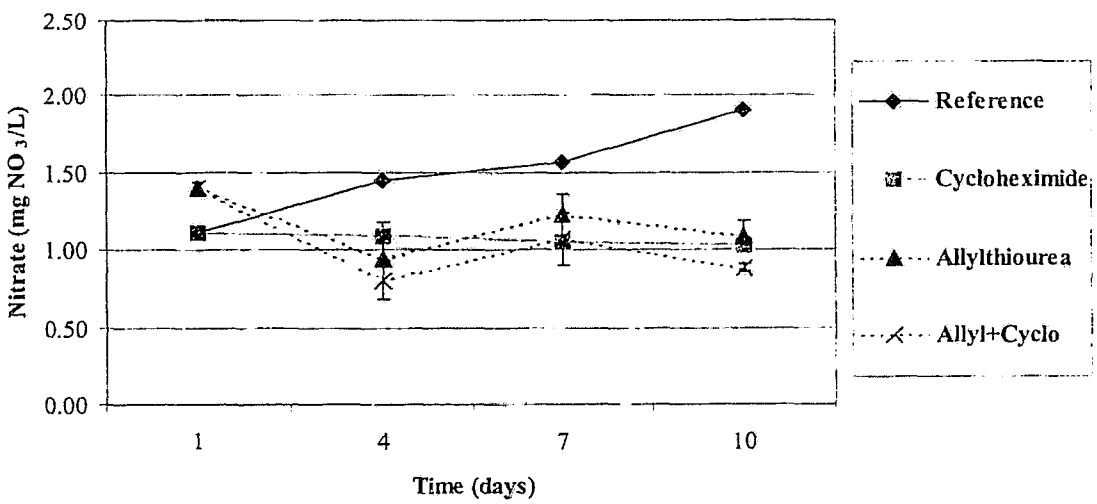
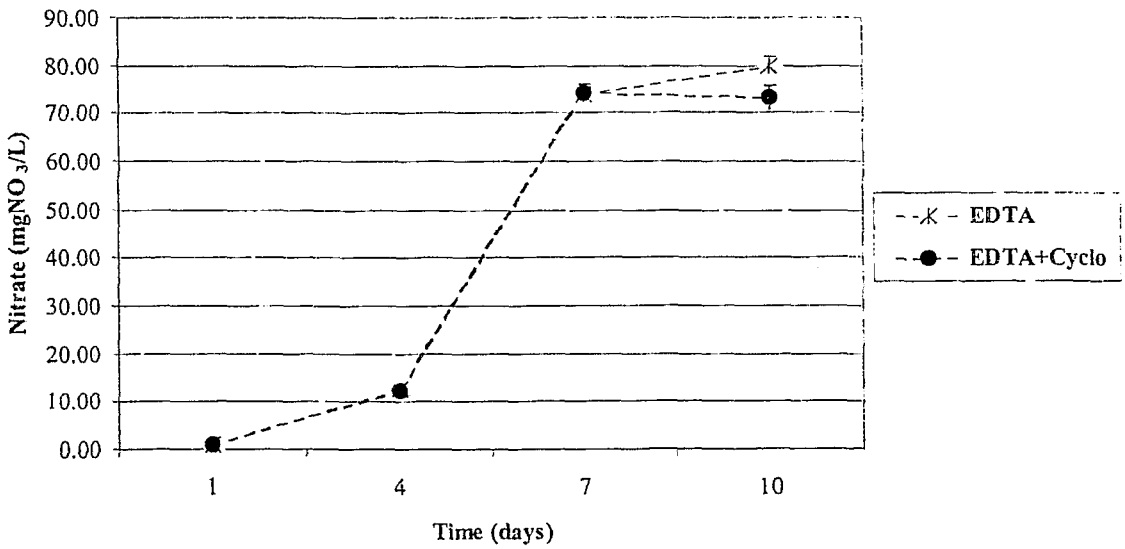


Figure 4.7B Nitrate Profile for EDTA and EDTA + Cycloheximide Treatments of 10-day Batch Reactors.

Error Bars = +/- 1SD

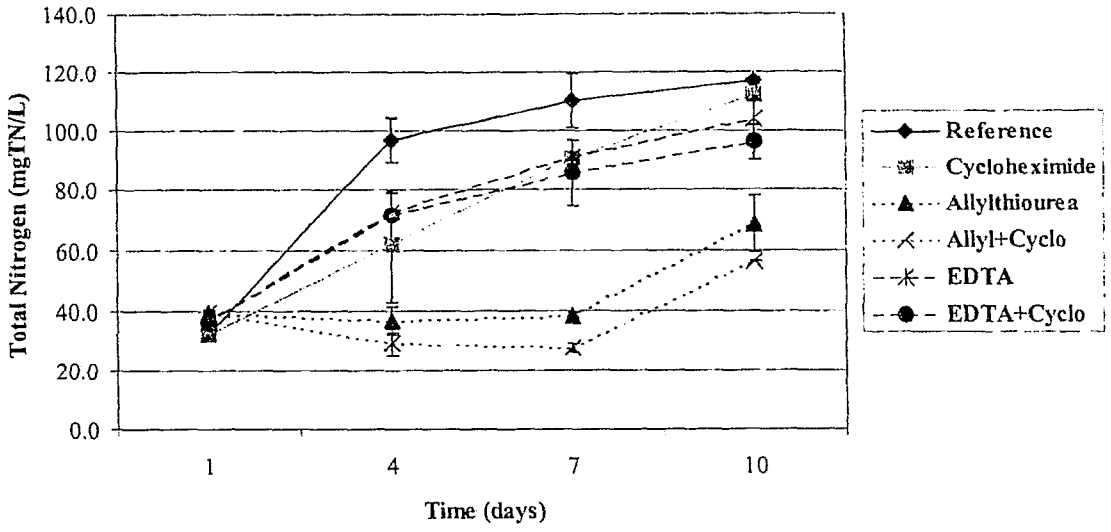


In the reference reactors, nitrate levels increased over the 10-day period from 1.11 mg NO_3^-/L on day 1 to 1.91 mg NO_3^-/L on day 10. In the cycloheximide treated reactors, nitrite levels remained fairly constant over the 10-day period, but there was an overall slight decrease from 1.11 mg NO_3^-/L on day1 to 1.03 mg NO_3^-/L on day 10. As was the case in the monitoring of nitrite levels, the levels of nitrate in the allylthiourea and allylthiourea + cyloheximide trials fluctuated throughout the trial. In both of these trials, there was an overall decrease in nitrate production from 1.41 mg NO_3^-/L on day 1 to 1.09 and 0.89 mg NO_3^-/L in the allylthiourea and allylthiourea + cycloheximide trials, respectively. Both trials were characterized by a decrease in nitrite levels on day 4, followed by an increase on day 7 and subsequent decrease to below day 1 levels by day 10. This pattern of nitrate fluctuation mirrors the pattern of nitrite fluctuation observed in the allylthiourea and allythiourea + cyloheximide trials. In the EDTA and EDTA + cycloheximide treated reactors, nitrate levels drastically increased over the 10-day period from a value of 1.00 mg NO_3^-/L on day 1 to 79.8 and 73.34 mg NO_3^-/L in the EDTA and EDTA + cycloheximide trials, respectively.

The total nitrogen (TN) parameter method measures all forms of nitrogen present in the wastewater including organic N, ammonia, ammonium, nitrite, and nitrate. Total nitrogen levels in the reactors were monitored using a colourimetric method (Figure 4.8).

Figure 4.8 Total Nitrogen Profile for all Treatments of 10-day Batch Reactors.

Error Bars* = +/- *1SD



Total nitrogen levels increased over the 10-day monitoring period in all trials. In both the reference and cycloheximide treated reactors, the total nitrogen increased from 32.2 mgTN/L on day 1 to 117.1 and 112.2 mgTN/L in the reference and cycloheximide reactors, respectively. In the allylthiourea and allylthiourea + cycloheximide treated reactors, total nitrogen levels decreased slightly from the initial level of 39.3 mgTN/L on day 4, but began increasing again and day 10 TN levels were 68.7 mg TN/L and 56.2 mg TN/L in the allylthiourea and allylthiourea + cycloheximide reactors, respectively. In the EDTA and EDTA + cycloheximide reactors, TN levels increased steadily from 36.4 mg TN/L on day 1 to 104.3 and 96.4 mg TN/L in EDTA and EDTA + cycloheximide reactors, respectively.

4.1.3 10-day Rates of Ammonia, Nitrite, Nitrate, and Total Nitrogen Production

In order to more accurately track nitrification activity, production rates of ammonia, nitrite, nitrate, and total nitrogen were calculated over the 10-day monitoring period. For details on how these rates were calculated the reader is referred to section 3.6 of this report. A positive production rate means that the amount of parameter increased over the 10-day period, while a negative production rate denotes a decrease in the amount of the parameter. A production rate of zero denotes that there was no change in the measured parameter over the 10 day period.

In order to compare the production rates of each parameter between the different treatments, a paired t-test was used. A significance level (α) of 0.05 (95% confidence limits) was selected for all statistical analyses. Table 4.1 summarizes the outcome of all the comparisons performed. A P-value of less than 0.05 denotes a statistically significant difference between samples.

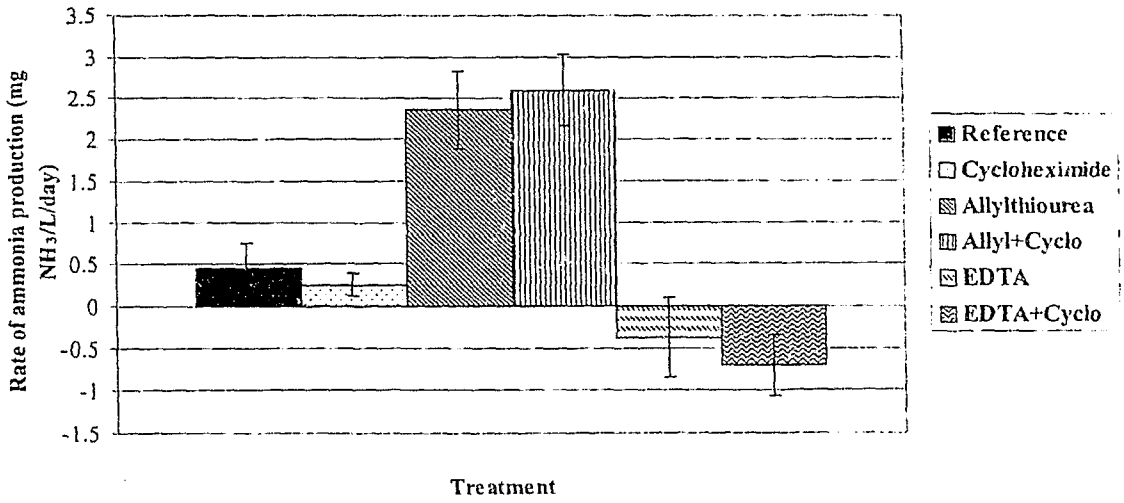
Table 4.1 Statistical t-test P-values (α=0.05) of Listed Treatment Comparisons For Rate of Ammonia, Nitrite, Nitrate, and Total Nitrogen Production. Statistically significant differences are noted with bold italic lettering.

	P-value at $\alpha = 0.05$			
Comparison:	NH_3^- Production Rate	NO_2^- Production Rate	NO_3^- Production Rate	TN Production Rate
Reference:cycloheximide	3.97×10^{-1}	***6.00×10^{-5}***	***4.24×10^{-8}***	***1.51×10^{-3}***
Reference:allylthiourea	***4.07×10^{-3}***	***3.32×10^{-6}***	***7.11×10^{-5}***	***5.06×10^{-4}***
Reference:allyl+cyclo	***2.01×10^{-3}***	***2.35×10^{-6}***	***4.44×10^{-7}***	***8.41×10^{-8}***
Reference:EDTA	6.86×10^{-2}	***2.70×10^{-7}***	***2.38×10^{-7}***	5.61×10^{-2}
Reference:EDTA+cyclo	***1.44×10^{-2}***	***4.43×10^{-6}***	***9.23×10^{-7}***	***3.09×10^{-3}***
Allylthiourea:allyl+cyclo	5.00×10^{-1}	***3.37×10^{-3}***	***4.00×10^{-2}***	8.00×10^{-2}
EDTA: EDTA+cyclo	4.00×10^{-1}	***5.70×10^{-3}***	***2.27×10^{-2}***	3.43×10^{-1}

The rates of ammonia production over the 10-day period in all treatments of the batch reactors is shown in figure 4.9. The rate of ammonia production in the cycloheximide trial was only slightly lower than in the control trial, and the difference was not significant. The highest rate of ammonia production occurred in the allylthiourea and the allythiourea + cycloheximide trials (2.37 and 2.60 mgNH_3/L/day, respectively). The rate of ammonia production in both of these was significantly higher than the rate of ammonia production in the reference trial. However, there was no significant difference in the ammonia production rates observed between the allylthiourea and allylthiourea + cycloheximide trials. The lowest ammonia production rates occurred in the trials containing EDTA (EDTA and EDTA + cycloheximide). The production rates in these trials were negative, indicating that the ammonia concentrations in the reactors decreased throughout the 10-day monitoring period. When the EDTA and EDTA + cycloheximide ammonia production rates were compared with the reference ammonia production rates, there was a significant difference only between the reference ammonia production rate and the EDTA + cycloheximide ammonia production rates. There was no significant difference in the rates of ammonia production between the EDTA and the EDTA + cycloheximide trials.

Figure 4.9 Rates of Ammonia Production for all Treatments of the 10-day Batch Reactors.

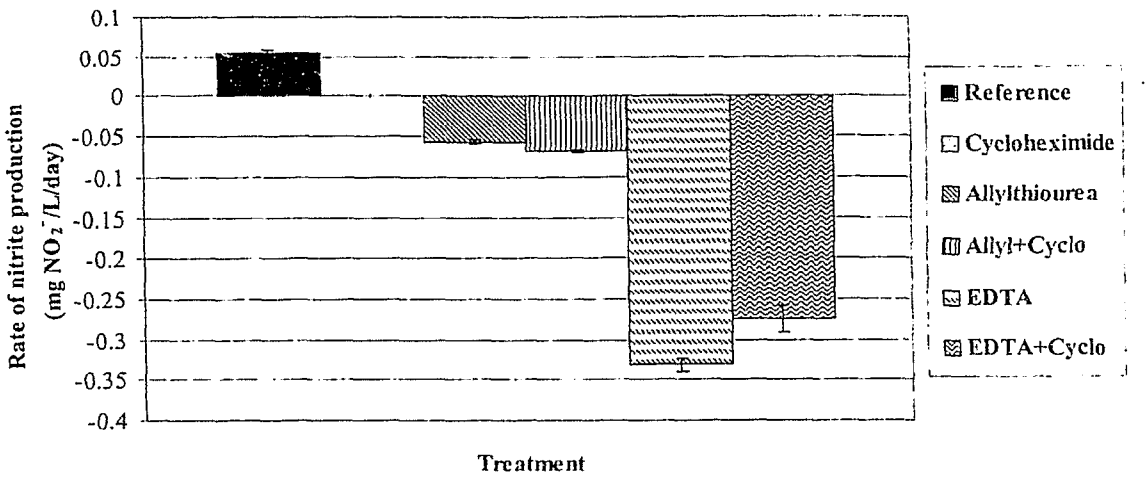
Error Bars = +/- 1SD



The rates of nitrite production over the 10-day monitoring period for all trials is shown in figure 4.10. The rates of nitrite production in the reference and cycloheximide trials were both positive (0.054 and 0.001 mg NO_2^-/L/day, respectively), and the rate of nitrite production in the cycloheximide trials was significantly lower than that of the reference trial. The rates of nitrite production in the allylthiourea and the allylthiourea + cycloheximide trials were negative (-0.056 and –0.068 mg NO_2^-/L/day, respectively) and significantly lower than the rate of nitrite production observed in the reference trial. The rate of nitrite production in the allylthiourea + cycloheximide trial was also found to be significantly lower than the rate of nitrite production in the allylthiourea trial. The lowest rates of nitrite production were observed in the EDTA and EDTA + cycloheximide trials (-0.33 and –0.27 mg NO_2^-/L/day, respectively). Both of these rates were significantly lower than the nitrite production rates observed in the reference trial, and the rate of nitrite production in the EDTA trial was significantly lower than that of the EDTA + cycloheximide trial.

Figure 4.10 Rates of Nitrite Production for all Treatments of the 10-day Batch Reactors.

Error Bars = +/- 1SD



The rates of nitrate production (Figure 4.11A and Figure 4.11B) varied greatly between each treatment. The rate of nitrate production in the reference trial was significantly greater than the rate of nitrate production in the cycloheximide trial (0.080 and –0.007 mg NO_3^-/L/day, respectively). The rates of nitrate production in the allylthiourea and in the allylthiourea + cycloheximide trial were both negative (-0.03 and 0.05 mg NO_3^-/L/day, respectively), and both were significantly lower than the rate of nitrate production in the control. The rate of nitrate production in the allylthiourea + cycloheximide trial was also found to be significantly lower than that of the trial treated with allylthiourea alone. The highest rates of nitrate production were found in the EDTA and the EDTA + cycloheximide trial (7.88 and 7.23 mg NO_3^-/L/day, respectively). Both of these rates were significantly higher than the rate of nitrate production in the reference trial, and the rate of nitrate production in the EDTA trial was significantly higher than that of the EDTA + cycloheximide trial.

Figure 4.11A Rates of Nitrate Production for Reference, Cycloheximide, Allylthiourea, and Allylthiourea + Cycloheximide Treatments of the 10-day Batch Reactors.

Error Bars = +/- 1SD

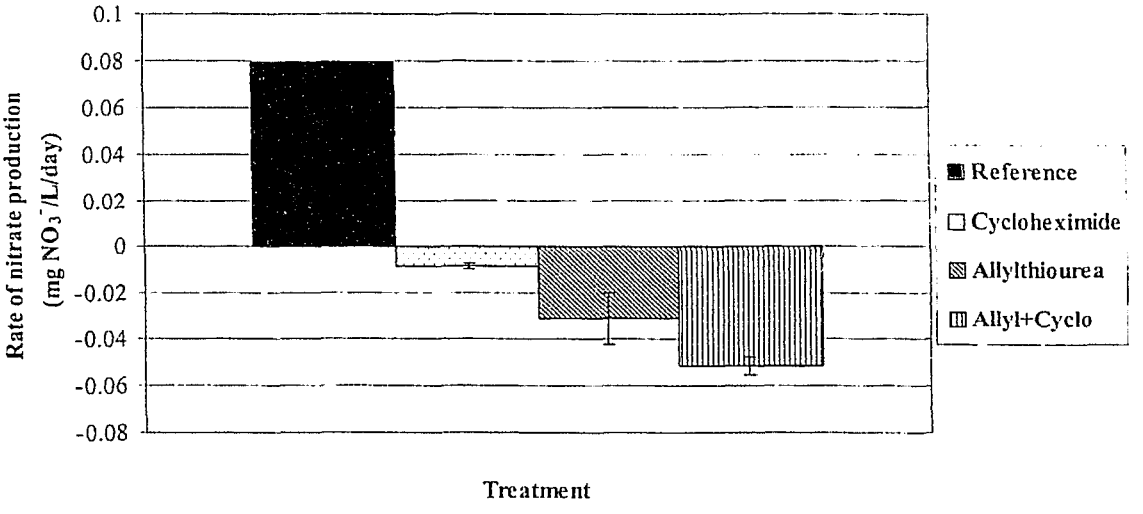
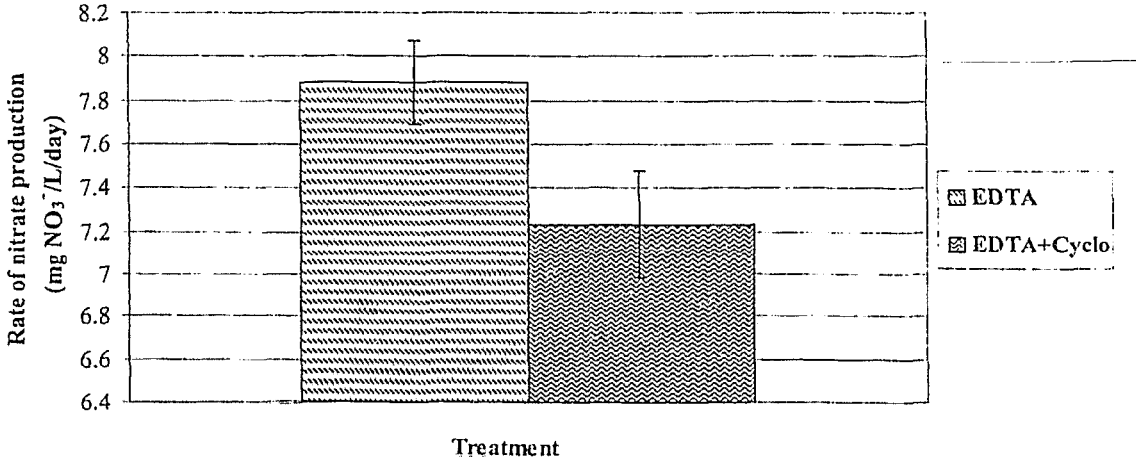
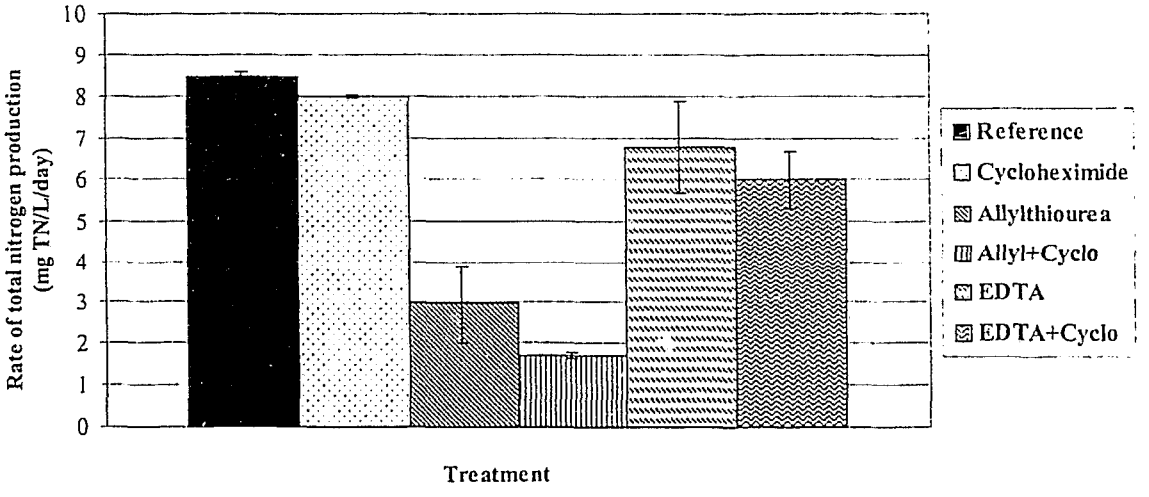


Figure 4.11B Rates of Nitrate Production for EDTA and EDTA + Cycloheximide Treatments of the 10-day Batch Reactors. *Error Bars = +/- 1SD*



The rates of total nitrogen production (Figure 4.12) were positive for every treatment, indicating that total nitrogen levels increased throughout each trial. The rates of total nitrogen production in the reference and cycloheximide reactors were the highest (8.49 and 8.00 mg TN/L/day, respectively), and the rate of total nitrogen production in the reference trial was significantly higher than that of the cycloheximide trial. The rate of total nitrogen production was the lowest in the allylthiourea and allylthiourea + cycloheximide trials (2.94 and 1.69 mg TN/L/day, respectively), and both of these rates were significantly lower than the rate of TN production observed in the reference trial. However, there was no significant difference between that TN production rate in the allylthiourea and allylthiourea + cycloheximide trials. The rate of TN production in the EDTA and EDTA + cycloheximide trials was 6.79 and 5.99 mg TN/L/day respectively. There was no significant difference between these two rates. However, the rate of TN production in the EDTA + cyloheximide trial was found to be significantly lower than that of the reference trial.

Figure 4.12 Rates of Total Nitrogen Production for all Treatments of the 10-day Batch Reactors. ***Error Bars = +/- 1SD***

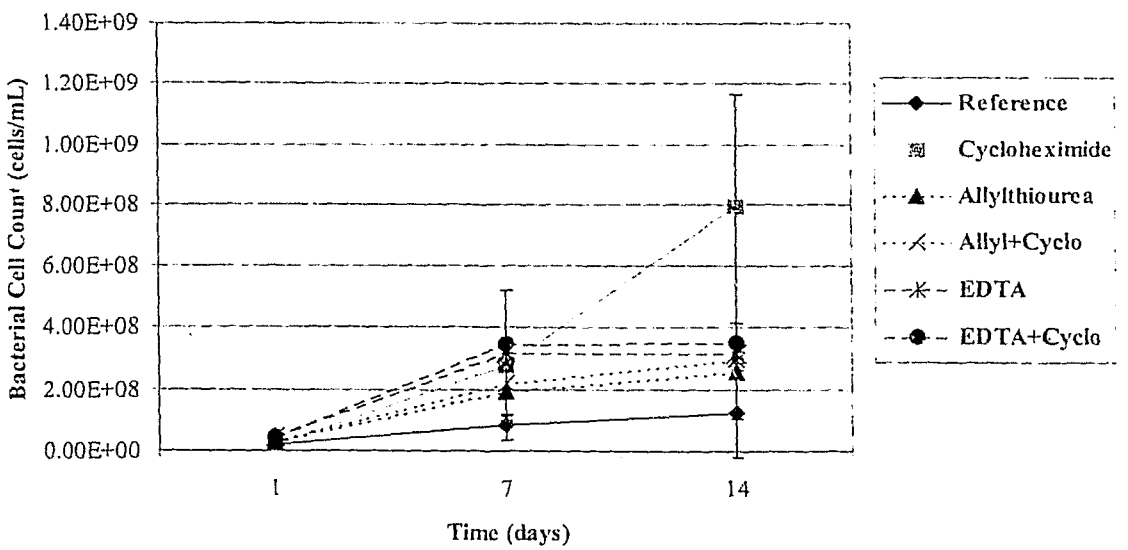


4.1.4 Enumeration of Bacterial Abundance

The enumeration of total bacterial abundance (both live and dead cells) was performed on day 1, day 7, and day 14 in each trial (Figure 4.13). Day 10 cell counts were not performed. Due to the low pH conditions present after day 10, it is not expected that an abundance of cell growth occurred between day 10 and day 14 of the trial. Thus, day 14 cell counts can be approximated to day 10 cell counts.

Figure 4.13 Profile of Bacterial Abundance (cells/mL) for all Treatments of Batch Reactors.

Error bars = +/- 1SD



In the reference trial, the bacterial abundance increased slightly from 1.77 X 10^{+7} on day 1 to 1.22 X 10^{+8} on day 14. In the cycloheximide trial, the bacterial abundance increased at a greater rate, rising from 1.77 X 10^{+7} on day 1 to 7.92 X 10^{+8} on day 14. In both the allylthiourea and allylthiourea + cycloheximide trials, the bacterial abundance increased slightly from 3.00 X10^{+7} on day 1 to 2.60 X 10^{+8} and 2.96 X10^{+8}, in the allylthiourea and allylthiourea + cycloheximide trials, respectively. In the EDTA and EDTA + cycloheximide trials, bacterial numbers increased from 4.46 X 10^{+7} on day 1, to 3.19 X 10^{+8} and 3.49 X 10^{+8} on day 7 in the EDTA and EDTA + cycloheximide trials, respectively. From day 10 to day 14, bacterial numbers only increased slightly to final values of 3.15 X 10^{+8} and 3.52 X 10^{+8}, in the EDTA and EDTA + cycloheximide trials,

respectively. Thus, in all trials total bacterial abundance increased throughout the trial. The final bacterial abundance was always higher in the trials containing cycloheximide as compared to the trials without cycloheximide.

A summary of the relative changes in COD, rate of ammonia production, rate of nitrite/nitrate production, rate of total nitrogen production, and bacterial cell abundance for each trial can be found in table 4.2.

Table 4.2. Relative changes in COD, rate of ammonia production, rate of nitrite/nitrate production, rate of total nitrogen production, and bacterial cell abundance over the 10-day monitoring period for each trial.

+ represents a positive increase over the 10-day period; ++ represents a large positive increase over the 10-day period; - indicates a decrease over the 10-day period; -- indicates a large decrease over the 10-day period.

Trial	COD (mgO_2/L)	Rate of NH_3 production ($mgNH_3/L/d$)	Rate of NO_2^- production ($mgNO_2^-/L/d$)	Rate of NO_3^- production ($mgNO_3^-/L/d$)	Rate of TN production (mgTN/L/d)	Bacterial Abundance (cells/mL)	Nitrification compared to reference?
Ref	-	+	+	+	++	+	N/A
Cyclo	+	+	+	-	++	++	Decrease
Allyl	-	++	-	--	+	+	Decrease
Allyl+Cyclo	-	++	-	--	+	+	Decrease
EDTA	+	-	--	++	++	+	Increase
EDTA+Cyclo	+	-	--	++	++	+	Increase

4.2 Fluorescent *In Situ* Hybridization (FISH) Combined With Confocal Scanning Laser Microscopy (CSLM) to Determine the Position and Abundance of Nitrifying Bacteria Within Activated Sludge Floc

The second phase of this study was concerned with determining the position and the relative abundance of nitrifying bacteria within the activated sludge floc. This was done by labelling nitrifying bacteria in the activated sludge samples with fluorescently tagged oligonucleotide probes through the process of *in situ* hybridization. After probes were hybridized to the bacteria within the sludge samples, flocs were examined using confocal

scanning laser microscopy (CSLM). All data collected during the FISH/CSLM portion of this study can be found in Appendix B of this report.

4.2.1 Floc Selection for Analysis

Flocs examined using the CSLM were selected randomly, with no preference given to flocs of certain size or depth proportions. A paired t-test with a significance level of $\alpha = 0.05$ was used to compare the floc size and diameter between treatments. Table 2 shows the P-values obtained in the t-tests performed on floc length and diameter data.

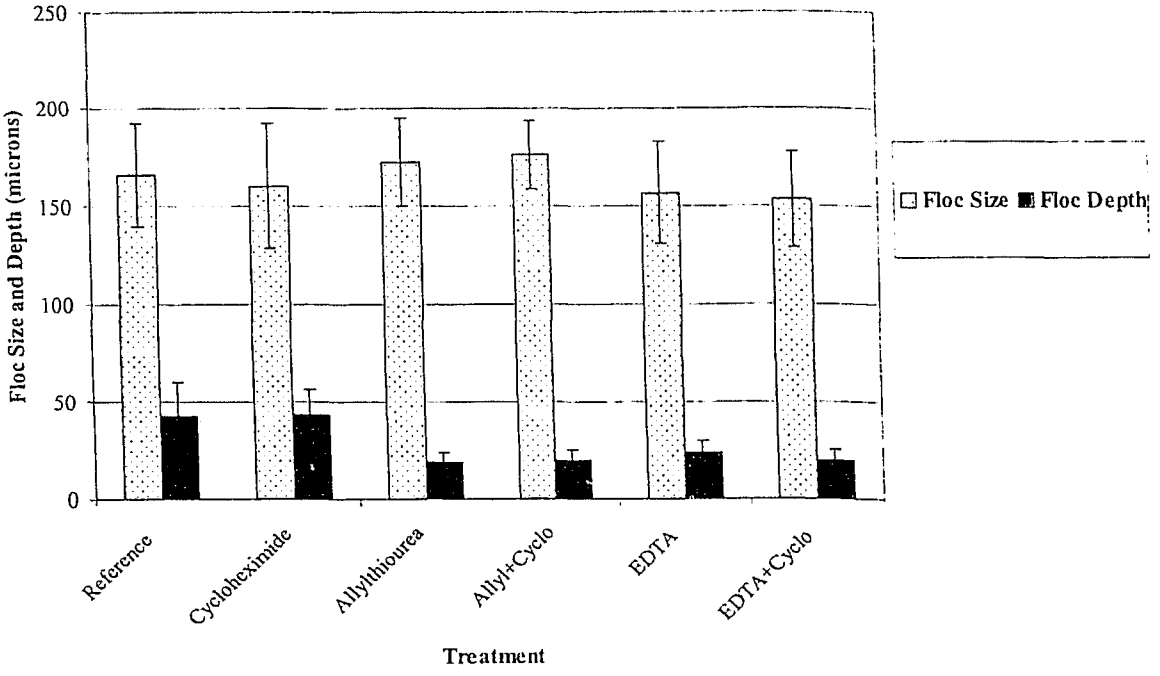
Table 4.3 Statistical t-test P-values (α=0.05) of Listed Treatment Comparisons For Floc Size and Floc Depth. Statistically significant differences are noted with bold italic lettering.

	P-value at $\alpha = 0.05$	
Comparison	**Floc Size (μm)**	**Floc Depth (μm)**
Reference:cycloheximide	4.16×10^{-1}	9.26×10^{-1}
Reference:allylthiourea	2.50×10^{-1}	***4.31×10^{-11}***
Reference:allyl+cyclo	5.98×10^{-1}	***1.79×10^{-10}***
Reference:EDTA	1.29×10^{-1}	***3.12×10^{-8}***
Reference:EDTA+cyclo	***3.45×10^{-2}***	***1.66×10^{-10}***
Allylthiourea:allyl+cyclo	4.78×10^{-1}	5.44×10^{-1}
EDTA: EDTA+cyclo	5.63×10^{-1}	***5.99×10^{-3}***

Figure 4.14 shows the average floc size and depth for the flocs examined in each trial.

Figure 4.14 Average Floc Size and Depth of Flocs Examined in Each Trial using CSLM.

Error bars=+/- SD



When floc size was compared among the trials, the only statistically significant difference was the difference in average floc size between the reference and the EDTA + cycloheximide trials.

Floc depth showed more variation, with smaller flocs measured in trials containing allylthiourea and EDTA. The average floc depth in both the allylthiourea and allylthiourea + cycloheximide treated trials was significantly smaller than the average floc depth observed in the reference trial. The average floc depth in the EDTA and EDTA + cycloheximide trials was also significantly smaller than in the reference reactors, and the average floc depth in the EDTA + cycloheximide trial was significantly smaller than in the trial containing EDTA only. A smaller floc depth in the EDTA trials is an expected result due to the deflocculating properties of EDTA. A smaller floc depth

in the allylthiourea trials could be a result of the increased in pH observed in the trial. It is important to note that floc depth may be altered during sample preparation procedures, as flocs may become compressed during sample preparation. Thus, some variation in floc depth is expected.

4.2.1 Observation of Probe Position Within Flocs and Quantification of Relative Probe Amounts

In order to determine whether the presence or absence of protozoa under the different treatment conditions affected the position and amount of nitrifying bacteria in the flocs, the relative position of the each probe within the flocs and the relative amount of each probe was determined for each trial on each sampling day (1,7, and 14).

In order to determine the relative position of each probe within the floc, the depth of the maximum binding of each probe (Dmax) was divided by the total floc depth for each probe in each floc observed. Thus, a relative position of 0.5 denotes a position of exactly mid-floc. Positions higher or lower than 0.5 denote positions closer to the outside of the floc. The depth of the maximum binding of each probe is the depth within the floc at which the highest fluorescence for that probe is observed. Figure 4.15, 4.16, and 4.17 show the relative position of each probe within the flocs for day 1, day 7, and day 14, respectively.

Figure 4.15 Average Relative Position of Each Probe Within Flocs in All Treatments of Batch Reactors on Day 1. ***Error bars=+/- SD***

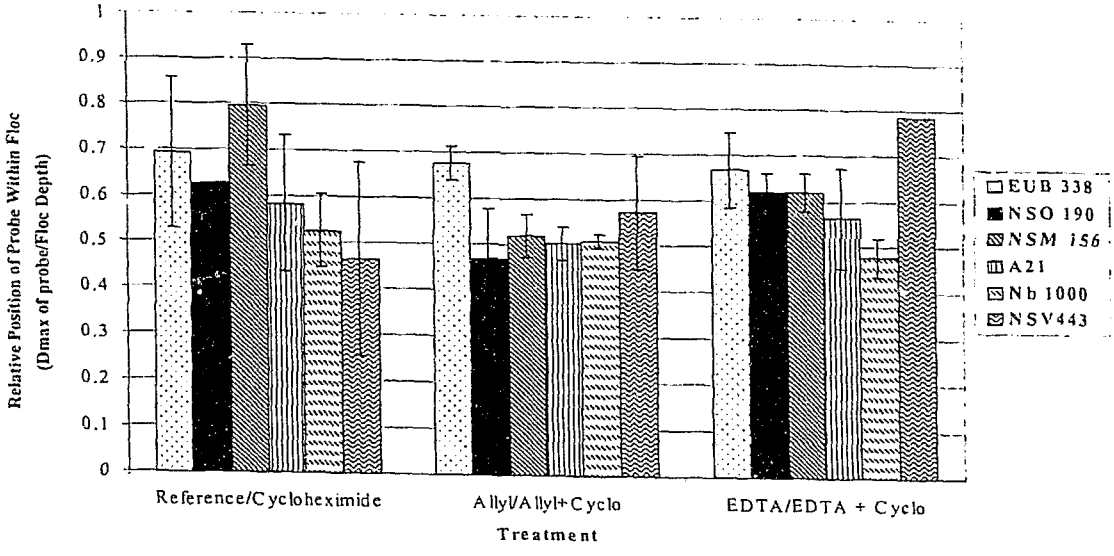


Figure 4.16 Average Relative Position of Each Probe Within Flocs in All Treatments of Batch Reactors on Day 7. ***Error bars=+/- SD***

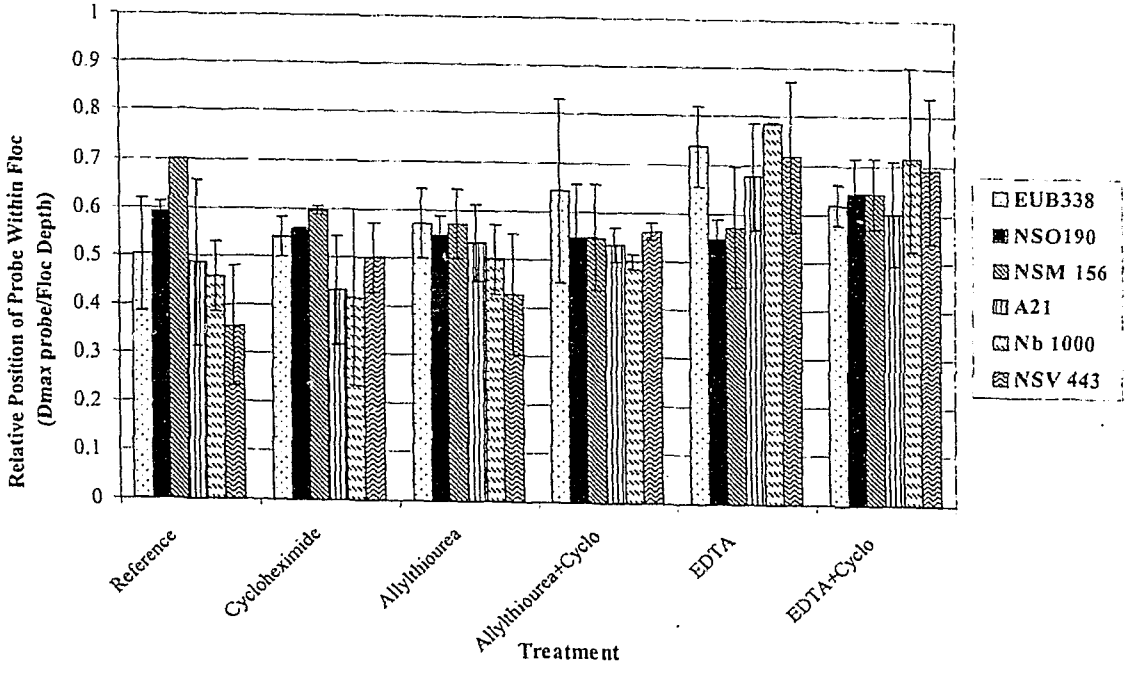
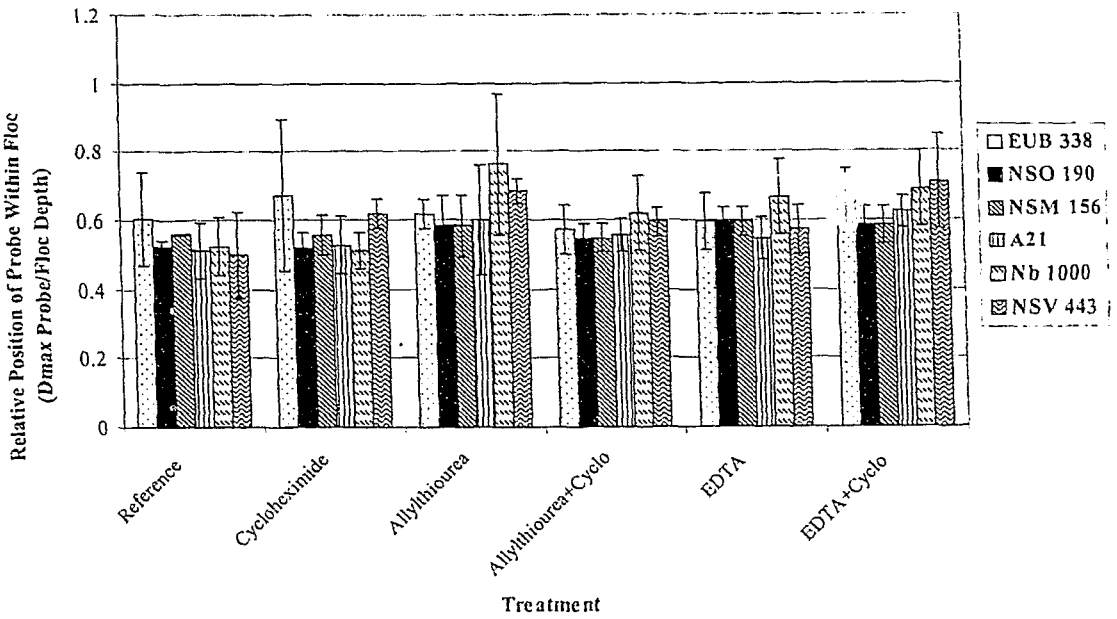


Figure 4.17 Average Relative Position of Each Probe Within Flocs in All Treatments of Batch Reactors on Day 14. ***Error bars*****=+/-** ***SD***



Through visual observation of the flocs and by examination of the figures above, some observations about the spatial organization of the bacteria within the floc can be made.

There was no noticeable difference in bacterial position within the floc between day 1, day 7, and day 14. Probe EUB 338 (most eubacteria) was found at all levels of floc, with the majority of EUB 338 binding occurring in the outer mid-depth of the floc (relative position of approximately 0.6). Probes NSO 190 and NSM 156 (AOB bacteria of β subclass *Proteobacteria* and AOB *Nitrosomonas* and *Nitrosococcus mobilis*, respectively) were found to be closely associated with each other in clusters, and the maximum amount of binding of these probes occurred in the mid –floc position (relative position of approximately 0.5). The β *Proteobacteria* and *Nitrosomonas* AOB were often found at multiple depths within the floc, with the majority of probe binding occurring mid-floc.

Probe A21 (NOB *Nitrospira*-like bacteria) was also observed in clusters and was most abundant at the mid-depth position of the floc. Probe Nb 1000 (NOB *Nitrobacter*) was

found closely associated with A21, but was usually observed slightly closer to the outside of the floc than the *Nitrospira*- like bacteria. Probe NSV 443 (NOB Nitrosospira spp.) was also found positioned mid-floc, but like *Nitrobacter* it was usually slightly closer to the outside of the floc than the *Nitrospira*-like bacteria. *Nitrospira*-like NOB were usually distributed throughout the floc, with the greatest abundance occurring in the mid-floc position. *Nitrosospira* and *Nitrobacter* were not observed to be distributed throughout the floc, and were often only found at a single depth.

Through visual examination of flocs using CSLM, an analysis of the relative amount of each type of probe used was performed. Probe amounts were ranked using a four- point scale. Table 4.3 summarizes the four-point scale used to quantify probe abundance, and figure 4.18 provides an example image of each of the four-point rankings on the scale.

Table 4.4 Scale for Quantification of Probe Abundance

Rank on Four-Point Scale	No. of Fluorescent Clusters
1	1-4
2	5-10
3	12-20
4	25 or more

The relative amounts of each probe observed in each trial on day 1, day 7, and day 14 can be found in Figures 4.19, 4.20, and 4.21.

Figure 4.18 Quantification of red fluorescence present in the images using the four-point ranking system.

(A) represents 1 ranking (B) represent 2 ranking (C) represents 3 (D) represents 4 ranking

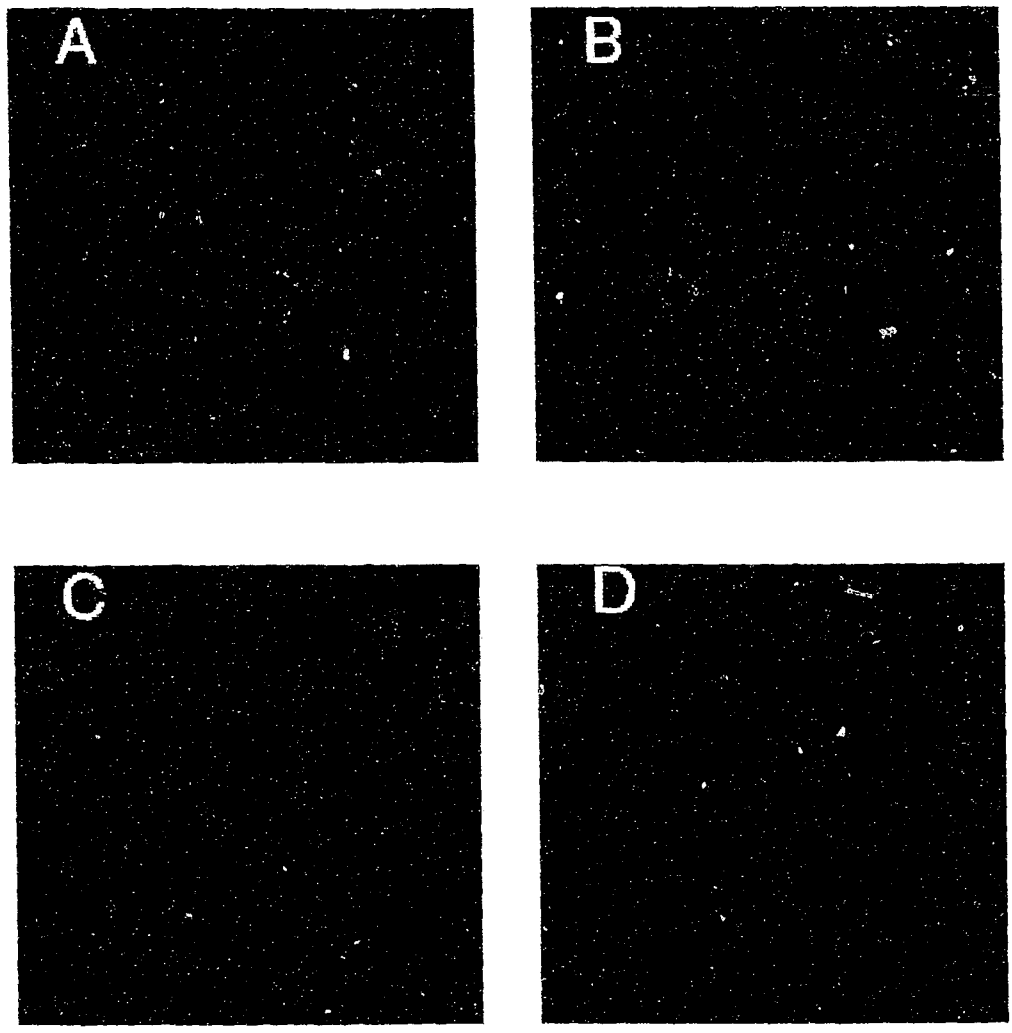


Figure 4.19 Average Relative Amount of Probe Based on Four-Point Ranking System for All Treatments of Batch Reactors on Day 1. ***Error bars=+/- SD***

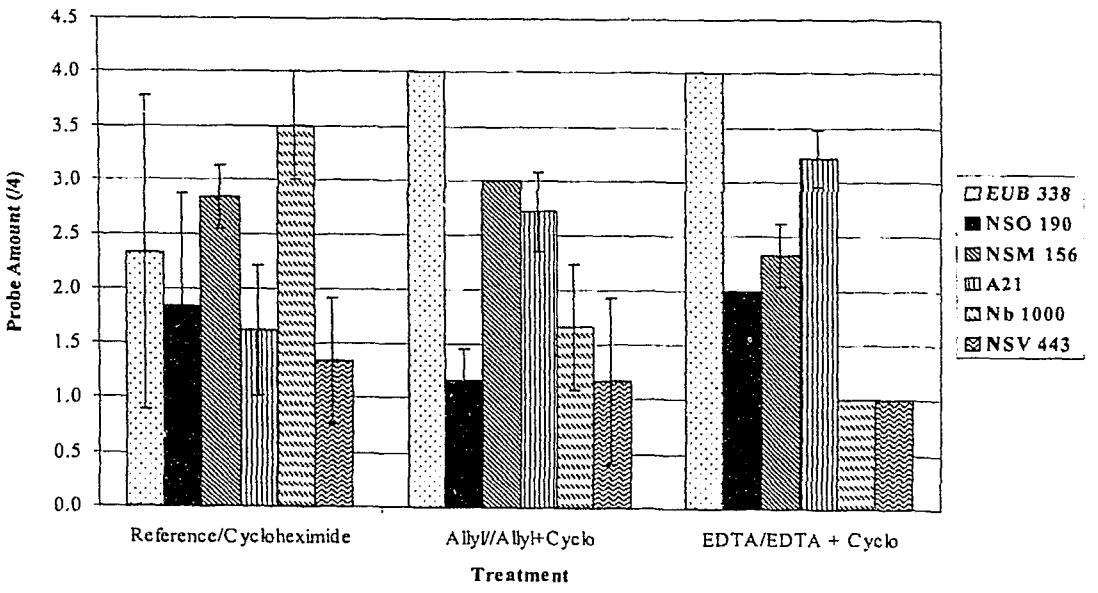


Figure 4.20 Average Relative Amount of Probe Based on Four-Point Ranking System for All Treatments of Batch Reactors on Day 7. ***Error bars=+/- SD***

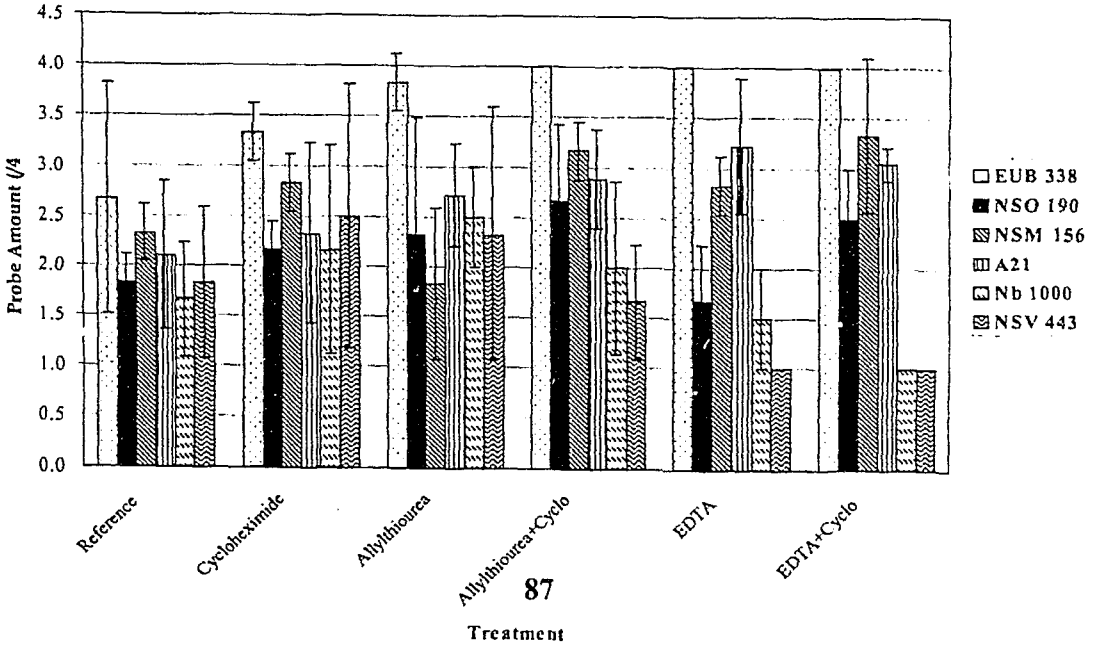
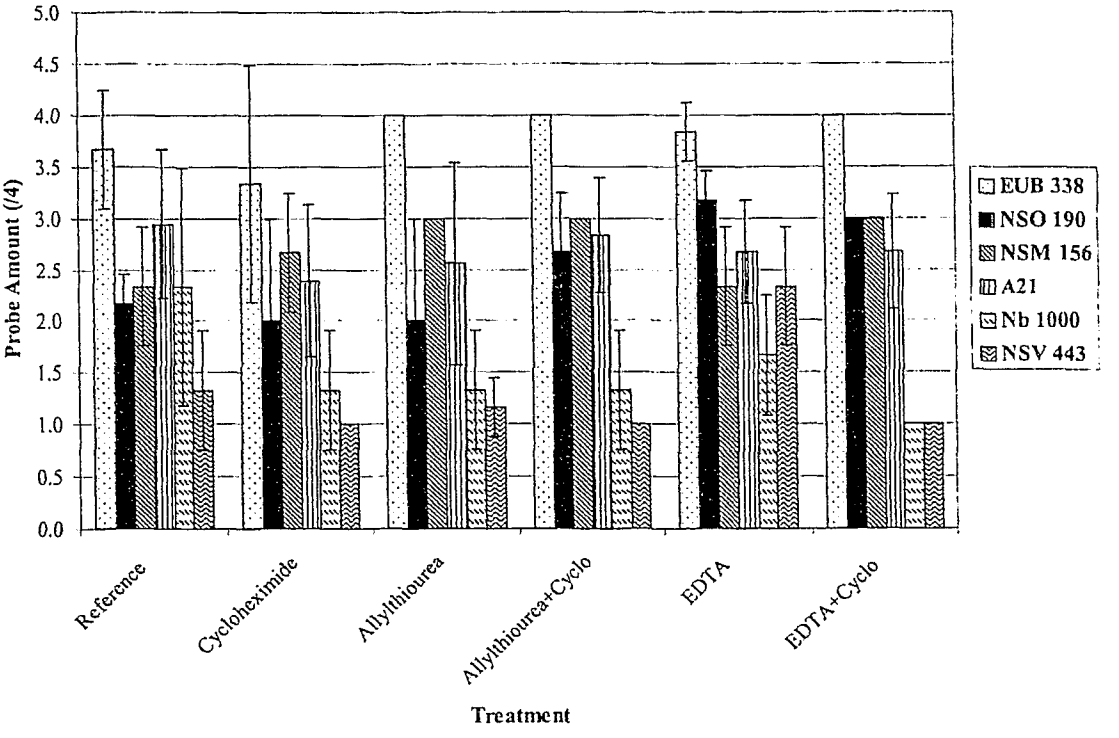


Figure 4.21 Average Relative Amount of Probe Based on Four-Point Ranking System for All Treatments of Batch Reactors on Day 14. ***Error bars=+/- SD***



EUB 338 was the most abundant probe in all trials on all days. This is to be expected since EUB 338 binds to most eubacterial cells. Overall, eubacteria were observed to be more abundant in the allylthiurea, allylthiourea + cycloheximide, EDTA, and EDTA + cycloheximide trials than in the reference and cycloheximide trials. However, the amount of eubacteria bound was highly variable, as can be seen by the large standard deviations shown on Figures 4.19, 4.20, and 4.21. *Betaproteobacterial* AOB, *Nitrosomonas* spp., and *Nitrosococcus mobilis* were moderately abundant, with *Nitrosomonas* spp. and *Nitrosococcus mobilis* often being present in greater relative abundance than *betaproteobacterial* AOB. *Nitrospira*-like NOB were the most abundant bacterial group observed for nitrite oxidizing bacteria. In almost every floc examined, they were more abundant than both *Nitrobacter* and *Nitrosospira* bacteria. The exception to this is flocs observed on day one for the reference/cycloheximide trials, where *Nitrobacter* was present in greater abundance than *Nitrospira*-like bacteria. The relative amounts of probes *Nitrobacter* and *Nitrosospira* were low (usually below a ranking value

of 2), with *Nitrosospira* usually being present in the least abundance of all bacterial species.

Overall, there was no noticeable difference in probe position or amount between the trials. The two probes used to detect ammonia oxidizing bacteria and the three probes used to detect nitrite oxidizing bacteria were both found in the greatest abundance positioned near the interior of the floc. As expected, eubacteria were observed in the greatest abundance of all the bacterial groups probed, used, and was also found distributed throughout the floc, although the maximum abundance did occur closer to the center of the floc. Of the AOBs, the *Nitrosomonas* spp. and *Nitrosococcus* mobilis were slightly more abundant than the *betaproteobacerial* AOB, although both were fairly abundant. All AOB probed were found concentrated mid-floc, but were present at other depths within the floc. For the nitrite oxidizing bacteria, there was a noticeable difference in the abundance of *Nitrospira*-like bacteria compared with *Nitrobacter* and *Nitrosospira* spp. , with *Nitrospira*-like bacteria being much more abundant than *Nitrobacter* and *Nitrosospira. Nitrospira*-like bacteria were also distributed at a number of depths throughout the floc, while *Nitrobacter* and *Nitrosospira* were usually only present at a single depth. When comparing the abundance of all probes used to detect AOBs to all probes used to detect NOBs, there did not appear to be a large difference in the relative abundance between these groups for any of the trials.

Figures 4.22 to 4.27 show examples of the images used for analysis and quantification.

Figures 4.22, 4.23, 4.24, and 4.25 show examples of each of the four probe combinations used in this study. It is apparent from Figure 4.22 that the eubacterial group was the most abundant group probed. It is also important to note the clustering nature of the nitrifying bacteria within the flocs.

Figures 4.26A, B, and C show the binding of probe EUB 338 + A21 to eubacteria and *Nitrospira*-like NOB on day 1, day 7, and day 14 of the allylthiourea trial. There was no noticeable difference in the position or abundance of probe binding between the sample days.

Figure 4.27 provides an example of a z-stacked floc containing eubacteria and *Nitrospira*-like NOB on day 14 of the allythiourea + cycloheximide trial. A z-stack

allows for a visual display of the floc at various depths. Probe EUB 338 (most bacteria) is present at all levels within the floc, while *Nitrospira*-like bacteria are only present at interior depths.

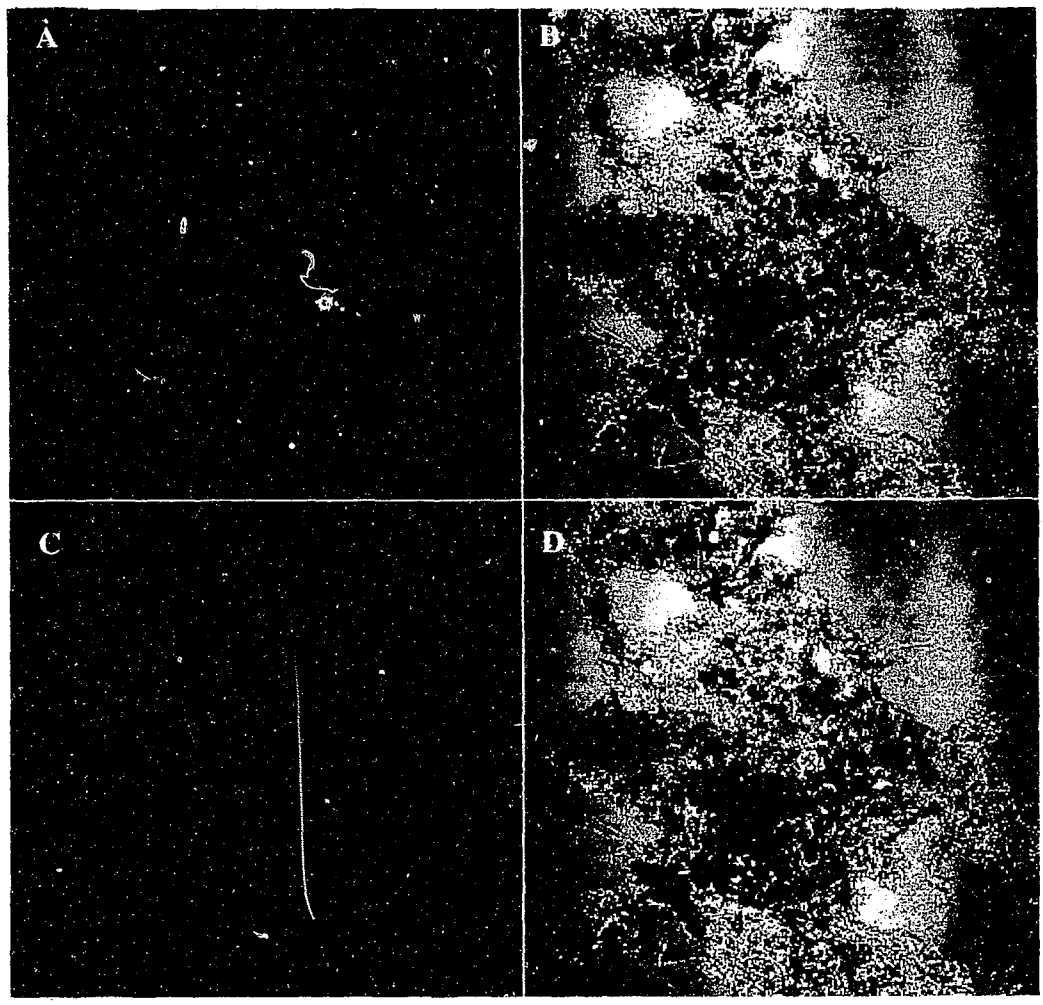


Figure 4.22 Binding of probes EUB 338 and A21 on day 7 of the Allylthiourea + Cycloheximide treatment.

(A) Green fluorescence indicates A21 binding (B) transmitted light component (C) Red fluorescence indicates EUB 338 binding (D) All components combined in one image. Binding shown at a depth of 11.1 microns deep in a floc of total depth 22.2 microns

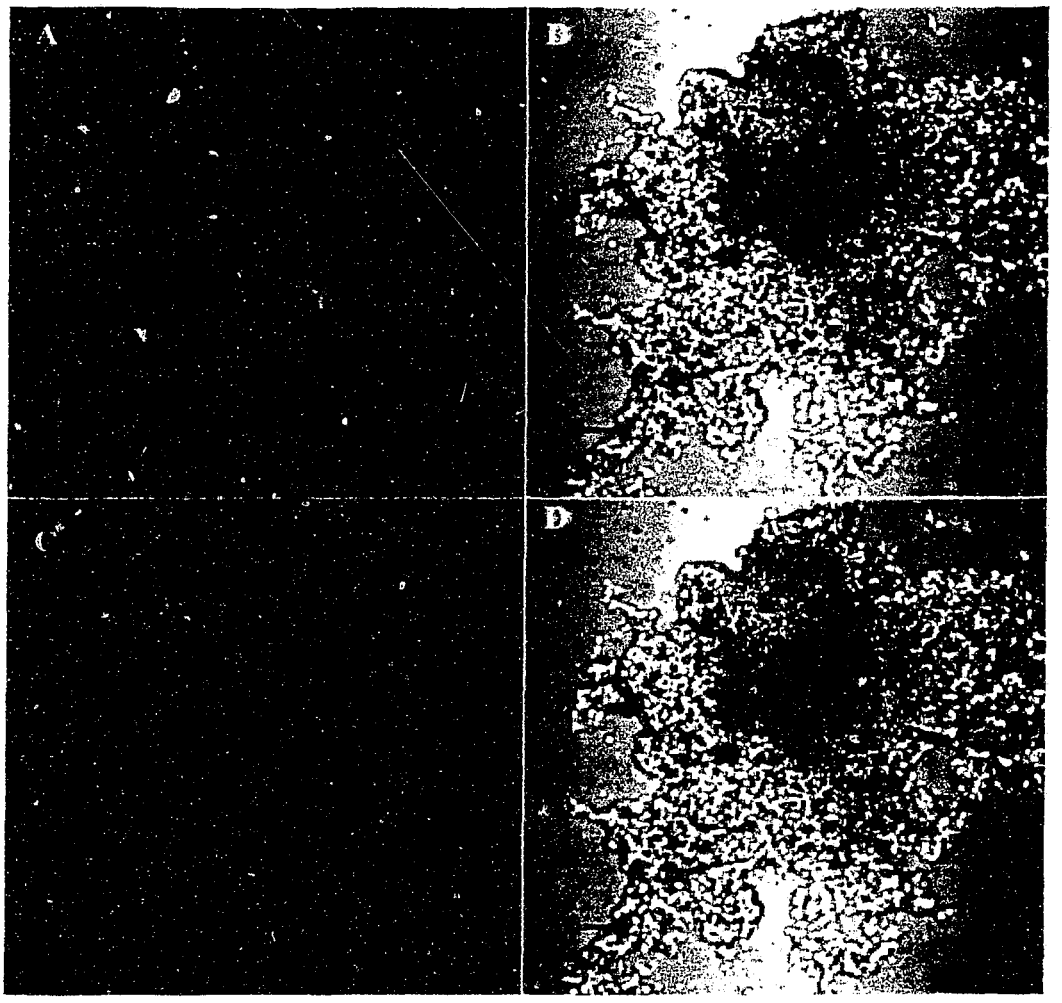


Figure 4.23 Binding of probes A21 and Nb1000 on Day 7 of the EDTA + Cycloheximide treatment.

(A) Red fluorescence indicates Nb1000 binding (B) transmitted light component (C) Green fluorescence indicates A21 binding (D) All components combined in one image. Binding shown at a depth of 13.52 microns in a total floc depth of 27.02 microns.

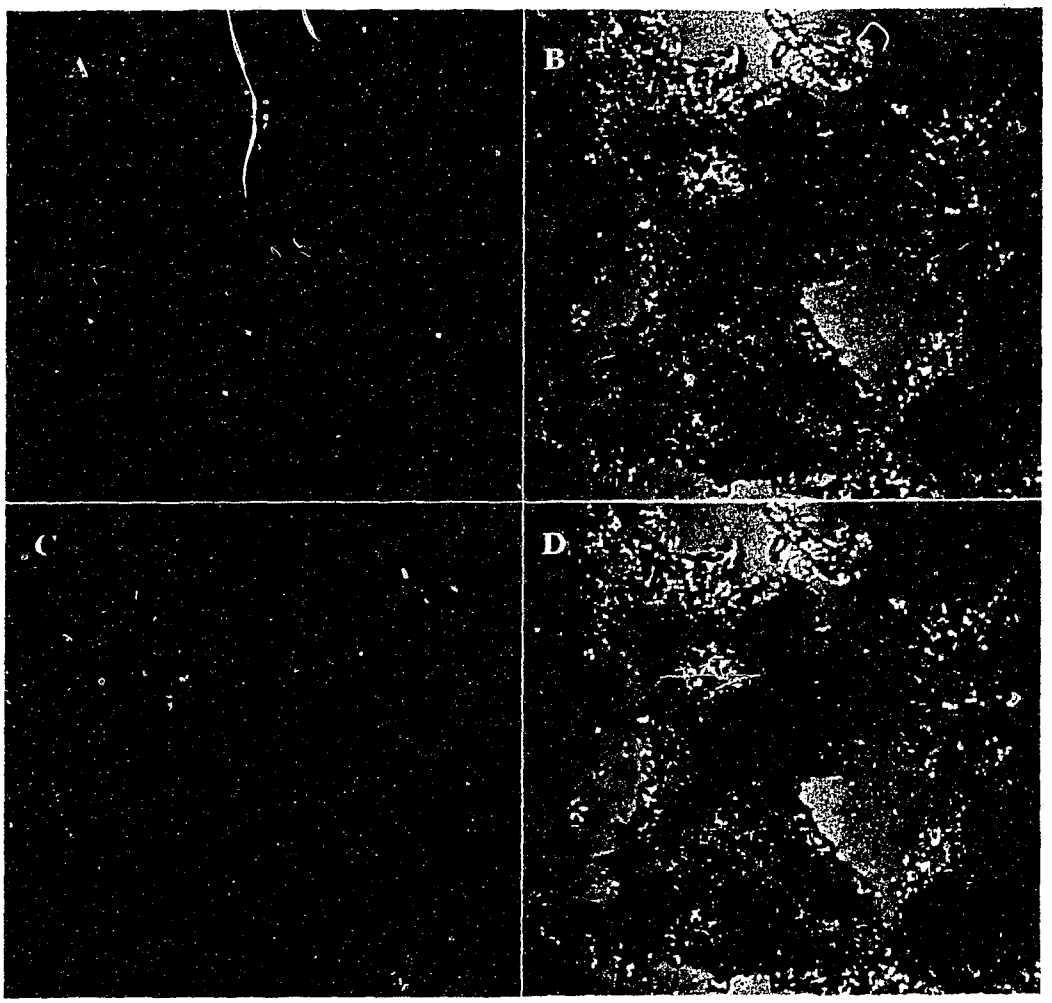


Figure 4.24 Binding of probes A21 and NSV 443 on Day 7 of the reference treatment.

(A) Red fluorescence indicates NSV 443 binding (B) transmitted light component (C) Green fluorescence indicates A21 binding (D) All components combined in one image. Binding shown at a depth of 20.8 microns in a total floc depth of 32.35 microns.

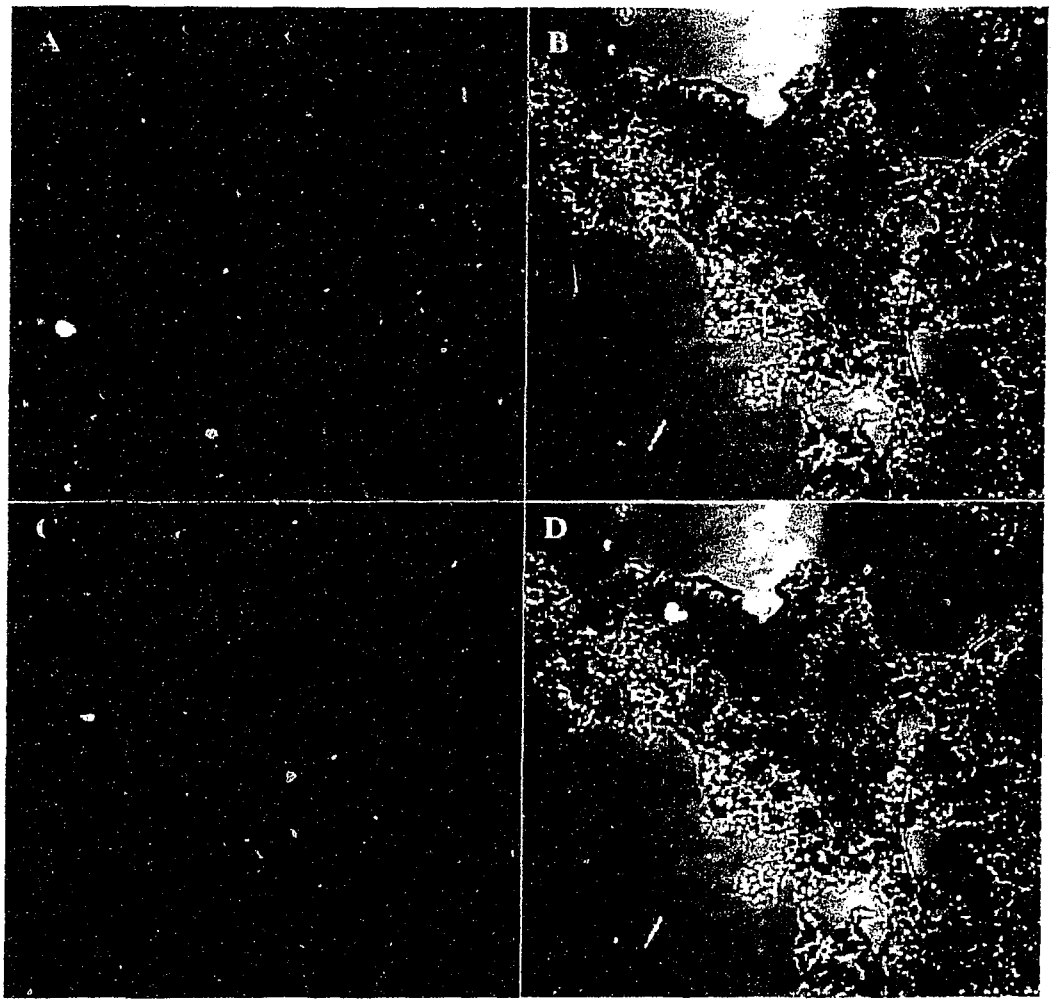


Figure 4.25. Binding of probes NSO 190 and NSM 156 on Day 7 of the allylthiourea treatment.

(A) Red fluorescence indicates NSM 156 binding (B) transmitted light component (C) Green fluorescence indicates NSO 190 binding (D) All components combined in one image. Binding shown at a depth of 10.6 microns in a total floc depth of 21.2 microns.

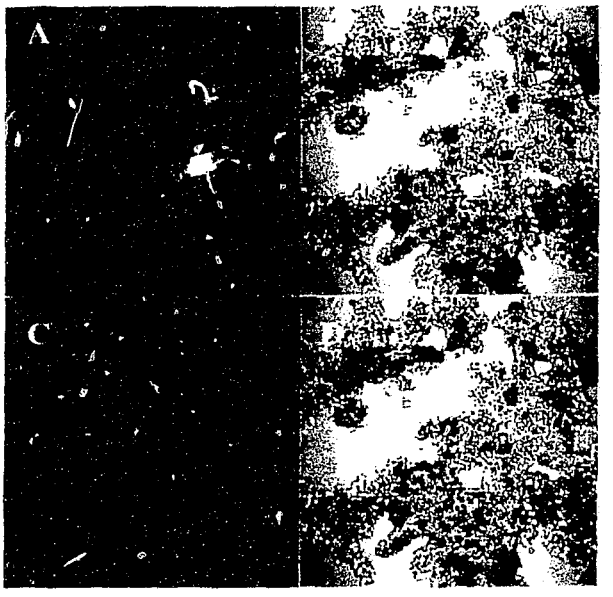


Figure 4.26A. Binding of probes EUB 338 and A21 on Day 1 of Allylthiourea treatment. Binding is shown at a depth of 10.45 in a total floc depth of 20.9 microns. (A) Green fluorescence indicates A21 binding (B) transmitted light component (C) Red fluorescence indicates EUB 338 binding (D) All components combined in one image.

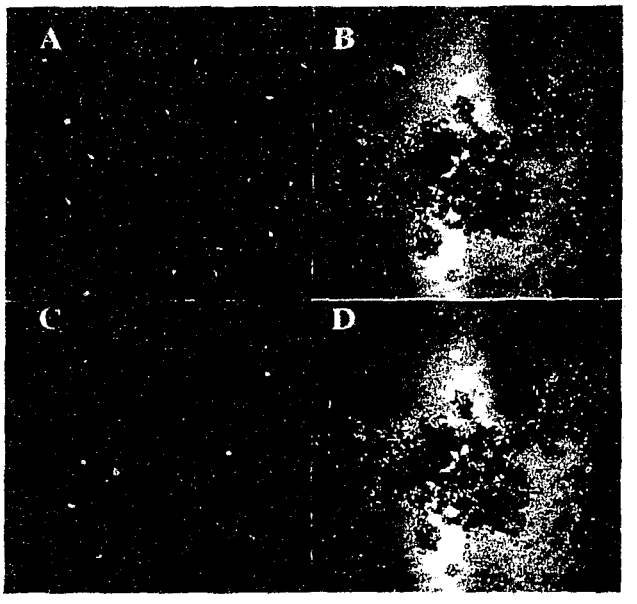


Figure 4.26 B Binding of probes EUB 338 and A21 on Day 7 of Allylthiourea treatment. Binding is shown at a depth of 9.57 microns in a total floc depth of 19.15 microns. (A,B,C,D same as for 4.26A)

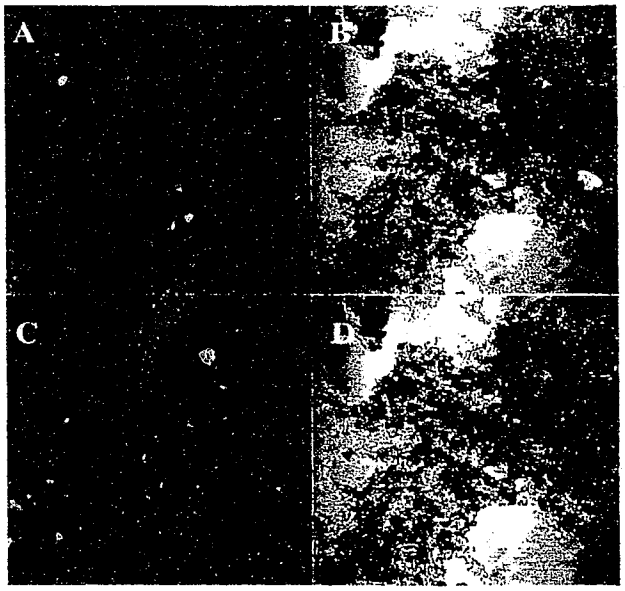


Figure 4.26C Binding of probes EUB 338 and A21 on Day 14 of Allylthiourea Treatment.

Binding is shown at a depth of 13.65 microns in a total floc depth of 27.30 microns. (A) Green fluorescence indicates A21 binding (B) transmitted light component (C) Red fluorescence indicates EUB 338 binding (D) All components combined in one image.

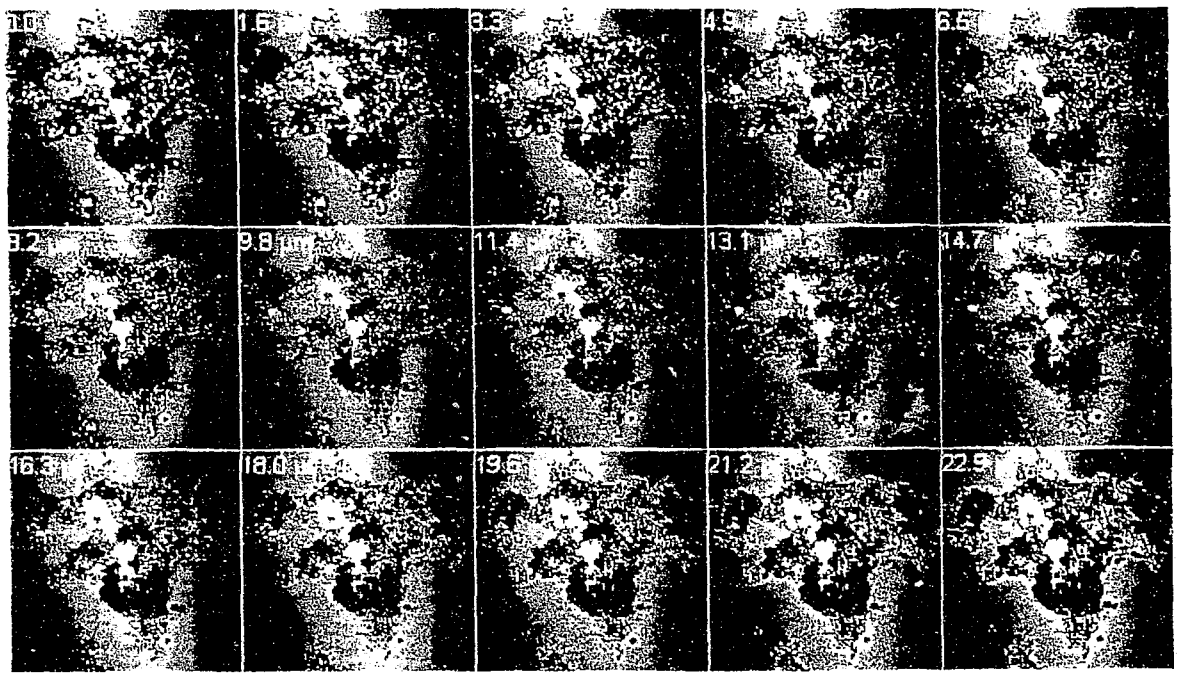


Figure 4.27 Example of a z-stacked Floc Showing Binding of Probes EUB 338 and A21 on day 14 of the Allylthiourea + Cycloheximide Treatment

Red binding indicates EUB 338 binding, green binding indicates A21 binding, yellow binding indicates binding of both probes. EUB 338 is present at all depths of the floc. The majority of the A21 binding is located in the interior of the flocs (stacks 9.8-13.1 microns).

5.0 DISCUSSION

5.1 The Role of Protozoa in Activated Sludge from Ashbridges Bay WWTP

It is generally agreed in the literature that protozoa exert an influence on the mineralization of organic compounds in three main ways:

1) By the excretion of mineral nutrients such as ammonia which results in an accelerated use of carbon-sources by bacteria (Strauss and Dodds, 1997; Verhagen and Laanbroek, 1992)

2) By the excretion of protozoa of growth-stimulating compounds that enhance bacterial activity (Nisbet, 1984).

3) By protozoan grazing on bacteria that may lead to the selection of certain fast growing bacterial species depending on the environmental conditions present (Sinclair and Alexander, 1989).

Several studies have attempted to directly link protozoan predation to nitrification in activated sludge systems. The outcome of these studies have suggested that the presence of protozoan grazing actually enhances nitrification rates in activated sludge systems (Strauss and Dodds, 1997; Clarholm, 1985; Verhagen and Laanbroek, 1992; Verhagen and Laanbroek, 1991). A very recent study by Petropoulos (2003) attempted to directly link the presence or absence of protozoa to nitrification within the activated sludge system by directly inhibiting protozoa activity and monitoring activated sludge parameters. Petropoulos (2003) found that the efficiency of nitrification increased in the presence of protozoan grazing and decreased in the absence of protozoa. Petropoulos' study also utilized activated sludge from Ashbridges Bay WWTP, and a protozoan species survey was carried out on sludge samples. The outcome of the protozoan species survey performed by Petropoulos is shown in Table 5.1.

Table 5.1 The total percentage abundances of protozoan species based on the 5% total dry-weight of the activated sludge system at the Ashbridges Bay Treatment Plant in Toronto (Petropoulos, 2003).

Protozoan Species	Abundance (%)
Amoeba	0.05
Ciliates	3.70
Flagellates	0.60
Rhizopods	0.65

This community composition is typical of the protozoan community existing in activated sludge environments (Al-Shawhani and Horan, 1991; Curds, 1982). Petropoulos (2003) also enumerated protozoa found in sludge from the Ashbridges Bay WWTP, and typical protozoan numbers were found to be approximately 9.50 X 10^6 cells/mL. The current study did not undertake a protozoan survey or count of the activated sludge from Ashbridges Bay WWTP. However, it can be assumed that the protozoan community composition and the protozoan abundance of the activated sludge used in this study is very similar to the community composition and abundance determined by Petropoulos (2003).

An idea of the role of protozoa in nutrient cycling in the activated sludge system as well as a knowledge of the protozoan community composition and abundance is important in order to assess the influence of protozoa on nitrification rates under the different conditions examined in this study.

5.2 Performance of the Batch Reactors

The batch system is a very popular system used to study interactions within the activated sludge environment for a variety of reasons. Batch reactors have short life spans, and no feed system needs to be implemented. The batch system is very short-lived (i.e. one to two weeks). If nutrients are not replaced, the system will eventually break down and the

biological community will be eliminated. In this study, no nutrient replacement fluid was used. This is because in order to track the change in parameters brought about by the different conditions implemented in this trial, nutrient replacement could not be performed. However, parameters such as temperature, pH, dissolved oxygen, and COD were monitored throughout the trial to ensure that the system was still operating and system break-down had not occurred.

Batch reactors were modified by inhibiting protozoan activity through the use of cycloheximide, inhibiting ammonia oxidation through the used of allylthiourea, and inducing deflocculation through the use of EDTA. The partial or complete inhibition of nitrification is a common and extremely serious problem in WWTPs. By several pathways (e.g. nondegradeable residues in sewage treatment plant, residues in manure or sludge used as fertilizer on fields or from surplus drugs in aquaculture) antibacterial agents that inhibit nitrification may reach the environment (Halling-Sorenson, 2001). Deflocculation is also a periodic problem in the activated sludge process and can be brought on by low pH, temperature shock, and by the sporadic input of toxic chemicals from industrial and commercial discharges (Bott and Love, 2002; Liao et al., 2002). The monitoring of the batch reactors under the various conditions in this trial was undertaken in order to assess how protozoan presence affects nitrification under each condition.

5.2.1 Temperature, pH, and Dissolved Oxygen

The general performance of the batch reactors was assessed by measurement of pH, temperature, dissolved oxygen, and chemical oxygen demand. Since the activated sludge process is a biotic process and relies heavily on the existence of healthy microbial communities, the maintenance of parameters within ranges that are conducive to microbial growth is essential. The pH, temperature, and dissolved oxygen content of batch reactors must be carefully monitored as the fluctuation of these parameters from normal values is indicative of a breakdown of the reactor ecosytem (Curds, 1972; Curds, 1974; Stout 1980).

The minimum temperature observed for all trials was 23.6°C and the maximum observed temperature for all trials was 26.4°C (Figure 4.1). Temperatures did not fall out of this range for any of the trials. The temperature of the batch reactors fluctuated with the

ambient temperature in the laboratory in which the experiments were performed. The vast majority of biological treatment systems operate within the temperature range of 20-40°C (Viessman and Hammer, 1998). Nitrifiers, and thus nitrification, are very sensitive to temperature. Nitrifying bacteria are particularly sensitive to low temperature (below 5°C), which leads to decreased growth and nitrification rates. Since batch temperatures did not fall below 23.6°C and did not rise above 26.4°C, temperature was not likely a limiting factor for microbial activity and nitrification.

In the reference, the cycloheximide, the EDTA, and the EDTA + cycloheximide trials the pH dropped from pH values commonly found in activated sludge systems (between pH 6.5 and pH 8.5), to acidic pH values by day 14. Since no volume replacement liquid was used in the batch reactors, a drop in pH as nitrification occurs is expected. For every milligram (mg) of nitrogen nitrified, 7.2 mg of alkalinity is consumed (Blackall and Burrell, 1999). The drop in pH is especially pronounced in water with high inputs of ammonium, such as wastewater, where higher rates of ammonia oxidation occur and the pH is lowered more rapidly (Princic et al., 1998). Up to and including day 10, the pH of the batch reactors had not dropped below a pH of 6.5 (Figure 4.2). However, from day 10 to day 14, a more rapid drop in pH was observed. Several studies have shown that nitrification is severely retarded in low pH environments, and nitrification does not occur at all below a pH of 6.5 (Blackall and Burrell, 1998; Princic et al., 1998; Viessman and Hammer, 1998). The pH range for growth of pure cultures of ammonia oxidizers is 5.8 to 8.5, and the pH range for the growth of nitrite oxidizers is 6.5 to 8.5 (Princic et al., 1998). Thus, after day 10 in the reference, cycloheximide, and allylthiourea trials it can be postulated that nitrification was not occurring at normal rates, since the nitrite oxidation step would be completely inhibited during this time. The rapid drop in pH after day 10 can be attributed to other decomposition processes, and also to the possible accumulation of nitrous acid in the system. Nitrous acid production is a function of pH and nitrite concentration. If nitrite oxidation is inhibited, then nitrite will accumulate in the system. If this occurs under low pH conditions, there is a tendency for nitrous acid to be produced, which further lowers the pH of the system (Garrido et al., 1998; Muller et al., 1998).

Interestingly, in the allylthiourea trials the pH actually increased throughout the trial, from a pH of 7.49 on day 1 to a pH of 8.87 and 8.60 in the allylthiourea and allylthiourea + cycloheximide trials, respectively (Figure 4.2). Since allylthiourea is an inhibitor of ammonia oxidation, its presence likely resulted in the build-up of ammonia in the system. When ammonia dissolves in water, a portion reacts with the water to form ammonium ions (NH_4^+) with the balance remaining as un-ionized ammonia (NH_3). The presence of NH_4^+ contributes to the alkalinity of the water, which explains why no drop in pH was observed in the reactors treated with allylthiourea. In addition to the direct effect on pH through nitrogen build-up, the presence of allylthiourea inhibits ammonia oxidation, and hence the entire nitrification process. Thus, the associated build-up of acidity associated with the nitrification process did not occur in the allylthiourea trials, and the pH did not drop as in the reactors not treated with allylthiourea. The pH in the allylthiourea trials never rose above a pH of 10, which is the threshold value for complete inhibition on nitrification (Princic et al., 1998; Blackall and Burrell, 1999).

Dissolved oxygen was monitored throughout the reference, cycloheximide, EDTA, and EDTA + cycloheximide trials (Figure 4.3). For the reference and cycloheximide trials, dissolved oxygen levels in the mixed liquor remained at approximately 8 mgO_2/L throughout the trial. In the EDTA and EDTA + cycloheximide trials, dissolved oxygen was slightly higher on day 1 and day 4 but then dropped down to 8 mg O_2/L on day 7, and remained at this level for the remainder of the trial. Nitrification is largely dependant on the oxygen available to nitrifying bacteria. It is important to note that the dissolved oxygen levels measured in mixed liquor (as measured in this study) are not necessarily the dissolved oxygen levels available to nitrifying bacteria within biological flocs. Dissolved oxygen levels directly available to nitrifiers below 0.3 mg O_2/L are known to inhibit nitrification, while at 1.0 mgO_2/L the nitrification rate is 90% of that observed in cells directly suspended in oxygen saturated water (Blackall and Burrell, 1999). Dissolved oxygen contents in mixed-liquor below 2 mg O_2/L are known to inhibit nitrification apparently because at below a mixed liquor dissolved oxygen of 2 mg O_2/L, dissolved oxygen available to nitrifiers within flocs is too low to allow for nitrification (i.e. below 0.3 mg O_2/L) (Princic et al., 1998). Since the mixed liquor dissolved oxygen

content in this study was well above 2 mg O_2/L, it can be assumed that adequate oxygen was available for nitrifying bacteria within the sludge floc.

5.2.2 The Monitoring of COD, Ammonia, Nitrite, Nitrate, and Total Nitrogen to Assess the Effect of Protozoan Grazing on Bacterially Mediated Nitrogen Cycling

One of the primary aims of this study was to determine if the presence or absence of protozoan grazing affected nitrification under different conditions. Monitoring NH_3 and NH_4^+ consumption rates or NO_2^- and NO_3^- production rates in activated sludge is one of the most popular ways to measure the nitrification activity of activated sludge samples (Gernaey et al., 1998). In this study, regular sampling and chemical analyses of the samples for ammonia, nitrite, nitrate, and total nitrogen was performed in order to attempt to measure nitrification activity. COD was also monitored in order to ensure that the organic material in the batch reactors was not completely depleted throughout the trial.

Chemical oxygen demand is the amount of oxygen necessary to oxidize all of the organic carbon completely to CO_2 and H_2O. Normally, the ratio of COD to BOD (biological oxgyen demand) is approximately 0.5. For wastewaters composed mainly of biodegradeable organic substances, the COD concentration approximates the ultimate carbonaceous BOD value, which is the plateau reached by the carbonaceous BOD due to depletion of the carbon source (Maier et al., 1999). In essence, the COD is a measure of the organic content of the wastewater. Typical COD values vary with the type of wastewater being processed and the seasonal or diurnal variation in wastewater composition. Data collected during 2004 from the Burlington Water Purification Plant shows that influent COD concentrations over a five month period from January to May fluctuated from as low as 100 mgO_2/L to as high as 600 mgO_2/L with an average of approximately 250 mg O_2/L. Effluent COD for the same treatment plant during the same period fluctuated between a maximum of 38 mgO_2/L and a minimum of 15 mg O_2/L, with an average value of approximately 25 mg O_2/L. In this study, the sewage sample came from the activated sludge aeration tank at the Ashbridges Bay WWTP. Thus, COD values are expected to be lower than typical influent COD concentrations. COD was measured on day 1, 4, 7, 10, and 14 in all trials (Figure 4.4). As organic matter in the activated sludge is oxidized, a decrease in COD is expected, and in fact this was the case

in the reference trial where the COD decreased from 120 mg/L on day 1 to 60 mg/L on day 10. This was also the case in both the allylthiourea and allylthiourea + cycloheximide where the COD decreased as expected over the 10-day period. In the cycloheximide trial, the COD was observed to increase over the 10-day period. The same phenomenon was observed in both the EDTA and the EDTA + cycloheximide trials. In the EDTA trial, COD increased only very slightly from 165 mg O_2/L on day 1 to 196 mg O_2/L on day 10. However in the EDTA + cycloheximide trial, the largest increase in COD was observed (from 165 mg/L on day 1 to 290 mg/L on day 10). An increase in the COD value in the EDTA trials can be explained by the deflocculating properties of EDTA. EDTA is a strong chelating agent that deflocculates activated sludge by breaking the salt bridges linking flocs in concentrations greater than 100 mg/L (Liao et al., 2002). Since the measurement of COD is a colourimetric method, activated sludge samples are filtered prior to sample digestion. Thus, a portion of organic material contained in flocs is removed in the filtering procedure. When EDTA is added to sludge samples, floc structure is disrupted, and organic nutrients contained within the floc structure are released to the surrounding environment. Thus, the concentration of organic matter available for biological degradation increases. It appears that the addition of cycloheximide also affected the COD values. In all trials containing cycloheximide, COD values sharply increased on day 4, followed by a subsequent decrease by day 7. Cycloheximide is a well known and widely used inhibitor of protozoan metabolic activity at concentrations of 100 mg/L (Tremaine and Mills, 1987; Kota et al., 1999; DeLorenzo et al., 2001). Cycloheximide could potentially increase the COD in two ways. The addition of cycloheximide inhibits protozoan metabolism and protozoa cease to feed, and eventually die. The build-up of dead protozoan cells and their eventual decay could potentially increase the chemical oxygen demand of the reactors. Another way that cycloheximide could increase the COD is by directly contributing to it since cycloheximide is an organic compound. However, this is unlikely since cycloheximide has been observed to be stable in aquatic environments for up to 30-days (DeLorenzo et al., 2001), and thus is presumably not readily biodegradable. An increase in COD in the absence of protozoan grazing was also observed by Curd et al. (1968).

Ammonia, nitrite, nitrate, and total nitrogen levels were measured on day 1, day 4, day 7, and day 10 in all trials using the phenate method (Figure 4.5-4.8). The purpose of measuring parameter concentrations was to calculate the rates of production for each of these over the 10-day period for each trial (Figure 4.9-4.12).

Ammonia concentrations in influent wastewater also vary seasonally and diurnally, but the typical influent (untreated) ammonia concentration in municipal wastewater is upwards of 25 mg/L (Viessman and Hammer, 1998). In the reference trial, the rate of ammonia production was positive, indicating a net production of ammonia throughout the 10-day period. The process of nitrification converts ammonia to nitrate, so in a nitrifying system one would expect the rate of ammonia production to be negative (i.e. a consumption of ammonia). However, the rate of ammonia production observed in the reference trial was only very slightly positive (0.44 mg NH_3/L/day). Also it must be remembered that other processes besides nitrification are occurring in the system. One major process that increases the ammonia concentration in biological systems is ammonification, the decomposition of organic nitrogen compounds to ammonia performed by many microoorganisms in activated sludge (Madigan et al., 2000). Thus, even in systems where nitrification is occurring, a positive ammonia production rate may often be observed. In the cycloheximide trial where protozoan grazing was inhibited, the rate of ammonia production was still positive (0.26 mg NH_3/L/day), but was smaller than in the reference trial, indicating that less ammonia was being produced, although the difference was not statistically significant. The production of less ammonia in the absence of protozoan grazing can be expected since in the absence of protozoan grazing fast-growing heterotrophic bacteria proliferate (Curds, 1982; Verhagen and Laanbroek, 1991; Verhagen and Laanbroek, 1992; Petropoulos, 2003). These fast-growing heterotrophic bacteria outcompete nitrifying bacteria for ammonia (Hanaki et al., 1990), and nitrification decreases. The assimilation of ammonia by heterotrophs has been observed to occur in preference to nitrification when ammonia is abundant and heterotrophic population densities are high (Hanaki et al., 1990). Whether the overall abundance of nitrifying bacteria decrease or the per-cell nitrification rate decreases is still not clear. Since ammonification is presumably still occurring, some ammonia is still being produced, but the overall rate of ammonia production decreases due to the decrease

in nitrification. The absence of protozoan grazing could also decrease the ammonia production rate because protozoa are no longer excreting ammonia as a by-product of metabolic activity. In the trials in which ammonia oxidation was inhibited in the presence of protozoan grazing the rates of ammonia production were significantly higher than in the reference trial (2.37 and 2.60 mg NH_3/L/day in the allylthiourea and allylthiourea + cycloheximide trials, respectively). This indicates that allylthiourea had a significant effect on nitrification. The rate of ammonia production in the allylthiourea + cycloheximide trial was slightly higher than that of the allylthiourea trial, but this difference was not significant. Allylthiourea is a widely used selective inhibitor of ammonia oxidation by *Nitrosomonas* at a concentration of 5 mg/L (Wood et al., 1981; Surmacz-Gorska et al., 1995; Ginestet et al., 1998). Allylthiourea operates to halt the oxidation of ammonia to nitrite by chelating the copper of the ammonia monooxygenase (AMO) active site (Ginestet et al., 1998). AMO is the enzyme that catalyzes the conversion of ammonia to nitrite, and once it is rendered inactive this conversion does not occur at all. Thus, in the trials containing allylthiourea, no ammonia oxidation was occurring, and this resulted in a subsequent build-up of ammonia in the system. In the trials containing both allylthiourea and cycloheximide, it was expected that the population of heterotrophic bacteria would proliferate due to lack of grazing. As in the trial containing allylthiourea only, nitrification is not occurring, thus ammonia is building up. However, due to the proliferation of heterotrophic bacteria, the assimilation of ammonia by heterotrophs should also increase. Along with this factor, there are also no protozoa present to excrete ammonia. Thus, it was expected that the rate of ammonia production would be lower in the allylthiourea + cycloheximide trial . This was not observed, and the rate of ammonia production was in fact slightly higher in the allylthiourea + cycloheximide trial as compared with the allylthiourea trial, although this difference was not significant. The lowest rates of ammonia production occurred in the EDTA and EDTA + cycloheximide trials (-0.37 and –0.71 mg NH_3/L, respectively). A negative ammonia production rate indicates that ammonia was being consumed throughout the 10-day trial. However, the difference in ammonia production rate from the reference was only significant in the EDTA + cycloheximide trial. In the presence of EDTA, it was expected that bacteria would deflocculate, and also that nutrients trapped

within the floc structure would be released. Since ammonia levels in EDTA trials decreased, it can be postulated that both nitrifying and heterotrophic bacteria were released from the floc structure, and the availability of the ammonia to these bacteria increased. Thus, the rates of nitrification and also heterotrophic ammonia assimilation both increased. When EDTA and cycloheximide were present, ammonia consumption rates were greater than in the presence of protozoan grazing. If protozoan grazing controls the populations of heterotrophic bacteria, we would expect that in the absence of protozoan grazing that nitrification rates would decrease, but that the assimilation of ammonia by heterotrophs may increase. It is important to remember that in the trials containing EDTA, it is possible that nitrifying bacteria were not in the floc structure, and nitrifiers and heterotrophs were likely equally grazed upon by protozoa. Since nitrifiers are slow growing compared to heterotrophs, in suspended systems where both bacteria are readily available as prey, grazing by protoza tends to eliminate slow-growing nitrifying bacteria (Sinclair and Alexander 1989; Verhagen and Laanbroek, 1992). Thus, when protozoan grazing is eliminated, it is likely that in the EDTA trials more nitrification occurs, rather than less. Thus, it seems reasonable that the rate of ammonia consumption was the greatest in the presence of EDTA and cycloheximide.

Nitrite is an intermediate in the nitrification process, and does not usually accumulate in wastewater since the nitrite oxidation step of nitrification is faster than the ammonia oxidation step (Keller and Blackall, 2002).). Still, in systems in which nitrification is occurring, there should be a net production of nitrite at any one instant in time, although depending on the amount of nitrification occurring and the types of bacteria present this may not always be the case (Gernaey et al., 1998; Keller and Blackall, 2002). Nitrate is the final product of nitrification, and when nitrification is occurring a net production nitrate is expected and nitrate will accumulate (in the absence of denitrification). Both nitrate and nitrite are present in influent wastewaters, but often only in very low concentrations (1-3 mg/L) (Viessman and Hammer, 1998). In the reference trial, a very small positive nitrite production rate of 0.05 mg NO_2/L/day was observed. This rate of nitrite production was significantly larger than any of the other trials. The rate of nitrate production in the reference trial was also positive (0.08 mgNO_3/L/day) and significantly larger than all of the trials except for the EDTA trials. This positive production values

for nitrite and nitrite indicate that nitrification was occurring in the reference trials. In the cycloheximide trial, the rate of nitrite production was significantly smaller than in the reference trial (0.001mg NO_2/L), as was the rate of nitrate production (-0.008 mgNO_3/L). This suggests that less nitrification was occurring in these trials, and concurs with the theory that the presence of protozoan grazing enhances nitrification (Clarholm, 1985; Verhagen and Laanbroek, 1992; Strauss and Dodds, 1997; Petropoulous, 2003). The rate of nitrite production in the allylthiourea and allylthiourea + cycloheximide trials were negative (-0.06 and –0.07 mg NO_2^-/L respectively), and significantly different from the nitrite production rate in the reference. The rates of nitrate production in these trials were also negative (-0.03 and –0.05 mg NO_3/L) and significantly different from the reference. The rate of nitrite and nitrate production in the allylthiourea + cycloheximide trial was significantly more negative than in allylthiourea trials, indicating that in the absence of protozoan grazing nitrite and nitrate consumption is higher. Since allylthiourea inhibits ammonia oxidation, very little nitrification was expected to occur in these trials and the negative production rates for nitrite and nitrate observed in these trials indicate that this was the case. The fact that production rates for nitrite and nitrate were negative may suggest that denitrification may have been occurring. Generally, denitrification requires anoxic conditions but in suspended growth systems such as activated sludge the possibility exists of both aerobic layers and anoxic layers situated next to each other in the floc and thus allowing for both nitrification and denitrification (Blackall and Burrell, 1999). The case for the occurrence of denitrification in the allylthiourea trials is also supported by the increasing pH observed in this trial as compared to the other trials. For every milligram of nitrogen denitrified, 3.6 mg of alkalinity is produced (Blackall and Burrell, 1999), so denitrification contributes to an increase in pH. The increased nitrite and nitrate consumption in the allylthiourea + cycloheximide trials may be explained by the possible increased growth of denitrifying bacteria in the absence or protozoan grazing. Another explanation is that the increased growth of heterotrophic bacteria brought on by the absence of protozoan grazing increased the occurrence of a process called assimililatory nitrate reduction, in which nitrate is taken up by bacterial cells (Maier et al., 2000). In the trials in which deflocculation was induced, the rate of nitrite production was negative (i.e. nitrite was being consumed). The rate of nitrite

consumption in the EDTA trials was greatest of all the trials and was significantly different from the reference (-0.33 and –0.27 mg NO_2/L in the EDTA and EDTA + cycloheximide, respectively). Interestingly, the rate of nitrate production in the EDTA and EDTA + cycloheximide trial was significantly higher than in any of the other trials. The rate of nitrate production was significantly higher in the EDTA trial than in the EDTA + cycloheximide trials. Thus, it appears that nitrification was occurring in the EDTA trials, and since the rate of nitrate production was greater in presence of protozoan grazing, this indicates that more nitrification was occurring in the presence of protozoan grazing, despite the deflocculating properties of EDTA. The high rates of nitrite consumption in the EDTA and EDTA + cycloheximide trial may be explained by a greater rate of nitrification at the end of the trial as compared to the beginning. This would cause a decrease in nitrite concentrations at the end of the trial as compared to the beginning.

The total nitrogen is the combined amount of all forms of nitrogen in the wastewater (ammonia, nitrite, nitrate, and organic nitrogen). The typical total nitrogen concentration in influent sewage is about 40 mg TN/L. After secondary treatment this value is usually reduced by about 50% (Viessman and Hammer, 1998). In all trials, the rate of TN production was positive, indicating a net build-up of nitrogen throughout the 10-day period. The only way that total nitrogen could decrease in a closed batch reactor is if a large amount of denitrification was occurring and nitrogen gas was being emitted. Since the batch reactor represents a closed system, an increase in total nitrogen concentrations can only be possible if more organic nitrogen was produced through increased cell growth, or if organisms that can fix nitrogen were present.

In summary, in the reference trial, the rate of ammonia production was small but positive, the rate of nitrite production was positive, indicating that nitrification was likely occurring. In the trial in which protozoan grazing was inhibited, the rate of nitrification as indicated by the ammonia, nitrite, and nitrate production rates was less than in the reference trials. In the trial in which ammonia oxidation was inhibited, no nitrification occurred, and ammonia build-up was observed. It was also found that nitrate was consumed throughout the trial, indicating the denitrification was occurring. In the trial in which ammonia oxidation and protozoan inhibition occurred, the rate of nitrate

consumption was significantly lower. may indicate that in the absence of protozoan grazing denitrifying bacteria proliferate. t also may indicate that in the absence of protozoan grazing certain species of bacteria capable of assimilatory nitrate reduction increased in numbers. The trial in which deflocculation was induced in the presence of protozoa, the rate of nitrate production was significantly higher than in the other trials. This could have been due to the increased substrate availability to deflocculated bacteria. The rate of ammonia consumption was also significantly lower than in the reference. This indicates that the rate of nitrification was likely high. In the trial in which deflocculation was induced in the absence of protozoa, the rate of nitrate production was still significantly higher than in the reference, but significantly lower than the trial containing EDTA in the presence of protozoa. This indicates a reduction in nitrification in the absence of protozoan grazing.

5.2.3 Enumeration of Absolute Bacterial Cell Numbers

Enumeration of bacterial abundance was carried out on day 1, day 7, and day 14 of each trial (Figure 4.13). The enumeration consisted of counting total bacterial cell numbers (both live and dead). The purpose of this enumeration was to attempt to determine if overall bacterial cell numbers increased throughout each trial in accordance with the theory that grazing by protozoa control the amount of non-nitrifying, heterotrophic bacterial populations. In the cycloheximide trial, we expected to see higher total bacterial numbers than in the reference trial due to the increased growth of fast-growing heterotrophs in the absence of protozoan grazing. This in fact was observed, and by day 14 of the cycloheximide trial the bacterial abundance was 7.92×10^{-8} cells/mL as compared to 1.22×10^{-8} cells/mL on day 14 of the reference trial.

In the allylthiourea trials, bacterial abundance also increased, by day 14 bacterial abundances were 2.60×10^{-8} and 2.96×10^{-8} cells/mL in the allylthiourea and allylthiourea + cycloheximide trials, respectively, from an initial day 1 cell abundance of 3.00×10^{-7} cells/mL.

An increase in bacterial cell abundance was also observed in the EDTA and EDTA + cycloheximide trials, from a day one abundance of 4.46×10^{-7} cells/mL to day 14 values of 3.15×10^{-8} and 3.52×10^{-8} cells/mL, respectively.

The reference trial had the lowest day 14 bacterial cell abundance, while the cycloheximide trial had the highest. Upon examination of Figure 4.13, it is obvious from the error bars on the graph that a huge amount of variation occurred between bacterial count samples (in the order of 10^{+8}). This large amount of variability brings into question the reliability of the cell count method as applied to flocculated activated sludge samples. The main problem lies in the fact that most bacteria in activated sludge systems are flocculated. Bacteria within flocs cannot be accurately counted using standard microscopic techniques. Even in the EDTA trial, where deflocculation occurred, a large proportion of bacteria was still observed to be in flocs. For this reason, bacterial cell abundances measured in this study are relative and not likely representative of actual bacterial cell numbers.

A more accurate way of enumerating total bacterial cells in activated sludge must be examined. Techniques of enumerating AOB and NOB numbers should also be investigated. Typical plate count techniques are not applicable to activated sludge samples since many of the bacteria present in activated sludge are as-yet uncultured. Counting techniques using the light microscope works well for some systems in which bacteria are freely suspended. However, in activated sludge systems, the majority of bacteria exist in flocs. In order to obtain proper counts, the flocs must first be completely broken up prior to staining samples. Several studies have attempted to break up flocs by forcing sludge samples through a small gauge needle, or by adding a detergent such as Tween 80 to samples (Lishman et al., 2000). These techniques were attempted in this study, and found to be less effective than the vortex technique used to break up the floc. However, the vortex technique was still not completely effective, and when counting samples many large flocs were observed. For a discussion of some possible techniques that could be used in future studies please refer to section 5.5.

5.3 The use of FISH Combined with CSLM to Determine the Position and Relative Amount of Nitrifying Bacteria

The second objective of this study was to determine if the presence or absence of protozoa under different conditions altered the spatial organization or relative amount of

nitrifying bacteria as determined by fluorescent *In situ* hybridization using rRNA-targeted oligonucleotide probes combined with confocal scanning laser microscopy.

In order to meet this objective, six different probes were used in order detect AOB, NOB, and eubacteria. For each floc examined, the relative position within the floc of fluorescently labelled bacteria was determined, as well as the relative abundance of the bacteria on a four point scale. The relative position and abundance of each type of bacteria was then compared between the trials. The average floc size and the average floc diameter were also compared between trials.

A total of four probe combinations were used in this study. Each combination consisted of two probes simultaneously hybridized to the sample. The purpose of combining probes is two-fold. Firstly, it cuts down on the number of slides that must be made and sample preparation time. Secondly, combining probes allows observations to be made about the position of different types of bacteria relative to one another.

Figure 4.14 represents the average floc size and floc depth of the flocs examined in each trial. Floc size was measured as the length of the floc at the widest point. When floc size was compared among trials, the only statistically significant difference was found between the reference and the EDTA + cycloheximide. The floc size in the EDTA + cycloheximide trial was significantly smaller than that of the reference trial. This could be explained by the combined effect of deflocculation by the EDTA and the lack of protozoan grazing. Protozoan grazing has been known to increase the flocculation of bacteria by grazing on free-floating species, and also by the excretion of compounds that promote flocculation (Curds, 1982; Ratsak et al., 1996).

Floc depth was significantly smaller in the allylthiourea, the allylthiourea + cycloheximide, the EDTA, and the EDTA + cyloheximide trials as compared to the reference trial. There was no significant difference in floc depth between the reference and cycloheximide trials. As was the case with floc size, the floc depth in the EDTA + cycloheximide trial was significantly smaller than in the EDTA trial. Again, this could be due to the combination of deflocculation by EDTA and a lack of grazing by protozoa. The smaller floc size in trials containing the deflocculating agent EDTA is expected, but the smaller floc size in the allylthiourea trials was not. The presence of smaller flocs in

the trials containing allylthiourea suggests that allylthiourea may also have some deflocculating characteristics. Another possibility is that the build-up of ammonia in these trials caused some amount of deflocculation due to cell death (Singh et al., 1994). An increase in pH in the allylthiourea trials was not likely to cause deflocculation as flocs dissociation constants have been found to be stable within the range of 4.5 to 9.5 (Liao et al., 2002).

It is important to note that floc size and floc depth are somewhat arbitrary designations, and entirely dependant on the position of the floc on the slide and in the plane of the microscope laser. It is also important to note that during sample preparation, and due to the use of a coverslip, flocs may become compressed. This has the effect of making the floc size as it is measured greater, and the floc depth as it is measured smaller. Thus, when making generalizations about floc size and depth some caution must be exercised.

The relative position within the floc of each type of bacteria examined in this study was obtained in each floc by locating the depth within each floc where the maximum amount of bacteria occurred and dividing this depth by the total floc depth. From these values, was possible to compare the relative positions of each type of bacteria within the flocs on day 1, day 7, and day 14. This is represented by Figure 4.15, 4.16, and 4.17. Through visual observation of the flocs, no obvious detectable difference in the bacterial position within the floc was observed between the trials. Also, there was no obvious detectable difference in the bacterial position within the floc between day 1, 7, and 14 of each trial. Probe EUB 338 detects most eubacteria. When used in combination with probes for AOB and NOB, it shows the position of AOB and NOB compared to other bacteria that are not AOB and NOB. Probe EUB was found at all levels of the activated sludge floc, indicating that bacteria are found at all levels within the activated sludge floc. However, the greatest amount of EUB 338 binding did occur in the center of the floc, indicating that in activated sludge a large number of bacteria are located within the activated sludge floc where they are protected from predation and make use of microenvironments and nutrient pockets (Droppo, 2001). This finding correlates with the findings of various earlier studies that used FISH combined with CSLM to determine the position of bacteria in activated sludge flocs (Wagner et al., 1994; Wagner et al., 1994(b); Schmid et al. 2003). The AOB of the β subclass *Proteobacteria*, *Nitrosomonas* species, and

Nitrosococcus mobilis were also found to be located in the middle of the activated sludge floc, and closely associated with each other in clusters. AOB were found at multiple depths within the floc, but the majority of binding was always found to occur mid-floc. This finding also is supported by the findings of Wagner et al. (1996; 1998) and by Mobarry et al., (1996) who also found AOB located in the interior of flocs, in closely associated clusters. The NOB *Nitrospira*-like group of bacteria was also found in clusters distributed throughout the floc, with the majority located mid-floc. *Nitrobacter* was found to be closely associated with the *Nitrospira*-like bacteria, but was usually located closer to the outside of the floc. *Nitrosospira* species were also found positioned mid-floc, but like *Nitrobacter* this species was usually located closer to the outside of the floc than the *Nitrospira*-like bacteria. Unlike *Nitrospira*-like bacteria, *Nitrobacter* and *Nitrosospira* were usually only found at a single floc depth and were not distributed throughout the floc. The position of NOB mid-floc is also supported by earlier findings by Mobarry et al., (1996), Wagner et al., (1996) and Daims et al., (2002a). The presence or absence of protozoan grazing did not appear to affect the position of nitrifying bacteria within the floc, since both AOB and NOB were found in the centre of the floc in all trials. Surprisingly, in the EDTA trials, flocs were still found to be intact, although the depth of flocs were smaller than in the reference trial. The position of nitrifying bacteria was not altered by deflocculation.

Perhaps of more interest than the position of nitrifying bacteria was the abundance of nitrifying bacteria within the floc. We were interested in determining whether or not the presence or absence of protozoa had an effect on the amount of nitrifying bacteria within the floc. We also wanted to determine if, when nitrification was halted through the use of allylthiourea, whether the abundance of nitrifying bacteria detected by FISH/CSLM decreased. The use of the probe EUB 338 that detects most bacteria was used in hope that an increase in heterotrophic bacteria would be detected using this probe. The quantification of bacterial abundance on a four point scale for each trial on each day is shown in Figure 4.19, 4.20, and 4.21. Probe EUB 338 was the most abundant probe in all trials on all days. This is to be expected since EUB 338 binds to most bacteria, and as such should bind to all nitrifying bacteria and all other bacteria present in the activated sludge floc. The amount of EUB 338 probe binding was highly variable, and more

bacteria appeared to be present in the trials containing allylthiourea and the trials containing EDTA than in the reference and cycloheximide trials. However, since the amount of EUB binding was so variable between floc samples, the presence of more bacteria in these trials cannot be stated with any certainty. For the ammonia oxidizing bacteria, both *betaproteobacterial* ammonia oxidizers and *Nitrosomonas* and *Nitrosococcus mobilis* were present in moderate amounts, with *Nitrosomonas* and *Nitrosococcus mobilis* slightly more abundant. This finding is in accordance with other studies that found that *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosococcus mobilis*, and members of the *Nitrosomonas marina* cluster are the most abundant ammonia oxidizers in activated sludge (Wagner and Loy, 2002). For the NOB, *Nitrospira*-like bacteria were present in the greatest abundance. *Nitrobacter* and *Nitrosospira* were also present in small amounts, with *Nitrosospira* being the least abundant. This is not surprising, since recent research determined that *Nitrospira*-like bacteria are the dominate nitrite oxidizers in WWTPs (Burrell et al., 1998; Juretschko et al., 1998; Okabe et al., 1999; Daims et al., 2000; Daims et al., 2001(a); Gieseke et al., 2001). The reason for this is that *Nitrospira-like* bacteria are K-strategists with high substrate affinities and low maximum growth rates and thus outcompete *Nitrobacter* under substrate-limiting conditions in WWTPs (Wagner and Loy, 2002).

The use of FISH combined with CSLM did not reveal a noticeable difference in either the position or abundance of nitrifying bacteria within the sludge flocs under the different conditions examined. When protozoan activity was inhibited, and increased growth of heterotropic bacteria was thought to increase, no subsequent increase in the abundance of probe EUB 338 was observed. In the presence of protozoan grazing, where increased nitrification was thought to occur as indicated by parameter measurements, no increase in the abundance of AOB or NOB was observed with FISH/CSLM. When ammonia oxidation was inhibited using allylthiourea, again, no noticeable change in the abundance of AOB or NOB was observed. This suggests that an increase in nitrification does not necessarily mean an increase in the abundance of nitrifying bacteria. Rather, an increase in nitrification might denote an increase in the per-cell nitrification rate.

There are several important limitations associated with the use of FISH combined with CSLM. One limitation is the difficulty in quantifying cell numbers using the

FISH/CSLM method. Another limitation is that both physiologically active and inactive cells are detected. Other limitations are methodological limitations, such as the occurrence of non-specific binding of probes that may distort abundance estimates.

It also should be noted that probe EUB 338 detects most bacteria, but in order to detect all bacteria should be used in conjunction with two newly developed probes: EUB 333II (*Planctomycetales* spp.) and EUB 338 III (*Verrucomicrobiales* spp.) (Loy et al., 2003). This omission could account for the failure to observe an increase in bacterial abundance in the trials lacking protozoan grazing.

The use of FISH/CSLM is an excellent starting point in observing the general position and relative amounts of nitrifying bacteria within activated sludge floc.

5.4 The Relevance of the Influence of Protozoan Grazing on Nutrient Cycling in the Activated Sludge Process

This study has established that in the presence of protozoan grazing, nitrification rates increase. This finding is supported by several other studies that also found enhanced nitrogen cycling in the presence of protozoan grazing in the activated sludge and other environments (Clarholm, 1984; Verhagen and Laanbroek, 1992; Strauss and Dodds, 1997; Petropoulos, 2003). What is still not clear is the exact mechanism by which protozoa affect nitrification. The possible interaction between protozoa can be summarized in two main ideas: the grazing effect, and the cycling effect.

The grazing effect entails the theory that protozoa may affect nitrification by controlling populations of heterotrophic bacteria which normally compete with nitrifying bacteria for ammonia. It has been shown that the assimilation of ammonia by heterotrophs happens in preference to nitrification and in fact reduces the available ammonia for nitrification (Hanaki et al., 1990). Thus, it follows that when heterotrophic bacterial numbers are controlled by protozoan grazing, that more ammonia will be available for nitrifying bacteria, and nitrification will increase. Several studies have shown that the presence of protozoan grazing in fact selects for fast growing heterotrophic bacteria and that slow growing nitrifying bacteria are eliminated (Sherr et al., 1988; Sinclair and Alexander, 1989). It is essential to note, however, that these studies did not involve bacterial populations that are highly flocculated, such as those present in activated sludge. In

activated sludge, the results of many studies, including the present study, have indicated that nitrifying bacteria are located within the interior of activated sludge flocs (Wagner et al., 1995; Mobarry et al., 1996; Wagner et al., 1996; Wagner et al., 1998; Daims et al., 2001(a)). Heterotrophic bacteria are located in all areas of the floc (Wagner et al., 1995). As such, nitrifying bacteria are not available to protozoa for predation under normal circumstances. Thus, the theory that protozoan grazing reduces numbers of slow-growing nitrifying bacteria is not applicable in activated sludge systems. Heterotrophic bacteria in activated sludge are also located in floc, and those that are not in flocs are consumed by protozoa, hence the basis of a self-regulating activated sludge system that selects for flocculating bacteria. In the absence of protozoan activity, bacterial populations are not controlled, and fast-growing heterotrophic bacteria proliferate, subsequently outcompeting the nitrifying bacteria for ammonia. The net result of this is a decrease in nitrification in the absence of protozoa due to the proliferation of heterotrophic bacteria.

The cycling theory of the effect of protozoa on nutrient cycling follows that protozoan grazing results in the excretion of mineral nutrients such as ammonia and phosphate, decreasing the C:N:P ratio and this results in an accelerated use of carbon resources by bacteria (enhanced carbon mineralization). A study by Sherr et al. (1998) found that in aquatic environments protozoa that consume living cells have the highest biomass specific excretion rates of inorganic nitrogen of all zooplankton groups. Protozoan grazing changes the C:N:P ratio by increasing the concentrations of mineral nitrogen (excreted by protozoa as ammonia) and phosphorous (excreted by protozoa as phosphate). The excretion of ammonia by protozoa provides more ammonia for oxidation by nitrifying bacteria, and also for assimilation by heterotrophs. One problem with the cycling theory applied to municipal activated sludge is that in such a system ammonia is often in abundant supply and is rarely limiting. Thus, the protozoan excretion of ammonia may have little affect on enhancing nitrification since it is probably already occurring at a high rate. However, it is possible that the influence of protozoan grazing on the C:N:P ratio is somewhat under-estimated because the composition of wastewater (i.e influent nitrogen and carbon loadings) is highly variable (Ratsak et al., 1996). For example, the N-concentration may be lowered by processes such as

denitrification and assimilation of ammonia by heterotrophic bacteria. Furthermore, some types of wastewater (such as pulp and paper) contain much higher levels of carbon in which case nitrogen may become limiting (Ratsak et al., 1996).

In reality, the exact mechanism by which protozoa effect nitrification is probably a combination of bacterial population control by grazing and enhanced nutrient cycling. Due to the complex nature of the activated sludge ecosystem, interactions are still poorly understood. However, there are several new techniques, such as the isotope array approach, that may give insights into interspecies metabolic interactions and even food webs.

5.5 Future Work and Recommendations

This study has established that while the presence of protozoa increases nitrification, it does not appear to affect the amount or position of nitrifying bacteria within the floc as determined by FISH combined with CSLM. This would suggest that that protozoan grazing does not promote the growth of more nitrifying bacteria, but does increase the per-cell nitrification rates. This finding is supported by earlier studies by Clarholm (1985), Verhagen and Laanbroek (1992), and Strauss and Dodds (1997), all of which determined that protozoan grazing increases nitrification rates in soil environments.

As mentioned above, there are several key limitations associated with the FISH/CSLM technique when it is used to examine nitrification activity in the activated sludge floc. One limitation is that the use of FISH/CSLM is not an ideal method to quantify bacteria, and to do so requires expensive and difficult to obtain specialized software. Another limitation this that rRNA is highly stable, and the use of FISH/CSLM does not allow one to differentiate between active and inactive cells. Even if nitrifying bacteria are not viable, cells will still fluoresce when examined using FISH/CSLM.

Future work should focus on first establishing whether or not protozoan grazing increases nitrification rates by increasing the number of nitrifying bacteria or by increasing per-cell nitrification rates. A study by Daims et al. (2001(c)) successfully utilized FISH combined with CSLM to determine absolute bacterial cell numbers (cells/volume). This technique involved spiking samples with internal standards comprised of *E. coli* cells. The absolute numbers of ammonia oxidizing bacteria were also obtained (Daims et al., 2001(c)). The

method used by Daims et al. (2001(c)) requires a specific type of software that was not available for use in this study. However, the acquisition of such software for quantification of absolute bacterial cell numbers in future studies would be beneficial.

There are several techniques available to determine the metabolic activity of bacterial cells. One such technique is the use of microautoradiography (MAR) combined with FISH. Microautoradiography is a method that can be used to study the microscale distribution or radiolabelled compounds (Nielsen et al., 1999). MAR combined with FISH can be used to study the *in situ* physiology of many microorganisms. Their ability to take up specific organic or inorganic compounds or their ability to be active under aerobic, anoxic, or anaerobic conditions can also be investigated (Nielsen et al., 1999). MAR also allows for the enumeration of certain functional groups of microorganisms that may be responsible for a certain process (i.e. nitrification) in a complex microbial system such as activated sludge. Accurate enumeration depends on finding a suitable radiotracer to track bacteria carrying out the process of interest. This number can be compared to the total population, e.g., from a DAPI count. Again, this requires specific software capable of quantifying fluorescence. MAR has been used *in situ* to detect ammonia oxidizing bacteria (Nielsen et al., 1999). One of the difficulties with the use of the MAR/FISH procedure to distinguish different functional groups of bacteria is that the radioactively labelled substrate has be exclusively consumed by the functional bacterial group being monitored. For example, if ammonia-oxidizing bacteria are investigated by the use of the radioactive label $^{14}C\text{-}HCO_3^-$, these are not distinguishable from other autotrophic bacteria (such as nitrite-oxidizing bacteria) unless nitrite is removed or specific inhibitors are used (Nielsen et al., 1999). Another drawback to the MAR/FISH technique is that it is extremely time consuming and calls for a large amount of sample preparation.

The isotope array method is a new method that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function (Adamczyk et al., 2003). The isotope array method allows for the simultaneous monitoring of the diversity and substrate incorporation of complex microbial communities by DNA microarray technology. A recent study by Adamczyk et al. (2003) used the isotope array technique to successfully monitor the CO_2 fixation activities of ammonia-oxidizing bacteria, using activated sludge amended with radioactive bicarbonate as the model

system (Adamczyk et al., 2003). After extraction and fluorescence labelling of community rRNA, the diversity and bicarbonate incorporation of AOB were measured by using a prototype DNA microarray for AOB detection (Adamczyk et al., 2003). An as yet unpublished paper by Wagner (2004), touts the isotope array method as a much less tedious and time consuming alternate to the MAR/FISH technique to decipher functions of complex microbial communities such as the activated sludge community.

To date, no studies have been done the attempt to directly link protozoan grazing to increased microbial nitrification as measured by substrate uptake. Performing such a study through the use of the MAR/FISH technique or the new isotope array method would provide some much-needed further insight into the relationship between protozoa and the bacterially mediated nitrogen cycling.

6.0 CONCLUSIONS

The purpose of this study was two-fold. First, it aimed to examine the effect of protozoan grazing on nitrification rates under different conditions. The second objective was to study the spatial distribution of ammonia- and nitrite-oxidizing bacteria (AOB and NOB) in activated sludge. In order to achieve this, batch trials were performed under six different treatment conditions: no treatment, inhibition of protozoan grazing, inhibition of ammonia oxidation, inhibition of both ammonia oxidation and protozoan grazing, deflocculation, and deflocculation combined with the inhibition of protozoa grazing. Parameters to assess nitrification activity were monitored throughout each trial. On day 1, 7, and 14 of each trial sludge samples were examined using fluorescent *In situ* hybridization combined with confocal scanning laser microscopy for the presence and spatial position of ammonia oxidizing and nitrite oxidizing bacteria.

In the absence of protozoan grazing, rates of nitrate, nitrite, and ammonia production were lower than in batches with protozoa. This shows that the nitrification rate is lower in the absence of protozoan grazing suggesting that the protozoa are important for converting the organic nitrogen in the biomass back into their inorganic forms.

When ammonia oxidation was inhibited, the rates of nitrate and nitrite production were also lower. It was also observed that there was a build-up of ammonia in the system.

The addition of EDTA as a deflocculant appeared to increase nitrification rates, perhaps by the increased contact of nitrifying bacteria with ammonia as a result of deflocculation. In the presence of protozoan grazing and EDTA, the nitrification rate appeared to be higher than without grazing, suggesting that grazing increases nitrification rates even under adverse conditions such as deflocculation.

In the reactors lacking both protozoan grazing and ammonia oxidation, the rates of nitrate and nitrite production were minimal. This suggests that the lack of protozoan grazing augments the effect of allylthiourea possibly by further reducing the inorganic nitrogen available for nitrification.

When sludge samples were examined using FISH/CSLM, both AOB and NOB were found clustered within the floc.

Neither inhibiting protozoa, inhibiting ammonia oxidation, or deflocculation appeared to lower the amount of AOB and NOB present or their position within the floc

Overall, this study suggests that protozoan grazing of bacteria is an important factor in nitrogen cycling within this system. It appears that their activity releases inorganic nitrogen into the system that can be used during nitrification. Since the amount of AOB and NOB were not affected by our manipulations, it suggests that the AOB and NOB are more active in the presence of protozoa (i.e. per cell nitrification increases). Further study using FISH combined with MAR or the new isotope array technique is needed to confirm this.

7.0 REFERENCES

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APPENDIX A: PARAMETERS OF BATCH MICROCOSMS

Table A.1. Daily Temperature Measurements and Standard Deviations

Trial	Number of Days	R1 (C)	R2 (C)	R3 (C)	Average (C)	s
Reference	1	26.0	26.2	26.0	26.1	0.00
Reference	2	25.6	25.6	25.4	25.5	0.12
Reference	3	25.5	25.7	25.6	25.6	0.10
Reference	4	24.8	24.9	24.8	24.8	0.06
Reference	5	25.0	25.0	25.1	25.0	0.06
Reference	6	25.5	25.5	25.3	25.4	0.12
Reference	7	25.8	25.8	25.8	25.8	0.00
Reference	8	25.7	25.9	25.9	25.8	0.12
Reference	9	25.6	25.7	25.7	25.7	0.06
Reference	10	25.5	25.6	25.5	25.5	0.06
Reference	11	25.4	25.5	25.5	25.5	0.06
Reference	12	25.3	25.3	25.3	25.3	0.00
Reference	13	25.3	25.4	25.4	25.4	0.06
Reference	14	25.5	25.5	25.5	25.5	0.00
Cycloheximide	1	26.0	26.0	26.1	26.0	0.00
Cycloheximide	2	25.3	25.3	25.4	25.3	0.06
Cycloheximide	3	25.5	25.4	25.3	25.4	0.10
Cycloheximide	4	24.7	24.6	24.6	24.6	0.06
Cycloheximide	5	25.1	25.0	25.1	25.1	0.06
Cycloheximide	6	25.5	25.3	25.3	25.4	0.12
Cycloheximide	7	25.7	25.8	25.8	25.8	0.06
Cycloheximide	8	26.0	26.1	26.2	26.1	0.10
Cycloheximide	9	25.7	25.7	25.6	25.7	0.06
Cycloheximide	10	25.4	25.3	25.3	25.3	0.06
Cycloheximide	11	25.4	25.4	25.5	25.4	0.06
Cycloheximide	12	25.4	25.3	25.3	25.3	0.06
Cycloheximide	13	25.3	25.4	25.4	25.4	0.06
Cycloheximide	14	25.5	25.5	25.4	25.5	0.06
Allylthiourea	1	26.4	26.3	26.4	26.4	0.06
Allylthiourea	2	25.9	25.3	25.3	25.5	0.35
Allylthiourea	3	24.5	24.5	24.6	24.5	0.06
Allylthiourea	4	24.2	24.3	24.5	24.3	0.15
Allylthiourea	5	25.0	24.3	24.2	24.5	0.44
Allylthiourea	6	24.5	24.2	24.2	24.3	0.17
Allylthiourea	7	25.3	24.8	24.3	24.8	0.50
Allylthiourea	8	23.9	23.8	23.7	23.8	0.10
Allylthiourea	9	24.1	24.3	24.3	24.2	0.12
Allylthiourea	10	24.4	24.3	24.2	24.3	0.10
Allylthiourea	11	24.3	24.4	24.2	24.3	0.10
Allylthiourea	12	24.9	24.5	23.4	24.3	0.78
Allylthiourea	13	23.2	23.4	23.2	23.3	0.12
Allylthiourea	14	23.6	23.7	23.6	23.6	0.06
Allylthiourea + Cycloheximide	1	26.4	26.3	26.4	26.4	0.06
Allylthiourea + Cycloheximide	2	25.6	25.3	25.2	25.4	0.21

Allylthiourea + Cycloheximide	3	24.3	24.5	24.1	24.3	0.20
Allylthiourea + Cycloheximide	4	24.2	24.0	23.9	24.0	0.15
Allylthiourea + Cycloheximide	5	24.2	24.0	24.0	24.1	0.12
Allylthiourea + Cycloheximide	6	24.3	24.2	24.1	24.2	0.10
Allylthiourea + Cycloheximide	7	24.2	24.4	24.3	24.3	0.10
Allylthiourea + Cycloheximide	8	23.8	23.5	23.5	23.6	0.17
Allylthiourea + Cycloheximide	9	24.2	24.2	24.3	24.2	0.06
Allylthiourea + Cycloheximide	10	24.4	24.3	24.2	24.3	0.10
Allylthiourea + Cycloheximide	11	24.3	24.4	24.1	24.3	0.15
Allylthiourea + Cycloheximide	12	23.3	23.2	23.3	23.3	0.06
Allylthiourea + Cycloheximide	13	23.1	23.4	23.0	23.2	0.21
Allylthiourea + Cycloheximide	14	23.5	23.5	23.2	23.4	0.17
EDTA	1	24.9	24.9	24.7	24.8	0.12
EDTA	2	24.4	23.9	24.2	24.2	0.25
EDTA	3	24.1	23.8	23.7	23.9	0.21
EDTA	4	25.2	24.7	25.2	25.0	0.29
EDTA	5	25.1	25.0	25.0	25.0	0.06
EDTA	6	25.2	24.9	24.6	24.9	0.30
EDTA	7	25.3	24.8	24.3	24.8	0.50
EDTA	8	24.8	24.5	24.2	24.5	0.30
EDTA	9	25.2	24.7	24.4	24.8	0.40
EDTA	10	25.7	25.3	24.9	25.3	0.40
EDTA	11	23.9	23.8	23.6	23.8	0.15
EDTA	12	24.0	24.1	23.9	24.0	0.10
EDTA	13	24.7	25.0	25.0	24.9	0.17
EDTA	14	24.9	25.2	25.1	25.1	0.15
EDTA+Cycloheximide	1	24.9	24.9	24.7	24.8	0.12
EDTA+Cycloheximide	2	24.0	23.8	23.9	23.9	0.10
EDTA+Cycloheximide	3	23.7	23.7	23.7	23.7	0.00
EDTA+Cycloheximide	4	25.0	24.9	24.7	24.9	0.15
EDTA+Cycloheximide	5	25.1	25.0	24.8	25.0	0.15
EDTA+Cycloheximide	6	25.2	24.5	24.6	24.8	0.38
EDTA+Cycloheximide	7	23.9	23.9	23.6	23.8	0.17
EDTA+Cycloheximide	8	24.1	23.9	23.7	23.9	0.20
EDTA+Cycloheximide	9	24.0	23.9	23.8	23.9	0.10
EDTA+Cycloheximide	10	24.6	24.6	24.4	24.5	0.12
EDTA+Cycloheximide	11	23.5	23.5	23.4	23.5	0.06
EDTA+Cycloheximide	12	24.0	24.0	23.6	23.9	0.23
EDTA+Cycloheximide	13	24.7	25.1	25.0	24.9	0.21
EDTA+Cycloheximide	14	25.0	25.0	24.9	25.0	0.06

Table A.2. Daily pH Measurements and Standard Deviations

Trial	Number of Days	R1	R2	R3	Average	s
Reference	1	7.52	7.52	7.52	7.52	0.000
Reference	2	7.50	7.50	7.50	7.50	0.000
Reference	3	7.50	7.50	7.50	7.50	0.000
Reference	4	7.50	7.50	7.30	7.43	0.115
Reference	5	7.30	7.40	7.20	7.30	0.100
Reference	6	7.00	7.10	7.00	7.03	0.058
Reference	7	6.89	7.00	6.70	6.86	0.152
Reference	8	6.89	7.00	6.60	6.83	0.207
Reference	9	6.70	6.98	6.50	6.73	0.241
Reference	10	6.50	6.80	6.40	6.57	0.208
Reference	11	6.40	6.50	6.40	6.43	0.058
Reference	12	5.60	6.00	6.00	5.87	0.231
Reference	13	5.10	5.54	5.50	5.38	0.243
Reference	14	4.85	5.10	4.76	4.90	0.176
Cycloheximide	1	7.52	7.52	7.52	7.52	0.000
Cycloheximide	2	7.50	7.50	7.50	7.50	0.000
Cycloheximide	3	7.45	7.50	7.50	7.48	0.029
Cycloheximide	4	7.40	7.30	7.20	7.30	0.100
Cycloheximide	5	7.40	7.00	7.00	7.13	0.231
Cycloheximide	6	7.30	7.00	7.10	7.13	0.153
Cycloheximide	7	7.00	7.00	6.90	6.97	0.058
Cycloheximide	8	7.00	7.00	6.80	6.93	0.115
Cycloheximide	9	6.80	6.90	7.00	6.90	0.100
Cycloheximide	10	6.70	6.70	6.50	6.63	0.115
Cycloheximide	11	6.50	6.50	5.75	6.25	0.433
Cycloheximide	12	6.20	6.00	5.45	5.88	0.388
Cycloheximide	13	5.43	5.30	4.98	5.24	0.232
Cycloheximide	14	4.81	4.81	4.76	4.79	0.029
Allylthiourea	1	7.49	7.50	7.49	7.49	0.006
Allylthiourea	2	7.92	7.91	7.90	7.91	0.010
Allylthiourea	3	8.10	8.21	8.14	8.15	0.056
Allylthiourea	4	8.18	8.10	8.23	8.17	0.066
Allylthiourea	5	8.19	8.09	8.10	8.13	0.055
Allylthiourea	6	8.38	8.31	8.20	8.30	0.091
Allylthiourea	7	8.56	8.59	8.60	8.58	0.021
Allylthiourea	8	8.64	8.54	8.71	8.63	0.085
Allylthiourea	9	8.64	8.55	8.70	8.63	0.075
Allylthiourea	10	8.66	8.56	8.70	8.64	0.072
Allylthiourea	11	8.67	8.59	8.61	8.62	0.042
Allylthiourea	12	8.69	8.59	8.58	8.62	0.061
Allylthiourea	13	8.71	8.72	8.69	8.71	0.015

Allylthiourea	14	8.81	8.89	8.91	8.87	0.053
Allylthiourea + Cycloheximide	1	7.49	7.50	7.49	7.49	0.006
Allylthiourea + Cycloheximide	2	7.92	7.93	7.96	7.94	0.021
Allylthiourea + Cycloheximide	3	8.11	8.00	8.26	8.12	0.131
Allylthiourea + Cycloheximide	4	8.32	8.45	8.32	8.36	0.075
Allylthiourea + Cycloheximide	5	7.91	8.01	8.02	7.98	0.061
Allylthiourea + Cycloheximide	6	8.30	8.31	8.27	8.29	0.021
Allylthiourea + Cycloheximide	7	8.62	8.65	8.59	8.62	0.030
Allylthiourea + Cycloheximide	8	8.58	8.67	8.62	8.62	0.045
Allylthiourea + Cycloheximide	9	8.57	8.75	8.65	8.66	0.090
Allylthiourea + Cycloheximide	10	8.50	8.67	8.51	8.56	0.095
Allylthiourea + Cycloheximide	11	8.55	8.54	8.60	8.56	0.032
Allylthiourea + Cycloheximide	12	8.44	8.45	8.45	8.45	0.006
Allylthiourea + Cycloheximide	13	8.56	8.60	8.41	8.52	0.100
Allylthiourea + Cycloheximide	14	8.69	8.78	8.32	8.60	0.244
EDTA	1	7.50	7.50	7.50	7.50	0.000
EDTA	2	7.50	7.40	7.50	7.47	0.058
EDTA	3	7.32	7.50	7.50	7.44	0.104
EDTA	4	7.00	6.99	7.20	7.06	0.118
EDTA	5	7.10	7.00	7.00	7.03	0.058
EDTA	6	7.00	6.80	7.00	6.93	0.115
EDTA	7	7.00	6.80	7.00	6.93	0.115
EDTA	8	7.00	6.80	7.00	6.93	0.115
EDTA	9	7.00	7.00	7.00	7.00	0.000
EDTA	10	6.80	6.70	6.90	6.80	0.100
EDTA	11	6.00	6.10	6.00	6.03	0.058
EDTA	12	5.02	5.61	5.50	5.38	0.314
EDTA	13	4.99	4.80	4.70	4.83	0.147
EDTA	14	4.73	4.73	4.66	4.71	0.040
EDTA+Cycloheximide	1	7.50	7.50	7.50	7.50	0.000
EDTA+Cycloheximide	2	7.70	7.50	7.30	7.50	0.200
EDTA+Cycloheximide	3	7.50	7.40	7.00	7.30	0.265
EDTA+Cycloheximide	4	7.30	7.50	7.10	7.30	0.200
EDTA+Cycloheximide	5	7.00	7.20	7.10	7.10	0.100
EDTA+Cycloheximide	6	7.00	7.00	7.00	7.00	0.000
EDTA+Cycloheximide	7	7.00	7.00	7.00	7.00	0.000
EDTA+Cycloheximide	8	6.90	6.90	7.00	6.93	0.058
EDTA+Cycloheximide	9	7.00	7.00	6.80	6.93	0.115
EDTA+Cycloheximide	10	6.60	6.70	6.50	6.60	0.100
EDTA+Cycloheximide	11	5.57	5.23	5.63	5.48	0.216
EDTA+Cycloheximide	12	4.83	5.12	5.13	5.03	0.170
EDTA+Cycloheximide	13	4.70	5.00	4.99	4.90	0.170
EDTA+Cycloheximide	14	4.64	4.71	4.85	4.73	0.107

Table A.3 Dissolved Oxygen Measurements and Standard Deviations

Trial	Number of Days	R1 (mg O_2/L)	R2 (mg O_2/L)	R3 (mg O_2/L)	Average (mg O_2/L)	s
Reference	1	8.0	8.0	8.0	8.0	0.0
Reference	4	8.0	8.0	8.0	8.0	0.0
Reference	7	8.0	8.0	8.0	8.0	0.0
Reference	10	8.0	8.0	8.0	8.0	0.0
Reference	14	8.0	8.0	8.0	8.0	0.0
Cycloheximide	1	8.0	8.0	8.0	8.0	0.0
Cycloheximide	4	8.0	8.0	8.0	8.0	0.0
Cycloheximide	7	8.0	8.0	8.0	8.0	0.0
Cycloheximide	10	8.0	8.0	8.0	8.0	0.0
Cycloheximide	14	8.0	8.0	8.0	8.0	0.0
Allylthiourea	1	n/a	n/a	n/a	n/a	n/a
Allylthiourea	4	n/a	n/a	n/a	n/a	n/a
Allylthiourea	7	n/a	n/a	n/a	n/a	n/a
Allylthiourea	10	n/a	n/a	n/a	n/a	n/a
Allylthiourea	14	n/a	n/a	n/a	n/a	n/a
Allylthiourea+Cycloheximide	1	n/a	n/a	n/a	n/a	n/a
Allylthiourea+Cycloheximide	4	n/a	n/a	n/a	n/a	n/a
Allylthiourea+Cycloheximide	7	n/a	n/a	n/a	n/a	n/a
Allylthiourea+Cycloheximide	10	n/a	n/a	n/a	n/a	n/a
Allylthiourea+Cycloheximide	14	n/a	n/a	n/a	n/a	n/a
EDTA	1	8.5	8.5	8.5	8.5	0.0
EDTA	4	8.5	8.5	8.4	8.5	0.1
EDTA	7	8.0	8.0	8.0	8.0	0.0
EDTA	10	8.0	8.0	8.0	8.0	0.0
EDTA	14	8.0	8.0	8.0	8.0	0.0
EDTA+Cycloheximide	1	8.5	8.5	8.5	8.5	0.0
EDTA+Cycloheximide	4	8.5	8.5	8.5	8.5	0.0
EDTA+Cycloheximide	7	8.0	8.0	8.0	8.0	0.0
EDTA+Cycloheximide	10	8.0	8.0	8.0	8.0	0.0
EDTA+Cycloheximide	14	8.0	8.0	8.0	8.0	0.0

Table A.4 Batch Reactor COD Measurements and Standard Deviations

Trial	Number of Days	R1 (mgO_2/L)	R2 (mgO_2/L)	R3 (mgO_2/L)	Average (mgO_2/L)	s
Reference	1	121	118	121	120	1.73
Reference	4	93	71	119	95	23.77
Reference	7	54	54	55	54	0.53
Reference	10	58	58	62	60	2.15
Reference	14	35	26	35	32	5.20
Cycloheximide	1	121	118	121	120	1.73
Cycloheximide	4	250	250	249	249	0.67
Cycloheximide	7	217	217	225	220	4.53
Cycloheximide	10	249	250	249	249	0.67
Cycloheximide	14	36	36	36	36	0.00
Allylthiourea	1	138	141	141	140	1.77
Allylthiourea	4	122	123	121	122	1.00
Allylthiourea	7	92	92	92	92	0.56
Allylthiourea	10	55	64	77	65	11.30
Allylthiourea	14	57	56	56	56	0.53
Allylthiourea+Cycloheximide	1	138	141	141	140	1.77
Allylthiourea+Cycloheximide	4	233	205	233	223	16.13
Allylthiourea+Cycloheximide	7	126	141	136	134	7.73
Allylthiourea+Cycloheximide	10	96	102	104	101	4.06
Allylthiourea+Cycloheximide	14	82	82	72	79	5.48
EDTA	1	170	164	162	165	4.07
EDTA	4	183	212	236	210	26.36
EDTA	7	165	170	189	175	12.81
EDTA	10	174	200	214	196	20.39
EDTA	14	149	171	216	179	33.85
EDTA+Cycloheximide	1	170	164	162	165	4.07
EDTA+Cycloheximide	4	420	420	357	399	36.75
EDTA+Cycloheximide	7	305	320	305	310	8.36
EDTA+Cycloheximide	10	320	270	284	291	25.71
EDTA+Cycloheximide	14	238	256	210	235	22.93

Table A.5 Batch Reactor Ammonia Measurements and Standard Deviations

Trial	Number of Days	R1 (mg NH_3/L)	R2 (mgNH_3/L)	R3 (mgNH_3/L)	Average (mgNH_3/L)	s
Reference	1	18.26	18.44	18.44	18.38	0.10
Reference	4	18.32	22.84	19.41	20.19	2.36
Reference	7	21.70	27.82	24.95	24.82	3.06
Reference	10	21.10	26.45	20.90	22.82	3.15
Reference	14	32.71	32.62	29.59	31.64	1.78
Cycloheximide	1	18.26	18.44	18.44	18.38	0.10
Cycloheximide	4	12.78	16.08	15.62	14.83	1.79
Cycloheximide	7	18.44	17.80	19.18	18.48	0.69
Cycloheximide	10	20.21	20.12	22.57	20.97	1.39
Cycloheximide	14	20.30	26.10	27.57	24.65	3.84
Allylthiourea	1	28.32	28.37	31.88	29.52	2.04
Allylthiourea	4	43.87	46.67	52.20	47.58	4.24
Allylthiourea	7	46.54	57.11	55.18	52.94	5.63
Allylthiourea	10	46.77	55.90	56.89	53.19	5.58
Allylthiourea	14	50.00	52.11	45.69	49.26	3.27
Allylthiourea+Cycloheximide	1	28.32	28.37	31.88	29.52	2.04
Allylthiourea+Cycloheximide	4	36.18	37.08	42.74	38.66	3.56
Allylthiourea+Cycloheximide	7	47.80	54.01	60.53	54.11	6.37
Allylthiourea+Cycloheximide	10	58.98	50.44	57.25	55.56	4.52
Allylthiourea+Cycloheximide	14	51.49	49.75	40.64	47.29	5.83
EDTA	1	29.40	32.84	30.71	30.98	1.74
EDTA	4	15.95	23.77	22.92	20.88	4.29
EDTA	7	24.66	21.15	26.19	24.00	2.58
EDTA	10	31.18	25.86	24.73	27.26	3.45
EDTA	14	26.27	31.60	32.81	30.23	3.48
EDTA+Cycloheximide	1	29.40	32.84	30.71	30.98	1.74
EDTA+Cycloheximide	4	18.70	19.00	11.73	16.47	4.11
EDTA+Cycloheximide	7	19.27	16.31	16.60	17.39	1.63
EDTA+Cycloheximide	10	25.19	27.07	19.50	23.92	3.94
EDTA+Cycloheximide	14	32.06	30.65	27.30	30.00	2.45

Table A.6 Batch Reactor Nitrite Measurements and Standard Deviations

Trial	Number of Days	R1 ($mgNO_2/L$)	R2 ($mgNO_2/L$)	R3 ($mgNO_2/L$)	Average ($mgNO_2/L$)	s
Reference	1	2.10	2.10	2.10	2.10	0.00
Reference	4	2.41	2.29	2.22	2.30	0.10
Reference	7	2.61	2.61	2.58	2.60	0.02
Reference	10	2.59	2.69	2.66	2.65	0.05
Reference	14	0.46	0.46	0.26	0.39	0.12
Cycloheximide	1	2.10	2.10	2.10	2.10	0.00
Cycloheximide	4	2.09	2.11	2.10	2.10	0.01
Cycloheximide	7	2.11	2.02	2.05	2.06	0.05
Cycloheximide	10	2.10	2.10	2.13	2.11	0.02
Cycloheximide	14	0.21	0.28	0.21	0.24	0.04
Allylthiourea	1	1.01	1.03	1.02	1.02	0.01
Allylthiourea	4	0.11	0.11	0.16	0.13	0.03
Allylthiourea	7	0.10	0.24	0.15	0.16	0.07
Allylthiourea	10	0.42	0.46	0.47	0.45	0.03
Allylthiourea	14	1.48	1.44	1.31	1.41	0.09
Allylthiourea+Cycloheximide	1	1.01	1.03	1.02	1.02	0.01
Allylthiourea+Cycloheximide	4	0.19	0.28	0.19	0.22	0.05
Allylthiourea+Cycloheximide	7	0.35	0.35	0.30	0.34	0.03
Allylthiourea+Cycloheximide	10	0.31	0.35	0.36	0.34	0.02
Allylthiourea+Cycloheximide	14	12.92	7.85	7.99	9.59	2.88
EDTA	1	3.66	3.71	3.63	3.67	0.04
EDTA	4	1.37	1.76	1.76	1.63	0.22
EDTA	7	3.84	4.30	4.23	4.12	0.24
EDTA	10	0.43	0.31	0.33	0.36	0.06
EDTA	14	0.83	1.11	0.95	0.96	0.14
EDTA+Cycloheximide	1	3.66	3.71	3.63	3.67	0.04
EDTA+Cycloheximide	4	1.95	1.94	1.57	1.82	0.21
EDTA+Cycloheximide	7	3.00	2.42	3.49	2.97	0.54
EDTA+Cycloheximide	10	0.90	0.81	1.05	0.92	0.12
EDTA+Cycloheximide	14	0.06	0.07	0.16	0.09	0.06

Table A.7 Batch Reactor Nitrate Measurements and Standard Deviations

Trial	Number of Days	R1 ($mgNO_3/L$)	R2 ($mgNO_3/L$)	R3 ($mgNO_3/L$)	Average ($mgNO_3/L$)	s
Reference	1	1.11	1.11	1.11	1.11	0.001
Reference	4	1.44	1.45	1.47	1.45	0.015
Reference	7	1.56	1.57	1.58	1.57	0.010
Reference	10	1.90	1.91	1.91	1.91	0.006
Reference	14	2.50	2.54	2.56	2.53	0.031
Cycloheximide	1	1.11	1.11	1.11	1.11	0.001
Cycloheximide	4	1.09	1.10	1.10	1.10	0.004
Cycloheximide	7	1.06	1.04	1.05	1.05	0.010
Cycloheximide	10	1.02	1.03	1.05	1.03	0.013
Cycloheximide	14	0.99	0.98	0.98	0.98	0.008
Allylthiourea	1	1.39	1.37	1.44	1.40	0.040
Allylthiourea	4	0.91	0.73	1.20	0.95	0.237
Allylthiourea	7	1.19	1.12	1.37	1.23	0.127
Allylthiourea	10	0.98	1.18	1.12	1.09	0.103
Allylthiourea	14	0.72	0.87	0.96	0.85	0.121
Allylthiourea+Cycloheximide	1	1.39	1.37	1.44	1.40	0.040
Allylthiourea+Cycloheximide	4	0.68	0.93	0.81	0.81	0.124
Allylthiourea+Cycloheximide	7	0.89	1.10	1.23	1.07	0.169
Allylthiourea+Cycloheximide	10	0.86	0.89	0.90	0.89	0.023
Allylthiourea+Cycloheximide	14	0.36	0.43	0.45	0.41	0.051
EDTA	1	1.03	1.02	0.95	1.00	0.046
EDTA	4	12.66	12.95	12.59	12.73	0.190
EDTA	7	74.04	74.37	73.64	74.02	0.369
EDTA	10	78.31	79.20	81.89	79.80	1.862
EDTA	14	49.51	52.08	50.89	50.82	1.287
EDTA+Cycloheximide	1	1.03	1.02	0.95	1.00	0.046
EDTA+Cycloheximide	4	11.88	12.28	12.49	12.21	0.309
EDTA+Cycloheximide	7	74.77	75.59	72.00	74.12	1.880
EDTA+Cycloheximide	10	75.85	70.93	73.24	73.34	2.461
EDTA+Cycloheximide	14	52.04	49.76	51.93	51.24	1.282

Table A.8 Batch Reactor Total Nitrogen Measurements and Standard Deviations

Trial	Number of Days	R1 (mgTN/L)	R2 (mgTN/L)	R3 (mgTN/L)	Average (mgTN/L)	s
control	1	32.4	32.1	32.1	32.2	0.17
control	4	90.4	105.4	95.5	97.1	7.62
control	7	101.5	109.7	120.1	110.4	9.29
control	10	117.2	116.0	118.0	117.1	1.02
control	14	122.1	155.1	136.1	137.8	16.53
Cycloheximide	1	32.4	32.1	32.1	32.2	0.17
Cycloheximide	4	48.3	74.9		61.6	18.78
Cycloheximide	7	91.1	89.3	92.2	90.9	1.50
Cycloheximide	10	112.5	112.5	111.7	112.2	0.47
Cycloheximide	14	100.2	127.1	110.5	112.6	13.56
allylthiourea	1	39.8	38.3	39.8	39.3	0.87
allylthiourea	4	36.9	32.2	41.2	36.8	4.51
allylthiourea	7	38.6	37.7	39.2	38.5	0.75
allylthiourea	10	60.7	66.3	79.1	68.7	9.45
allylthiourea	14	92.7	92.4	101.1	95.4	4.91
allylthiourea+cycloheximide	1	39.8	38.3	39.8	39.3	0.87
allylthiourea+cycloheximide	4	31.1	24.6	31.1	28.9	3.77
allylthiourea+cycloheximide	7	27.7	26.2	29.2	27.7	1.47
allylthiourea+cycloheximide	10	56.5	56.1	56.1	56.2	0.19
allylthiourea+cycloheximide	14	53.1	43.5	48.9	48.5	4.80
EDTA	1	38.6	33.1	37.4	36.4	2.87
EDTA	4	72.0	71.7	74.6	72.8	1.61
EDTA	7	89.9	89.9	93.9	91.2	2.29
EDTA	10	93.9	107.0	112.1	104.3	9.42
EDTA	14	108.3	127.2	135.1	123.5	13.80
EDTA+cycloheximide	1	38.6	33.1	37.4	36.4	2.87
EDTA+cycloheximide	4	64.7	79.3	70.9	71.6	7.33
EDTA+cycloheximide	7	92.4	72.8	92.4	85.8	11.29
EDTA+cycloheximide	10	100.6	98.5	89.9	96.4	5.67
EDTA+cycloheximide	14	102.3	127.2	118.5	116.0	12.65

Table A.9 Bacterial Cell Counts and Standard Deviations

Trial	Number of Days	R1 (cells/mL)	R2 (cells/mL)	3 (cells/mL)	Average (cells/mL)	s
Reference	1	1.71E+07	1.75E+07	1.84E+07	1.77E+07	6.66E+05
Reference	7	9.90E+07	8.60E+07	7.50E+07	8.67E+07	1.20E+07
Reference	14	2.86E+08	3.50E+07	4.50E+07	1.22E+08	1.42E+08
Cycloheximide	1	1.71E+07	1.75E+07	1.84E+07	1.77E+07	6.66E+05
Cycloheximide	7	1.36E+08	1.40E+08	5.57E+08	2.78E+08	2.42E+08
Cycloheximide	14	1.15E+09	8.25E+08	4.01E+08	7.92E+08	3.76E+08
Allylthiourea	1	2.00E+07	4.60E+07	2.40E+07	3.00E+07	1.40E+07
Allylthiourea	7	2.80E+08	1.60E+08	1.40E+08	1.93E+08	7.57E+07
Allylthiourea	14	1.60E+08	4.40E+08	1.80E+08	2.60E+08	1.56E+08
Allylthiourea+Cycloheximide	1	2.00E+07	4.60E+07	2.40E+07	3.00E+07	1.40E+07
Allylthiourea+Cycloheximide	7	1.62E+08	3.36E+08	1.52E+08	2.17E+08	1.03E+08
Allylthiourea+Cycloheximide	14	3.02E+08	2.90E+08		2.96E+08	8.49E+06
EDTA	1	6.40E+07	4.00E+07	3.00E+07	4.47E+07	1.75E+07
EDTA	7	3.14E+08	3.08E+08	3.36E+08	3.19E+08	1.47E+07
EDTA	14	3.20E+08	3.06E+08	3.20E+08	3.15E+08	8.08E+06
EDTA+Cycloheximide	1	6.40E+07	4.00E+07	3.00E+07	4.47E+07	1.75E+07
EDTA+Cycloheximide	7	3.40E+08	3.60E+08	3.46E+08	3.49E+08	1.03E+07
EDTA+Cycloheximide	14	3.50E+08	3.50E+08	3.56E+08	3.52E+08	3.46E+06

APPENDIX B: FLUORESCENT IN SITU HYBRIDIZATION COMBINED WITH CONFOCAL LASER MICROSCOPY DATA

Table B.1 FISH/CSLM Data for Probe Combo EUB+A21

Trial	Day	A21 Dmax (μm)	EUB 338 Dmax (μm)	A21Amount (/4)	EUB Amount (/4)	Floc depth (μm)	Floc Diam (μm)
Control	1	40.97	40.97	2	4	55.1	198.75
		14.86	14.86	1	1.5	18.05	85.24
		22.54	24.3	1	1.5	47.9	177.75
	7	13.31	13.31	1	2	31.95	165.62
		29.1	27.28	2	2	43.05	193.1
		43.34	25.09	3	4	54.75	151.49
	14	38.75	26.25	3	4	57.75	141.39
		31.52	45.04	3	4	63.05	140.98
		25.8	33.17	2.5	3	51.6	182.6
Cycloheximide	1	40.97	40.97	2	4	55.1	198.75
		14.86	14.86	1	1.5	18.05	85.24
		22.54	24.3	1	1.5	47.9	177.75
	7	23.12	23.12	2	3.5	46.25	167.25
		23.35	27.6	2	3.5	50.95	159.97
		31.15	31.15	3	3	53.4	185.42
	14	38.25	51	2	4	59.5	188.65
		19.56	19.56	1	2	45.65	138.56
		38.25	43.5	2	4	59.5	179.37
Allylthiourea	1	10.45	13.44	2.5	4	20.9	188.65
		11.57	14.45	2.5	4	20.25	157.54
		11.78	15.2	2.5	4	22.8	154.3
	7	11.9	14.66	3	4	22.8	188.25
		10.94	9.57	2	4	19.15	95.34
		8.53	9.74	2.5	3.5	17.05	149.46
	14	13.65	15.6	3.5	4	27.3	166.43
		17.2	19.35	3.5	4	30.1	176.13
		13.43	15.11	3	4	23.5	191.48
Allyl+Cyclo	1	10.45	13.44	2.5	4	20.9	188.65
		11.57	14.45	2.5	4	20.25	157.54
		11.78	15.2	2.5	4	22.8	154.3
	7	7.14	7.14	2.5	4	12.5	155.98
		15.45	15.47	3	4	30.95	142.61
		20.31	31.97	2	4	37.3	188.65
	14	12.69	14.27	3.5	4	22.2	171.68
		9.32	9.32	3.5	4	18.65	165.62
		11.45	13.06	3	4	22.85	189.96
EDTA	1	18.57	23.21	3.5	4	32.5	119.6
		13.97	15.97	3.5	4	27.95	178.95
		18.51	20.57	3.5	4	28.8	151.09
	7	19.9	21.89	4	4	27.86	146.64
		19.75	21.73	4	4	27.65	148.25
		10.48	10.48	4	4	16.3	136.96
	14	16.89	19	3	3.5	29.55	155.93
		11.68	13.62	3	4	27.25	154.26
		12.55	16.14	2	4	25.1	185.42
EDTA+Cyclo	1	18.57	23.21	3.5	4	32.5	119.6
		13.97	15.97	3.5	4	27.95	178.95
		18.51	20.57	3.5	4	28.8	151.09
	7	9.51	10.7	3	4	16.6	178.55
		7.69	10.26	3	4	17.95	101.8
		11.37	12.79	3	4	19.9	138.98
	14	10.86	10.86	2.5	4	19	153.91
		11.38	12.64	3.5	4	17.7	182.59
		10.99	12.21	3	4	17.1	180.97

Table B.2 FISH/CSLM Data for Probe Combo A21+ NB1000

Trial	Day	A21 Dmax (Å m)	Nb1000 Dmax (Å m)	A21 Amount (/4)	Nb 1000 Amount (/4)	Floc Depth (Å m)	Floc Diameter (Å m)
Reference	1	23.1	25.42	2	3	41.49	185.01
		22.5	22.5	2.5	3.5	49	173.31
		28.28	22.63	2	4	45.25	183.4
	7	39.87	39.87	2	2	79.95	174.51
		18.79	28.18	1	1	75.15	185.82
		16.78	18.3	2	2	36.6	141.79
	14	27.89	26.89	4	3	62.75	154.31
		31.8	31.8	4	3	55.63	125.63
		35.53	40.6	2	1	71.05	164.82
Cycloheximide	1	23.1	25.42	2	3	41.49	185.01
		22.5	22.5	2.5	3.5	49	173.31
		28.28	22.63	2	4	45.25	183.4
	7	28.9	30.97	1	2.5	49.55	141.79
		18.49	18.49	2.5	3	63.4	134.12
		8.82	8.82	1	1	26.45	134.11
	14	21.26	21.26	2.5	1	45.52	170.89
		33.07	33.07	2.5	1	66.15	102.64
		31.91	31.91	3.5	2	55.85	147.04
Allylthiourea	1	11.38	11.38	3	2	22.75	189.46
		12.05	12.05	2	1	24.1	188.65
		15	15	3	2	28.6	188.32
	7	10.56	10.56	2.5	2.5	24.65	188.29
		9.75	9.75	3	3	19.5	189.89
		8.71	8.71	2	2	15.25	153.11
	14	11	11	2	2	11	188.65
		9.69	10.9	1	1	16.95	197.16
		8.26	8.26	1	1	12.85	157.97
Allyl+Cyclo	1	11.38	11.38	3	2	22.75	189.46
		12.05	12.05	2	1	24.1	188.65
		15	15	3	2	28.6	188.32
	7	10.58	10.58	3	2.5	21.15	182.59
		7.61	7.61	3	1	14.8	104.6
		10.51	10.51	3	2.5	21.7	180.6
	14	7.8	9.75	2.5	2	13.66	189.08
		8.71	9.8	3	1	15.25	188.65
		9.13	9.13	3	1	18.25	172.19
EDTA	1	8.1	8.1	3	1	16	130
		6.3	6.3	3.5	1	12.6	155.93
		13.97	11.98	3	1	27.95	177.75
	7	11.94	16.42	3	2	20.9	179.97
		13.11	14.42	3	1.5	18.35	170.47
		16.71	18.39	3	1	23.4	170.08
	14	13.95	17.05	3	2	21.7	182.21
		14.8	14.8	3	1	25.9	169.26
		12.74	14.34	3	2	22.3	97.76
EDTA+Cyclo	1	8.1	8.1	3	1	16	130
		6.3	6.3	3.5	1	12.6	155.93
		13.97	11.98	3	1	27.95	177.75
	7	15.7	17.19	3.5	1	20.06	157.14
		13.52	13.52	3	1	27.05	142.62
		11.71	12.89	3	1	16.4	148.25
	14	10	12.22	3	1	15.55	139.37
		5.89	5.89	3	1	10.3	156.35
		16.04	16.04	2	1	22.45	179.38

Table B.3 FISH/CSLM Data for Probe Combo A21 + NSV443

Trial	Day	A21 Dmax (μm)	NSV 443 Dmax (μm)	A21 amount (/4)	NSV443 amount (/4)	Floc Depth (μm)	Floc Diamter (μm)
Reference	1	22.99	14.63	2	2	50.15	184.61
		24.49	24.49	1	1	35.04	182.5
		8.97	8.91	1	1	22.15	188.24
	7	13.21	13.2	2.5	2.5	46.25	164.41
		15.25	8.71	2.5	2	30.5	178.15
		15.1	15.1	3	1	30.2	127.65
	14	33.72	33.72	3	1	78.69	190.26
		18.49	20.8	3	2	32.35	125.64
		20.94	20.94	2	1	48.85	189.86
Cycloheximide	1	22.99	14.63	2	2	50.15	184.61
		24.49	24.49	1	1	35.04	182.5
		8.97	8.91	1	1	22.15	188.24
	7	11.07	22.14	3	1	38.75	96.55
		15.41	17.98	3.5	3.5	35.95	145.43
		15.92	15.92	3	3	37.15	187.03
	14	32.94	37.06	3	1	57.65	182.19
		26.91	35.89	3	1	62.8	100.18
		27.02	34.75	2	1	54.05	98.57
Allylthiourea	1	11	14.14	3	0.5	22	173.71
		13.3	17.1	3	2	26.6	189.87
		6.81	6.8	3	1	15.9	185.42
	7	11.18	4.47	3	1	15.65	170.07
		5.67	5.67	3	2.5	11.35	132.91
		6.57	6.57	3.5	3.5	13.15	164.41
	14	8.05	10.35	3	1.5	16.1	187.03
		6.28	8.96	3	1	12.55	181.88
		7.1	9.15	3	1	13.19	186.64
Allyl+Cyclo	1	11	14.14	3	0.5	22	173.71
		13.3	17.1	3	2	26.6	189.87
		6.81	6.8	3	1	15.9	185.42
	7	12.2	12.2	3.5	2	21.35	166.03
		8.88	8.88	3.5	2	16.39	175.73
		13.46	13.46	2.5	1	23.55	186.25
	14	4.71	4.71	2	1	8.25	162.52
		9.67	9.67	3	1	15.05	189.06
		6.09	6.09	2	1	10.65	165.62
EDTA	1	21.61	21.61	3	1	27.5	181.38
		11.27	20.66	3	1	26.3	184.62
		13.92	17.01	3	1	21.65	180.57
	7	12.03	13.74	3	1	22.33	173.5
		12.86	14.5	3	1	22.55	185.82
		19.13	19.13	2	1	21.35	185.82
	14	19.54	21.99	2	3	34.2	148.66
		18	18	3	2	36	135.73
		14.51	14.51	2	2	25.4	171.3
EDTA+Cyclo	1	21.61	21.61	3	1	27.5	181.38
		11.27	20.66	3	1	26.3	184.62
		13.92	17.01	3	1	21.65	180.57
	7	10.29	13.71	3	1	16	130.89
		11.41	11.41	3	1	17.75	156.75
		7.83	7.83	3	1	13.7	154.73
	14	12.51	12.51	2	1	21.9	113.93
		10.91	15.01	2	1	17.75	94.53
		11.89	13.21	3	1	18.5	135.33

Table B.4 FISH/CSLM Data for Probe Combo NSO190+NSM156

Trial	Day	NSO190 Dmax (μm)	NSM 156 Dmax (μm)	NSO190 Amount (/4)	NSM 156 Amount (/4)	Floc Depth (μm)	Floc Diameter (μm)
Control	1	19.81	27.7	1.5	2.5	31.7	178.9
		22.4	23	1	3	35.8	180.3
		13.6	18.9	3	3	21.7	186.3
	7	12.3	14.42	2	2.5	20.6	129.7
		12.2	15.1	1.5	2	21.6	176.3
		13.4	15.4	2	2.5	22	180.4
	14	11.7	12.6	2	2	22.6	129.08
		12.9	14.4	2	2	25.7	177.9
		14.7	15.2	2.5	3	27.1	180.06
Cycloheximide	1	19.81	27.7	1.5	2.5	31.7	178.9
		22.4	23	1	3	35.8	180.3
		13.6	18.9	3	3	21.7	186.3
	7	13.1	14.2	2	3	23.4	187.9
		14.8	15.9	2	3	26.7	165.3
		31.5	33.1	2.5	2.5	56.2	175.2
	14	19.3	21	3	3	34.1	180.98
		14.1	15.2	1	2	27.3	134.2
		12.2	13	2	3	25.9	167.9
Allylthiourea	1	4.61	6.45	1.5	3	12.9	177.75
		12.86	12.86	1	3	22.5	184.66
		9.89	9.89	1	3	20.5	187.54
	7	11.89	11.89	3	2.5	20.8	183.4
		7.03	7.91	3	2	12.3	136.14
		7.35	7.35	1	1	14.7	134.12
	14	9.35	9.35	2	3	18.7	188.65
		8.57	8.57	3	3	15	185.82
		13.89	13.89	1	3	20.45	153.96
Allyl+Cyclo	1	4.61	6.45	1.5	3	12.9	177.75
		12.86	12.86	1	3	22.5	184.66
		9.89	9.89	1	3	20.5	187.54
	7	9.66	9.66	2.5	3	22.55	185.08
		11.51	11.51	2	3	20.15	186.24
		13.63	13.63	3.5	3.5	21.2	168.45
	14	7.62	7.62	3	3	15.25	189.49
		8.49	8.49	3	3	14.85	179.76
		7.97	7.97	2	3	13.95	181.76
EDTA	1	10.96	10.96	2	2.5	17.05	130.2
		9.51	9.51	2	2.5	16.65	137.75
		9.64	9.64	2	2	15	161.99
	7	8.67	11.15	2	3	17.35	179.55
		18.6	13.95	2	3	32.55	155.53
		9.37	10.54	1	2.5	16.4	118.36
	14	11.34	11.34	3	2	19.85	184.22
		12.54	12.54	3	2	21.95	110.68
		15.65	15.65	3.5	3	24.35	104.63
EDTA+Cyclo	1	10.96	10.96	2	2.5	17.05	130.2
		9.51	9.51	2	2.5	16.65	137.75
		9.64	9.64	2	2	15	161.99
	7	10.63	10.63	2.5	2.5	18.6	170.07
		14.75	14.75	3	3.5	22.95	156.74
		20.79	20.79	2	4	29.1	182.6
	14	12.92	12.92	3	3	20.1	181.79
		8.2	8.2	3	3	15.23	148.68
		8.71	8.71	3	3	15.25	142.6