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Evaluation of effluent organic nitrogen and its impacts on receiving water bodies

Item Type	Master Projects
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DOI	10.7275/5RVR-6A55
Download date	2026-04-10 12:52:26
Link to Item	https://hdl.handle.net/20.500.14394/4798

Evaluation of effluent organic nitrogen and its impacts on receiving water bodies

by

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A project report submitted in partial fulfillment of the
requirements for the degree of

Master of Science in Civil Engineering

Department of Civil & Environmental Engineering
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February 2012

Evaluation of Effluent Organic Nitrogen and Its Impacts on Receiving Water Bodies

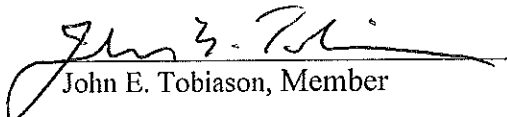
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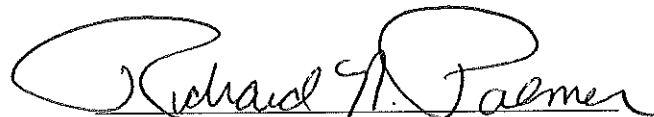
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ACKNOWLEDGEMENTS

I want to gratefully acknowledge the support and encouragement of my advisor, Dr. Chul Park. His enthusiasm for research is inspiring and contagious. I would also like to thank all the other professors in our program for their support and assistance.

I would like to thank Dr. Douglas Borgatti, Jane Brooks, and Mickey Nowak for their continued commitment to and financial support of this project. I also want to thank all of the technicians who work in the wastewater treatment plants that provided assistance in sampling.

A huge thank you to all of the graduate students in the program who have been my compatriots and colleagues these last two years; especially Pamela Westgate for training me in the lab, and assisting in any way she could. Without her help, I could not have achieved what I have today.

Last but not least, I would like to acknowledge and thank my family and friends for making this possible. Without their support and sacrifices I could not have done the work necessary to do this research and earn this degree. I love you all, and am looking forward to the fruits of our labor and sacrifice. I hope to spend more time with my parents in the future and enjoy the beauty of life.

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LIST OF ABBREVIATIONS

AM	Amherst wastewater treatment facilities
BT	Belchertown
SF	Springfield
WL	Winsor Lock
TN	total nitrogen
TOC	total organic carbon
TP	total phosphorus
COD	chemical oxygen demand
TSS	total suspended solids
VSS	volatile suspended solids
DO	dissolved oxygen
LIS	Long Island Sound
NPDES	national pollutant discharge elimination system
SDS-PAGE	sodium dodecyl Sulfate polyacrylamide gel electrophoresis

ABSTRACT

This research characterizes different wastewater treatment plant effluent nitrogen species in order to further understand the organic fraction of effluent nitrogen. Four effluents from biological nutrient removal (BNR) treatment and conventional activated sludge (CAS) treatment processes were investigated. Proteomic tools were applied in characterizing effluent protein. The protein profiles show the presence of active enzymes in all effluents and the organic nitrogen in the BNR effluent is more diverse than that in the CAS effluent, especially in the size range of 50-75 kDa. Size fractionation of the effluents suggests that over 70% of the effluent protein is soluble and most of the soluble proteins have a size smaller than 1kDa.

Laboratory incubation was performed to analyze the impacts of treated effluent on receiving water bodies. Freshwater and saline water were incubated with different effluents in this study. River water and ocean water represented the freshwater and saline waters respectively in the real environments. The fate of nitrogen species were closely monitored along with other parameters, including COD, VSS, TSS and TOC. Incubation result indicates that the filtration (<0.45 μm) of the effluent lowers the impact of effluent nitrogen loading on the receiving waters. Another important finding is that the patterns of biomass generation during the incubation are different between river and ocean waters. In addition, the BNR effluent shows higher potential than CAS effluent for both river and ocean water incubation, in terms of TSS, VSS and protein production.

1. INTRODUCTION

Harmful algal blooms are a worldwide phenomenon and have posed a significant challenge to fisheries, public health, and economies. It is reported that the Long Island Sound (LIS) has experienced algal blooms since the 1950's. In the spring of 2008, a large bloom occurred across Long Island's south shore bays during the spring, summer, and fall. Hypoxia, or low dissolved oxygen, appeared in the bottom water of western Long Island Sound. Extensive monitoring and modeling of the Long Island Sound have revealed excessive discharge of nitrogen from human activities as the primary pollutant causing hypoxia (Branca and Focazio, 2009). Nitrogen stimulates the growth of algae in the Sound, which eventually decompose in the nutrient limited environments. This decomposition would reduce the dissolved oxygen. There is enough nitrogen released in terms of point source and nonpoint source pollution, causing a hypoxia problem each year. In order to reduce the nitrogen impact, stringent discharge limits have been imposed on wastewater treatment plants which discharge large volumes of treated effluent to the LIS. National Pollutant Discharge Elimination System (NPDES) permits were established, which numerically specified the amount of suspended solids, coliform bacteria, and biochemical oxygen demand (BOD) that facilities are allowed to discharge. However, the nutrients released from the treated effluent are not fully regulated in all state permits. States along the Connecticut River, such as New York and Connecticut, are having more and more stringent effluent limits while states along the upper Connecticut River have less stringent limits. Strict concentration limits on nitrogen release require downstream states to operate the facilities to reach optimal goals.

Many of the wastewater treatment facilities along the Connecticut River are making efforts to reduce the nutrient loading in the final effluent by upgrading the current treatment processes to more advanced treatment processes, typically to a biological nutrient removal (BNR) process. Recently, the Mattabasset District in Connecticut decided to invest in a three-year, \$100 million upgrade project for its facility in order to meet new state requirements for nitrogen removal (Mill, 2011). The investment and efforts put into the upgrading projects are substantial.

In 2002, the Connecticut Department of Environmental Protection established the Nitrogen Credit Exchange program, which is one management strategy to reduce nitrogen loading (EPA, 2002). Seventy nine sewage treatment plants located throughout Connecticut were involved in this program. Facilities that reduced their nitrogen loading could trade their discharge credits with those that could not at the end of year. In addition to this program, other strategies for nitrogen control are applied to protect the cherished LIS natural water resources in the state.

The nitrogen that is discharged from wastewater treatment plant may contain organic and inorganic forms. Inorganic forms include ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-), while organic forms include proteins, amino acids, urea, amino sugars, and humic substances. A large variety of studies investigated the modification and optimization of different treatment processes in order to reduce the total concentration of nitrogen in the final effluent. Current technologies are able to reduce the soluble inorganic nitrogen ionic species substantially and achieve high removal rates by transforming them to free nitrogen and release to the atmosphere.

However, the organic nitrogen species in the treated effluent are not completely understood. There was a hypothesis that the organic nitrogen in the final effluent was recalcitrant and not bioavailable since the effluent underwent biological treatment. Recent researchers are providing more evidence that shows that not all of the organic nitrogen is inert after it is discharged to receiving waters (Bronk, D A et al., 2010). Nevertheless, the composition of organic nitrogen in the final effluent of different treatment facilities remains uncertain. Studies of nitrogen-containing organic matter in wastewater effluent have measured the quantity of various components (Pehlivanoglu-Mantas and Sedlak, 2008). Proteins, considered as one major group of effluent organic nitrogen, are likely to be diverse and include the recalcitrant protein in primary influent, as well as soluble microbial products (SMPs) that are generated by organisms in the biological secondary treatment process (Westgate and Park, 2010). The current knowledge on organic nitrogen in effluent is not enough to identify the organic composition in different treatment plants. Further characterization and comparison of organic nitrogen species should be performed.

Moreover, the real benefits of treatment upgrades have not been thoroughly assessed from a receiving water environment perspective. The bioavailability of the effluent organic nitrogen has been poorly investigated. The effluent discharges from wastewater treatment facilities contain a large group of organic nitrogen, whose information is barely provided when the effluents enter the receiving waters. This unknown fraction of nitrogen could react with the microbial community after entering the receiving water body. A nitrogen limited water body has a wealth of microbes which may uptake and utilize the nitrogen vigorously for their growth, since they survive in a salty and nutrient limited environment.

Potential ways that organic nitrogen in treated wastewater contributes to algal growth has been studied (Berman and Chava, 1999; Bronk, D et al., 2007; Bronk, D A, et al., 2010). Algal growth bioassays were conducted on denitrified wastewater effluent samples, in the presence and absence of bacteria isolated from effluent-receiving surface water (Pehlivanoglu and Sedlak, 2004). Nevertheless, the lack of information about the change of nitrogen in real environment conditions would inhibit the understanding of the impacts of the treated effluents on the receiving water bodies.

My study is innovatively designed to incubate secondary effluent with actual receiving water samples in the laboratory. Further characterization of treated effluent and receiving water is provides more information about the nitrogen species before incubation. The goal of this research was to evaluate proteins and organic nitrogen in secondary wastewater effluents and investigate the impact of nitrogen species from different plants on two receiving waters, the Connecticut River and Long Island Sound. The specific objectives of this research were to:

1. Gain a better understanding of effluent nitrogen, especially organic nitrogen in secondary effluent.
2. Study the environmental impacts of secondary effluents on receiving waters, especially the fate of nitrogen after entering receiving water bodies.

Treated effluents were collected from four wastewater treatment facilities that discharge to the Connecticut River. These facilities were the Amherst Wastewater Treatment Plant, the Belchertown Wastewater Treatment Plant, the Springfield Regional Wastewater Treatment Facility, and the Winsor Locks Wastewater Treatment Plant. Connecticut River water was collected in Northampton, MA, and LIS ocean water was collected in Old Lyme, CT.

Bioassays were performed by incubation of the effluents with receiving water samples under controlled conditions. Three of the effluents were incubated with the river and ocean water samples. Results presented in this thesis are the typical incubations of all incubations conducted during last two years.

2. BACKGROUND AND RELATED RESEARCH

This review starts with an overview of the nitrogen transformation in wastewater treatment processes and the effects of increasing nitrogen loadings in aquatic environments. Investigation of the fate of effluent nitrogen and its influence on receiving water bodies is also included.

2.1 The Nitrogen Transformation in Wastewater Treatment Plants

In typical wastewater treatment facilities, the effluent from the primary clarifier is exposed to oxygen and activated sludge in aerobic treatment; the biological conversion of Ammonium to nitrate in the aerobic treatment unit is defined as nitrification. Nitrification is a two-step process. The conversion of ammonia and ammonium to nitrite is performed by ammonia-oxidizing bacteria. And then, the nitrite-oxidizing bacteria complete the conversion of nitrite to nitrate. If the wastewater is subsequently subjected to anaerobic conditions, denitrification may occur. The biological reduction of nitrate to nitrogen gas by facultative heterotrophic bacteria is defined as denitrification. Denitrification occurs when oxygen levels are depleted and nitrate becomes the primary electron acceptor for microorganisms. Some nitrogen is converted into nitrogen oxide (NO) or nitrous oxide (N₂O) and leaves the system in the gas phase. The emission of this gaseous nitrogen lowers the nitrogen concentration in the treatment system. BNR processes lower the concentration of total nitrogen because of nitrification and denitrification occurrence (Tchobanoglous et al., 2003). The nitrogen that is discharged from conventional wastewater treatment plants is generally in the inorganic form of ammonium and nitrate, and in the organic form of dissolved organic nitrogen and particulate organic nitrogen. Treatment facilities that have nitrification/denitrification as a treatment process generally have low levels of ionic nitrate (NO₃⁻) in the effluent; most of the nitrogen from these plants is in the form of soluble organic nitrogen (Pehlivanoglu-Mantas and Sedlak, 2008).

2.2 Organic Nitrogen in Final Effluent

Organic nitrogen in the final effluent is mainly divided into two groups: dissolved organic nitrogen (DON) and particulate organic nitrogen (PON). Organic nitrogen in the wastewater effluent is diverse, and includes protein, urea, amino acids and humic substances (Berman and Bronk, 2003). For the advanced treatment processes, dissolved organic nitrogen took up 20% of the total nitrogen. It was the major component in low level total nitrogen effluent (Pagilla et al., 2006). DON is one of the largest fractions of effluent organic nitrogen (Barker and Stuckey, 1999; Holbrook et al., 2005; Pehlivanoglu-Mantas and Sedlak, 2006). Due to the diversity of organic nitrogen, not all of the organic nitrogen species in the final treated effluent are inert or biologically refractory.

Dissolved organic nitrogen in the effluent is also believed to be composed of two distinct groups: a large refractory group, and a labile group. Recalcitrant organic nitrogen is not biologically active in the receiving water body, while the other fraction is composed of highly labile compounds, such as amino acids and urea. However, the available information about the effluent organic nitrogen is limited. The component in the final effluent still remains uncertain. In the a study of effluent organic nitrogen, the author pointed out that up to 70% of the DON was not identifiable based on available methods (Bronk, D, et al., 2007).

Some studies were done recently to get a better understanding of effluent organic nitrogen. Research findings showed that the remaining soluble organic nitrogen was characterized as dissolved free and combined amino acids which were considered as the identifiable soluble organic nitrogen (Berman and Bronk, 2003). The unidentified part of the soluble organic nitrogen mainly consisted of hydrophilic, low-molecular weight compounds capable of passing through a 1000 Dalton ultrafilter (Pehlivanoglu-Mantas and Sedlak, 2008). Sattayatewa et al also reported that approximately 28–57% of the effluent DON was bioavailable (Sattayatewa et al., 2009). Further investigation of the effluent organic nitrogen revealed that in primary and secondary effluents, the protein concentration had a strong correlation with organic nitrogen and could take up to 60% of effluent organic nitrogen (Westgate and Park, 2010).

The concerns about the discharge of effluent organic nitrogen to the environment stimulate the research on the effluent organic nitrogen, especially the soluble organic nitrogen. Soluble organic nitrogen could be utilized for bacterial growth, phytoplankton growth, photochemical decomposition, and abiotic adsorption (Berman and Bronk, 2003). Another recent study focused on enzymes, which accounted for a large portion of soluble organic nitrogen, suggested that potential hydrolysis rate constants for ectohydrolases varied largely over time and among sampling stations in the Hudson River and western Long Island Sound. Aminopeptidase, which generally reacted with proteins, was consistently the largest portion of total hydrolytic activity (Taylor et al., 2003).

The insufficient information about the effluent organic nitrogen is the main motivation for this study. Instead of identifying single nitrogen species, the size fractionation is the technique used in the study for the characterization of the effluent organic nitrogen. Size separation could provide more useful information of the composition of the unknown group of organic nitrogen. Based on this, proper techniques could be applied to reduce the impacts of effluent organic nitrogen on the environment. In addition, the varying sizes of organic nitrogen also reflect the diversity of organic nitrogen in the domestic treated effluent.

2.3 The Impact of Organic Nitrogen on Receiving Water Environments

Recent investigations on effluent dissolved organic nitrogen (DON) utilization strongly suggest that DON should be included in nitrogen loading budgets to estuaries instead of the narrow focus on freshwater because of their bioavailability in the marine system. The concentrations of DON are often high, even in regions considered to be nitrogen-limited (Berman and Bronk, 2003). Studies showed that DON accounted for $38 \pm 22\%$ and $36 \pm 17\%$ of the total dissolved nitrogen in Yealm and Plym estuaries. Fresh water samples were observed to have a lower DON compared to saline samples (Badr et al., 2008). It was also reported that the DON concentrations were comprised 8 to 94% of the total dissolved nitrogen in the rivers. One of the bioassays results suggested that DON decreases were still observed in the low concentration river water by conducting a bioassay. And 23% of the DON was bioavailable in most of the rivers while the rest of them showed no change in DON concentration (Wiegner et al., 2006).

Bioassays became an important way to study the organic nitrogen and its impacts on receiving water. A series of bioassays were designed to analyze different nitrogen species' behaviors in the laboratory. The bioassays were conducted in different condition for specific purposes.

Bronk et al examined ways to differentiate between autotrophic and heterotrophic utilization under a dark bioassay. The collected effluents were concentrated by 0.2 μ m cartridge filtering and incubated with the James River water in a 13.5/10.5 h light/dark cycle. In the flasks were capped with aluminum foil and incubated in the dark at 20°C with gentle stirring (Bronk, D A, et al., 2010). In Urgan-Demirtas et al study, nitrified effluent and denitrified effluent were examined by The Printz Algal Assay Bottle Test in eight different scenarios. After the addition of the algae and bacteria inoculums, flasks were incubated at 20°C with exposure to fluorescent light (Urgan-Demirtas et al., 2008). Another researcher designed the bioassay to evaluate the role of bacteria in labilization of effluent organic nitrogen. All alga cultures were incubated on a shaker in triplicate 500 mL-Erlenmeyer flasks, at 20–22°C, with a 12-h light/dark cycle (Pehlivanoglu and Sedlak, 2004). The same bioassays protocol was found in the study of organic nitrogen bioavailability in the absence and presence of nitrate (Sattayatewa, et al., 2009). In A batch culture experiment, the cultures were covered with dark foil and incubated close to *in situ* temperature for 72 hours (Joergensen et al., 1999).

As the studies listed above, the light condition is one of factors that would affect the bioavailability of effluent organic nitrogen. Recent findings in freshwater and marine systems suggested that photochemical processes can affect the release of labile nitrogen from dissolved organic matter. DON from a freshwater is a source of labile nitrogen for microbial processes after the photochemical reaction occurrence associated with organic nitrogen (Berman and Bronk, 2003). The photochemical reactivity can alter the bioavailability of DON and these photochemical reactions can affect the lability of organic material along estuarine gradients (Minor et al., 2006). In addition, exposure to light during the incubation performed in the laboratory has impacts on the nutrient assimilation pattern (Bronk, D, et al., 2007). A recent paper shows that recalcitrant DOM can be converted to bioavailable forms via photochemical

reactions. The concentration of particulate nitrogen, chlorophyll a, and the biomass of phytoplankton and protozoa increased more in light exposure than in dark controls (Vähätalo and Järvinen, 2007). Another study also indicated that this release of labile nitrogen could be the reason why bacterial growth efficiency, bacterial nutrient demand, and bacterial biomass and respiration rates are influenced by light (McCallister et al., 2005). The effluent organic nitrogen (EON) bioavailability study also implies the similar light exposure influence on the organic nitrogen conversion (Murthy et al., 2006).

In order to simulate the real condition in the environment, the bioassay conducted in this study is exposed to the natural sunlight. All of the incubation bottles are subject to the natural day and night light cycle at 20°C. There is no specific control of the light condition. Overall impact of the discharged effluent on receiving water bodies is obtained. Therefore, the conditions from this study are close to the real condition with continuous mixing.

Besides the light condition, another leading factor that should be considered is the microbial community in the effluent and receiving water. The microbial community present in the aquatic system affects the bioavailability of organic compounds and the composition of microbial community varies along the ambient salinity gradients (Fisher et al., 1999; Marshall et al., 2005). Various bacteria and phytoplankton species have different transport and enzyme systems that allow them to take up a range of nitrogen substrates (Mulholland et al., 2004). The composition of DON is also believed to be affected by the bacteria and algae in the aquatic system. The growth of bacteria and algae altered the composition and even the bioavailability of DON (Hopkinson et al., 1998; Wiegner, et al., 2006). In the case of phytoplankton, the consumption of dissolved combined amino acid by the phytoplankton was observed. This amino acid stimulated the growth of phytoplankton as a nitrogen source in a series of studies (Bronk, D, et al., 2007). Bronk et al also reported that soluble organic nitrogen fraction of wastewater treatment plant (WWTP) effluent could contribute to coastal eutrophication, direct biological removal, and photochemical release of labile compounds in incubation condition (Bronk, D A, et al., 2010).

A number of organic nitrogen compounds, such as urea, dipeptides and cyanate, were utilized by microbial communities as a nitrogen provider (Berman and Bronk, 2003; Mulholland and Lee,

2009; Palenik et al., 2003). In addition, bacterial activity assisted the uptake of organic nitrogen and altered the biodegradability of recalcitrant organic nitrogen for algae generation (Berg and Jørgensen, 2006). Besides the specific organic nitrogen mentioned above, the soluble microbial products, such as enzymes and other extracellular polymer substances, led to the conversion of high molecule weight DON into low molecule weight labile organic forms (Mulholland et al., 2002; Palenik, et al., 2003; Stoecker and Gustafson, 2003). Urgan-Demirtas et al used a protocol containing bacterial and algal inocula to assess the bioavailability of DON. *Selenastrum capricornutum* algal culture was used for the algal inoculums. The results showed an increase in both algal chlorophyll a concentration and bacterial counts along with a decrease in DON concentration over time (Urgan-Demirtas, et al., 2008). In another study, algal growth bioassays were conducted on denitrified wastewater effluent samples in the presence and absence of bacteria isolated from effluent-receiving surface water. A Truckee River bacterial inoculum was concentrated and used to assess the role of bacteria in labilization of wastewater DON. *Selenastrum Capricornutum* was chosen as the algal species for the bioassay experiments. Bioassay results indicated that effluent DON is not bioavailable to the algae *Selenastrum Capricornutum* in the presence of bacteria. However, Pehlivanoglu et al noted that approximately half of the wastewater-derived organic nitrogen was available to the algae in the presence of bacteria.

The presence of bacteria assisted the consumption of effluent DON for algal biomass growth (Pehlivanoglu and Sedlak, 2004). Sattayatewa et al reported that approximately 28–57% of the effluent DON was bioavailable. Bioavailable (to algae and bacteria) DON (ABDON) and biodegradable (to bacteria) DON (BDON) results did not show significant differences in terms of quantity, but DON utilization rates by ABDON were higher than that of the BDON in the nitrate-removal samples. Therefore, ABDON requires a shorter time to exert the bioavailable fraction due to a symbiotic relationship between algae and bacteria (Sattayatewa, et al., 2009). Bacterial uptake or release of dissolved nitrogen compounds, including amino nitrogen, urea, ammonium and nitrate, were examined in the seawater from an estuary (Santa Rosa Sound, northwestern Florida) and an open-water location in the Gulf of Mexico. This study suggested that the bacterial nutrient dynamics were strongly related to oxygen consumption and the activity of enzymes in nitrogen assimilation. In addition, this study indicated that bacteria served more as

mediators of nitrogen in eutrophic conditions than those in oligotrophic conditions (Joergensen, et al., 1999). Another study was conducted to examine utilization of river DON by bacteria from an estuary. Concentrates of estuarine bacteria were obtained using water from a small estuary, Barnegat Bay, New Jersey. The results suggested that decreases in DON during incubation were accounted for by increases in microbial biomass plus conversion to inorganic nitrogen. Organic nitrogen inputs may contribute more to estuarine and shelf eutrophication than was previously suspected. These experiments demonstrated that the inorganic nitrogen species input was underestimated (Seitzinger and Sanders, 1997).

Studies have pointed out the importance of the nitrogen composition in incubation. The current studies use a highly selected bacteria and algae as inoculums to assess the impacts of organic nitrogen. However, no bioassay was conducted by incubating real effluent and receiving water directly. The concentrated inoculums might change the original microbial community in the collected sample. The extra pressure provided during filtration is likely to alter the microbes in the incubation inoculums. Also, a single species of algae and synthetic samples are widely used in the investigation of effluent organic nitrogen bioavailability. Study results from these bioassays are limited to the role of a small group of algae and bacteria separately. The overall interaction of other microbes present in the real environment is underestimated. Therefore, in this study, whole receiving water and effluent samples are examined to gain a better understanding of the overall impacts of the effluent organic nitrogen on the environment. All the incubation samples are not subjected to concentrate techniques. Effluent and receiving waters are mixed homogeneously as the bioassays get started. This is the ideal condition at the wastewater effluent discharge outlet.

As in the bioassay discussed above, salinity is also one of the factors that change the bioavailability of dissolved organic substance, and consequently affect photochemical reactions. The saline conditions in different salinity levels alter the organic carbon and organic nitrogen availability (McCallister, et al., 2005; Minor, et al., 2006). Also, due to the saline condition, the microbial community (bacteria and phytoplankton) was different in the estuary area (Marshall, et al., 2005). Differences in microbial community affect nutrient utilization in both the abiotic and biotic reactivity organic matter, including humic substances (Baalousha et al., 2006). Recent

studies show that ammonium ion could be associated with humic substance cation binding sites in freshwater, and ammonium then was transported to estuary where the cation binding sites were replaced by other cations in the saline water (Bronk, D A, et al., 2010). As the humic materials move downriver, the salt assisted the release of ammonium in the estuary area which was found in laboratory experiments with humic isolated from three different rivers (Wiegner, et al., 2006).

Ocean water is used to represent saline water in this study. The water quality of ocean water is different from fresh water in many ways. Salinity is much higher than that in the river water. Microbes in the ocean could be unique and the dominant species are not the same as those in the freshwater. Effluents are designed to incubate with the real ocean water and exposed to the same condition as the river water condition.

3. MATERIALS AND METHODS

3.1 Source of effluent and receiving waters

Effluents collected in this study were the treated wastewater from local treatment plants, included wastewater treatment facilities in Amherst (AM), Springfield (SF) and Belchertown (BT) of Massachusetts plus one in Winsor Locks (WL), CT. Amherst wastewater treatment plant deals with 7.1 million gallon wastewater per day, most of which is domestic wastewater. The primary effluent undergoes traditional activated sludge wastewater treatment, whose sludge retention time is around 4-5 days. The final effluent has a high TN concentration, which mainly consists of ammonium. There is minimal nitrification or denitrification occurrence in the treatment process. Therefore, the nitrite and nitrate concentrations in the final effluent are usually low or even undetectable. The total nitrogen concentration is always the highest among the facilities investigated in this study.

Springfield wastewater treatment plant treats up to 67 million gallons of wastewater per day for the Springfield region. In its primary influent, the industrial part comprises up to 20% of the total influent which is different from AM wastewater treatment plant. Ludtzac Ettinger (LE) treatment is applied in this treatment facility without internal recycle of wastewater. Generally, nitrification and denitrification occurs in the aeration and anoxic zones (Tchobanoglous, et al., 2003). As a result, nitrogen is removed efficiently when the nitrification and denitrification occurs. Even without the internal recycle of nitrified wastewater, the concentration of TN in the SF effluent reaches a level below 5mg/L.

The effluent from Windsor Locks wastewater treatment plant has similar characteristics as Springfield effluent. The advanced biological nutrient removal technologies in this plant substantially decrease the effluent nitrogen concentration and result in extremely low ammonium concentration in the effluents. Modified Ludtzac Ettinger (MLE) process applied in the Windsor Locks contributes greatly to the reduction of nitrogen in the final effluent. The sludge retention time is around 15 days.

The Belchertown wastewater treatment effluent is currently using the sequencing batch reactor to treat up to 1.3 million gallons wastewater per day, which is the smallest amount of wastewater among all the plants in this study. Overall, the effluent from four different plants are typical effluent, representing the final effluent generated from the technologies mentioned above. The Table 1 below is a summary of the wastewater treatment plants and the samples collection dates.

Table 3.1: The information about the wastewater treatment plants and sample collection in this study

Wastewater treatment plant	Treatment process	Treatment Capacity (MGD)	Sludge Retention Time (days)	Nitrogen species in final effluent	Sample collection date
Amherst	CAS	7.1	4-5	Ammonium, nitrate, organic N	06/07/10, 09/21/10, 12/16/10, 03/01/11
Springfield	LE	67	25	Nitrate, nitrite, ammonium, organic N	06/07/10, 09/21/10, 12/16/10, 03/01/11
Windsor Locks	MLE	2.1	15	Ammonium, nitrate, organic N	03/01/11
Belchertown	SBR	1.3	15	Ammonium, nitrate, organic N	09/21/10

For the receiving water samples, Connecticut River and estuary water samples were respectively collected near *J. Elwell Conservation Area*, MA and *White Sand Beach* in Old Lyme, CT. As expected, the salinity in the estuary sample was much higher than all the other samples.

3.2 Chemical analyses

Effluents and water samples were collected in plastic containers and kept in a 4°C constant temperature room for analysis before the incubation started. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined immediately after the samples were taken on the sampling day, while some samples were frozen for later measurement of protein, TN, ammonium, nitrate and nitrite concentrations.

Zymogram analysis was performed for effluent samples. The goal was to determine if they contained active proteolytic enzymes. The casein infused gel was able to separate the protein by using the electrophoresis. The enzyme activity was determined by the protein profile on the gel (Bio-Rad, Hercules, CA, USA). Pretreatment for the sample should be done before electrophoresis. All of the samples were combined with zymogram buffer (Bio-Rad, Hercules, CA, USA) and centrifuged at 12,000 rpm for 3 minutes. The supernatant after the centrifugation was collected and injected into the wells on the gel for the zymogram analysis. After this, the gel was stained by solution (Bio-Rad, Hercules, CA, USA) at a constant temperature at around 37°C for 12 hours.

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by following the method of Laemmli (Laemmli, 1970). In order to concentrate the much diluted sample, ammonium sulfate was used to precipitate the target substance. Since the gel electrophoresis required certain amount of protein in a limited volume well, the concentration of protein in samples should be high enough before analysis. Heating was applied when the protein concentrations were at a low level. Samples were pretreated by incubation with sample buffer consisting of XT Mops sample buffer and a reducing agent (Bio-Rad, Hercules, CA, USA) before concentrated samples were ready for size separation on polyacrylamide gels. Following heat concentration, samples were centrifuged at 12,000 rpm for 3 minutes and the supernatant was used for SDS-PAGE. Prepared samples were loaded onto pre-cast Criterion XT 4-12% gradient gels (Bio-Rad, Hercules, CA, USA). The proteins in the sample were separated on the gels by a potential of 80V for 20 minutes, followed by 100V for two hours (Westgate and Park,

2010). After electrophoresis, gels were stained with coomassie brilliant blue using Bio-Rad's Silver Stain Kit or Bio-Safe stain (Bio-Rad, Hercules, CA, USA).

Total protein concentrations in each of the effluents were measured using the Lowry method (Lowry et al., 1951). The concentration of standard protein substance, which in this study was bovine serum albumin (Fisherbrand Scientific, Pittsburg, PA, USA), was determined using Lowry Method. A series of calibration curves were obtained from this standard substance measurement.

On the day of sample collection, TSS and VSS were measured for primary and secondary effluents according to *Standard Methods* (APHA, 2005). Light absorbance for COD tests was determined using a Thermospectronic Genesys 10 UV Spectrophotometer (Thermo Spectronic, Madison, WI, USA).

Enzyme activity was determined by following the Hoppe method (Hoppe, 1983). The fluorescent products generated from enzymatic hydrolysis of fluorogenic model substrate model substrates were determined to represent enzyme activity. The L-leucine 7-amino-4-methylcoumarin was the substrate for Leucine-aminopeptidase. 5 mmol/L stock solution of L-leucine 7-amido-4-methylcoumarin was prepared before the sample was added. Samples were combined with the stock solution and incubated for 3 hours in darkness at room temperature. A pH 10 BDH buffer was mixed with post-incubated sample to obtain fluorescence activity at 455 nm, under excitation at 366 nm (Chappell and Goulder, 1994). Specific experiment procedure the calibration curves of protease and phosphate concentration are shown in Appendix G and H, respectively.

Total nitrogen concentrations were determined using the persulfate method (Hach, Loveland, CO, USA) and a Shimadzu TN analyzer (Shimadzu TOC-VCPH with TNM-1, Shimadzu North America, SSI Inc., Columbia, MD, USA). Ammonium, nitrate and nitrite ion concentrations were measured after the samples were filtered through a 0.45 μ m membrane. A Metrohm ion chromatograph (Metrohm, Herisau, Sz) was used to obtain the inorganic ion concentrations. The

standard curve of different inorganic nitrogen was shown in Appendix E. Organic nitrogen was the difference between total nitrogen and the soluble inorganic nitrogen ion concentration.

In order to perform size fractionation for all the effluent and receiving water samples, filtration was performed using 1kDa ultrafiltration membranes (Millipore, Billerica, MA, USA). Amicon stirred cells (Millipore, Billerica, MA, USA) were used under 50 psi pressure to filter out the materials that were larger than 1kDa. Prefiltration was done by filtering the river and ocean samples through 100 μ m mesh size membrane which was glycerol controlled pore glass membrane (Millipore, Billerica, MA, USA). Only materials that had sizes smaller than 1kDa in the effluent were capable of going through the ultrafiltration membrane.

3.3 Laboratory Bioassay

In this study, Connecticut River water samples and LIS estuary water samples were incubated with effluents from Amherst, Springfield and Winsor Locks wastewater treatment plants respectively. A clear two-liter Pyrex glassware bottle was used as an incubator which was autoclaved before incubation. Receiving water samples were filtered (<100 μ m) to remove large particles in the natural water body before incubation. Receiving water samples were incubated with each filtered (<0.45 μ m) and whole effluent from the AM and SF plants at room temperature. In this study, the whole effluent was defined as the effluent that did not undergo 0.45 μ m filtration. Whole effluent sample therefore included soluble and particulate fractions. For each scenario, one liter of receiving water sample was incubated with one liter of effluent for at least 7 days. The incubation was subjected to the natural light/dark cycles and continuous stirring during the incubation.

Killed control incubation was prepared separately. After the effluent was combined with receiving water in the bottle, they were subjected to autoclaving at 120°C for 15-20 min. After

cooling samples to room temperature, the killed control sample was incubated under the same condition as other regular samples.

4. RESULTS AND DISCUSSION

In this section, the chemical analysis results for the effluents are presented. Image results from proteomic analysis are also included. Important incubation results and discussion are also presented in this section. River and ocean water incubations are divided into two major parts in the section. The most significant findings from the incubation are shown and thoroughly discussed.

4.1 Effluent and Receiving Waters Analysis

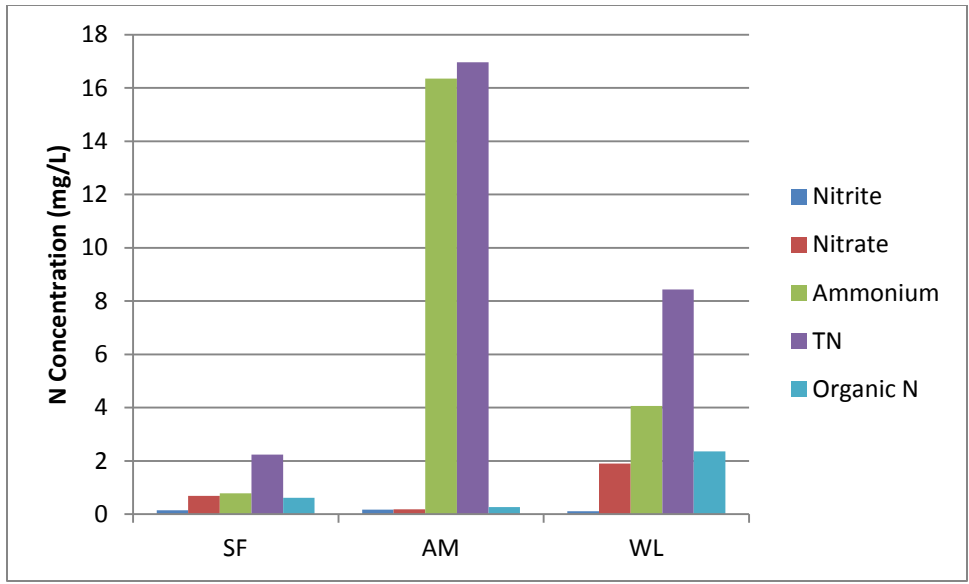
The characteristics of effluent and receiving water samples were thoroughly analyzed before starting incubation. The composition of different nitrogen species in both effluent and receiving water were intended to be assessed. Both chemical and proteomic analysis were performed for all the samples collected from the sites. The results from chemical and proteomic analysis for the effluent provided the information of the samples before incubation.

4.1.1 Chemical analysis

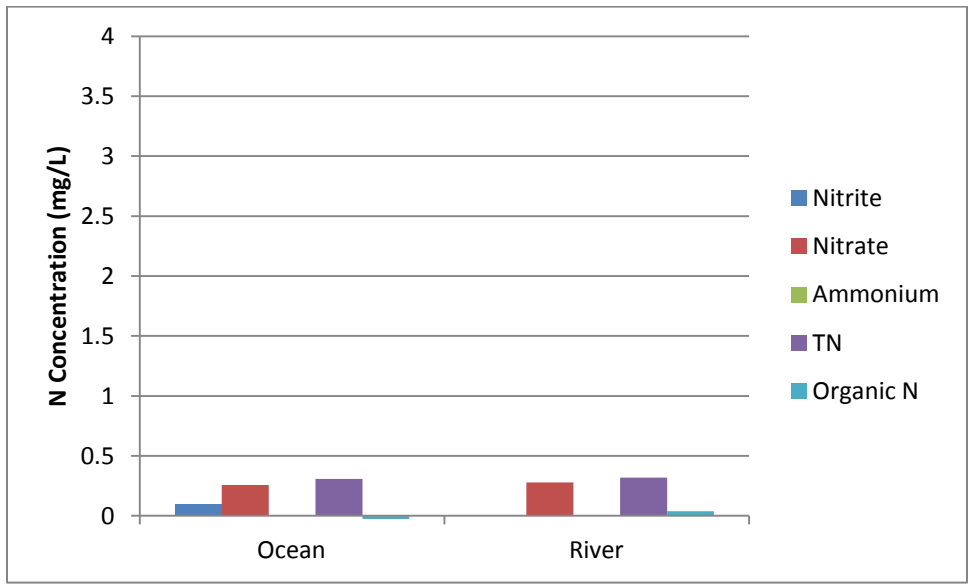
The chemical analysis was conducted for the all the effluent and receiving water samples, which included particulate and dissolved fraction of the effluent, collected in June and December of 2010, and March of 2011. The results showed different compositions of nitrogen species in the effluents (Figure 1). AM effluent had the greatest concentration of soluble nitrogen which was up to 17.1 mg/L. Ammonium was the major component and the concentrations of nitrite and nitrate were low in the effluent. SF and WL effluent contained less total soluble nitrogen compared to AM effluent, at 2.2 and 8.4 mg/L respectively. The nitrate concentrations in these two effluents were greater than the AM effluent. The organic nitrogen concentration was obtained by subtracting inorganic nitrogen from the total nitrogen concentration. The average soluble fraction of organic nitrogen in the AM, SF and WL effluents were 0.6, 0.3, 2.4mg/L. Phosphate concentration in the AM, SF and WL effluents varied, which were 0.34, 0.24 and 0.10

mg/L respectively. The phosphate concentrations in this study are all considered low in the effluents compared to the other investigations of effluent DON and dissolved phosphorus fraction removal, in which the effluent contained 0.25 mg/L phosphorous after enhance coagulation and microfiltration (Arnaldos and Pagilla, 2010).

River and ocean waters contained a low level of nitrogen, which were both below 0.5 mg/L. The inorganic nitrogen species in these receiving waters had significantly low concentration compared to the effluent samples.



(a)



(b)

Figure 1: Concentration of nitrogen species in effluents and receiving water (a) effluent samples; (b) receiving waters samples

The differences in total nitrogen concentration and compositions among the three effluents are mainly attributed to the treatment processes in these facilities. Advanced treatment processes in SF and WL are effective at removing nitrogen from the effluent. The BNR treatment process is effective with inorganic nitrogen removal and therefore would contribute to lower total nitrogen concentration in the final effluent (Tchobanoglous, et al., 2003). Nitrification and denitrification

occur in the two advanced treatment facilities and the effluent nitrate concentrations are both much higher than that in the CAS effluent. The results presented here agree with previous findings (Westgate and Park, 2010). Soluble organic nitrogen accounted for less than 2% of the total soluble nitrogen in the AM effluent while the WL and SF effluents were 27% and 28% of total nitrogen, respectively. This finding is in the range that reported in Pagilla et al study, which indicated that the dissolved organic nitrogen was above 20% in the low level total nitrogen effluent. The organic nitrogen remained in the BNR effluent became the challenge to achieve higher nitrogen removal rate for the wastewater treatment plants (Pagilla, et al., 2006).

Further information about the effluent nitrogen is critical because the effluent nitrogen composition would result in the different response in the receiving water bodies. Investigations on effluent organic nitrogen are still far from enough. The composition of organic nitrogen is more important than the concentration. The identification of organic nitrogen species in the effluent would benefit the evaluation of their impacts on receiving water body. One of the tools used to characterize the organic nitrogen is size fractionation. Effluents and receiving waters were filtered through a membrane filter ($<0.45 \mu\text{m}$) to eliminate the particulate nitrogen. Ultrafiltration was also performed for the effluents to filter out material greater than 1 kDa from effluent samples. The determination of protein concentration was conducted for the whole, soluble and 1kDa filtrate fractions of the effluent samples.

The results in Figure 2 shown below suggest the highest protein concentration in the AM effluent compared to the other two effluents. The soluble fraction which remained in the effluent after $0.45\mu\text{m}$ filtration in the AM, BT and SF final effluents accounted for 90%, 73%, and 87% of the total nitrogen respectively. This result was comparable to the findings in a prior DON study (Urgun-Demirtas, et al., 2008). For the 1kDa filtrate fraction of the effluents, over 60% of the soluble proteins were capable of passing through the 1000 Dalton membrane in all the effluent samples. A study of effluent organic nitrogen (Bronk, D A, et al., 2010) confirmed the presence free amino acids and small peptides in the effluent. Therefore, 1kDa filtrate fraction of effluents mainly consists of free amino acids and peptides due to the size fractionation analysis.

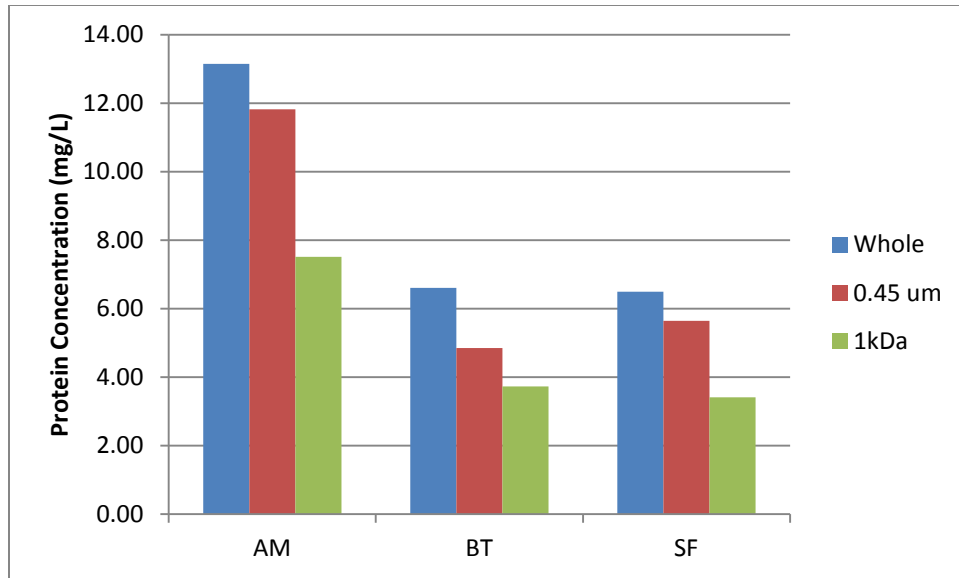


Figure 2: Protein concentrations of different effluent fractions (Sept, 2010)

It is not surprising to find that the effluents contained large amount of small proteins since biological treatment generates a variety of organic nitrogen substances, including soluble microbial products generated in upstream treatment (Barker and Stuckey, 1999). For the low molecule weight organic nitrogen part of effluent, further investigation should be performed in order to understand the identifiable species concentrations in these wastewater treatment plants. The protein concentrations obtained in this study are still not enough to identify the components in low molecule weight organic nitrogen group.

However, it is a challenge to identify the effluent organic nitrogen species although a prior study noted that the protein concentration in the effluent is strongly correlated with organic nitrogen (Westgate and Park, 2010). Similar to protein, the total amino acids and EDTA accounted approximately for less than 30% of the DON leaving 70% of the DON unidentified. It is likely that these compounds consist of a complex suite of partially metabolized compounds of biogenic origin (Pehlivanoglu-Mantas and Sedlak, 2008). Therefore, the advanced analysis techniques should benefit the effluent organic nitrogen study, especially the small size organic nitrogen in the effluents.

4.1.2 Proteomic analysis

The Zymogram profiles for three effluents are shown in Figure 3. The presence of active enzymes in the final effluent in the AM, BT and SF are indicated with clear white bands. The band intensity in each of the three lanes also reveals the enzymes present in the final effluent. The bands located in each lane suggest that active enzymes are contained not only in the effluent of the CAS and BNR processes but also in the SBR treatment processes. It is noted that the bands for the AM effluent lane were wider than those for BT and SF. It is reasonable to assume that the concentration of active enzyme in the AM effluent is the highest among all the effluents. Different from the finding in Westgate study (Westgate and Park, 2010), which the SF secondary effluent showed a different sets of bands compared to other local secondary effluents, there is no significantly different enzyme band appearing in SF secondary effluent sample.

Main finding in this proteomic study provided more information from different wastewater treatment plants. The presence of enzymes in the final effluents is confirmed by zymogram profiles. The variation of enzymes in the secondary effluents indicates that the impact of treated effluent on the receiving water bodies could be different. Thorough study of the of enzyme categories in current wastewater treatment plants would provide more information about the enzymes based on this size fractionation. Consequently, the impact of different enzymes from treated effluent on the real water body could be further investigated.

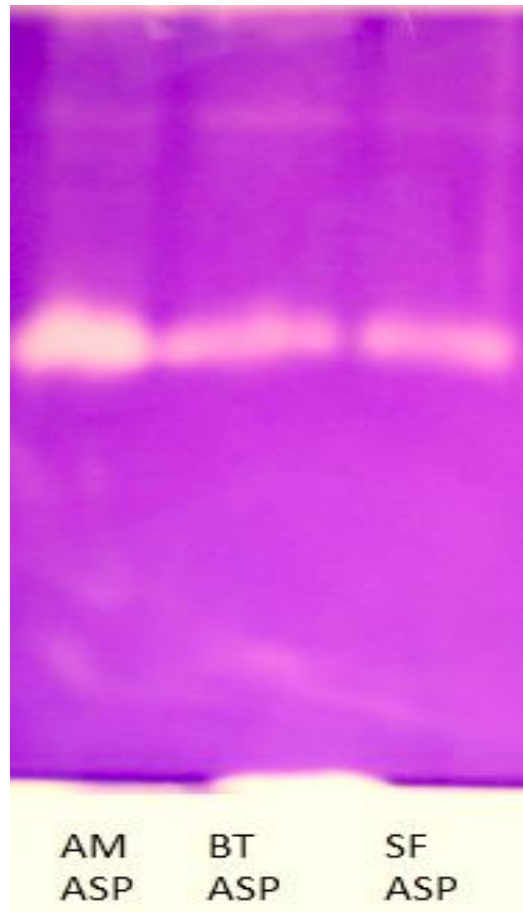


Figure 3: Zymogram of ammonium sulfate precipitated (ASP) AM, BT and SF effluent (Sept, 2010)

SDS-PAGE protein profiles in Figure 4 below demonstrate the different sizes of protein in the AM and SF primary and secondary effluents. Both of the treatment processes were able to largely reduce the nitrogen level, including proteins in the primary effluents. It is apparent that nitrogen level in the secondary effluent was substantially low compared to primary influent. Also, AM and SF shared several bands in their primary effluent lanes, which suggested similar protein contained in the primary effluent. In the secondary effluent lanes, some of the bands disappeared due to the treatment process, which had the molecule weight between 50-75 kDa. The disappearing bands on the secondary effluent lane indicated that some of the proteins in primary effluent were biodegradable while the recalcitrant proteins still shown on the profile.

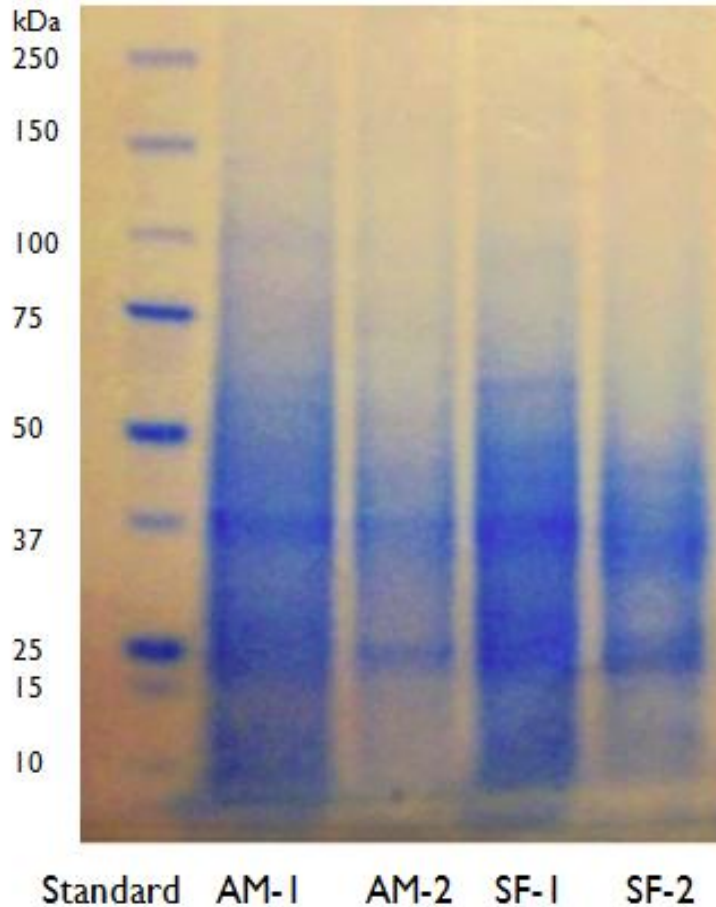


Figure 4: SDS-PAGE of primary (-1) and secondary (-2) effluent for AM and SF (Sept, 2010)

Moreover, the secondary effluent lanes also show the differences of the protein composition in the final effluent. The SF effluent had more bands in the range of 25-50 kDa compared to the bands in the AM effluent. The diversity of proteins in the SF effluent might be related to the treatment processes in the plant as discussed before. In addition, sludge retention time (SRT) in the SF is usually as long as 25 days while the AM just around 10 days. The long SRT increase the chance that new protein would be generated (Barker and Stuckey, 1999). These new proteins in SF could be the products from the metabolism of biomass. The SDS-PAGE profile obtained from the effluent was not always the same. The newly generated proteins have varying size. It is reported that the composition of the protein as shown above might be alter by the operational conditions and the seasonal variability of influent in each treatment plant (Westgate and Park, 2010).

4.2 Laboratory Bioassay

Incubation was performed to investigate the impacts of effluent nitrogen on the receiving water body. Effluent was incubated with receiving water under a controlled condition, which was designed to mimic the real environment. The incubation time varied from 1 to 3 weeks. Both biotic and abiotic reactions occurring inside the incubation bottle might change the concentration of nitrogen species. The fate of nitrogen was monitored throughout the whole incubation.

4.2.1 Killed Control Incubation

Killed control (KC) incubation was performed in order to study the change of nitrogen fate caused by abiotic reaction. Before the incubation started, all the incubation bottles were autoclaved at 120°C for 20 min. They were all exposed to the same conditions in the laboratory as the other regular incubation bottles. The change of the fate of nitrogen in the killed control bottle was only attributed to the abiotic reaction if it was seen during the incubation.

As Figure 5 shows, without the participation of active algae and bacteria in the river water incubation, there is only small change in TSS, VSS and protein concentration. In addition, the small change in protein concentration indicates that organic nitrogen turnover was not apparent during this river water incubations conducted. Protein concentration in both the AM and SF incubation were almost at the same level at the end of incubation as the starting point. Therefore, the abiotic reaction is not the main factor that contributes to the biomass growth and the change of the fate of effluent nitrogen.

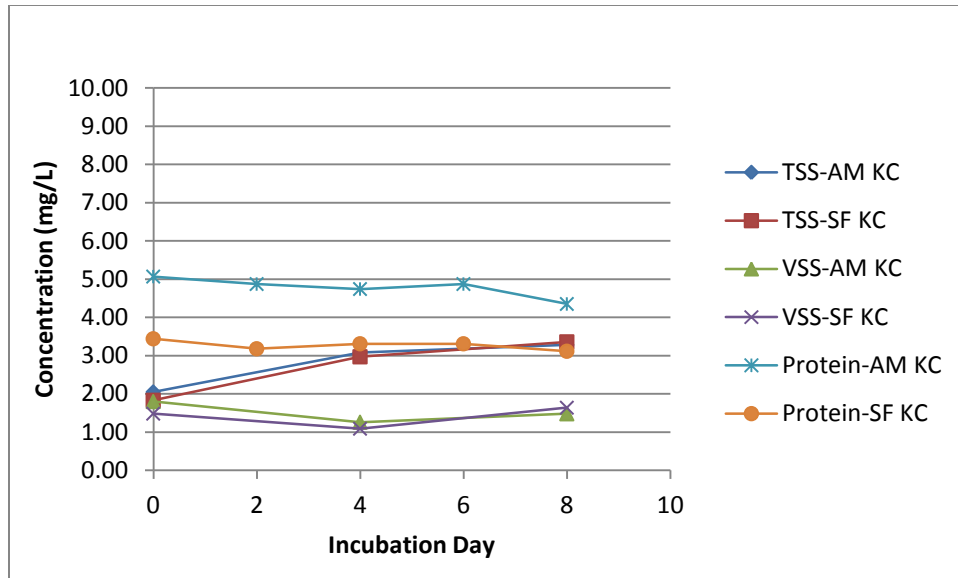


Figure 5: TSS, VSS and protein concentration of killed control samples over incubation time (Jun, 2010)

4.2.2 River Water Incubation

Most wastewater treatment plants discharge effluent to river or lake, which are defined as freshwater. The impacts of treated effluent on the river water were investigated by performing river water incubation under lab-controlled conditions. In this river water incubation section, the results from the three different incubation bioassay sets are discussed.

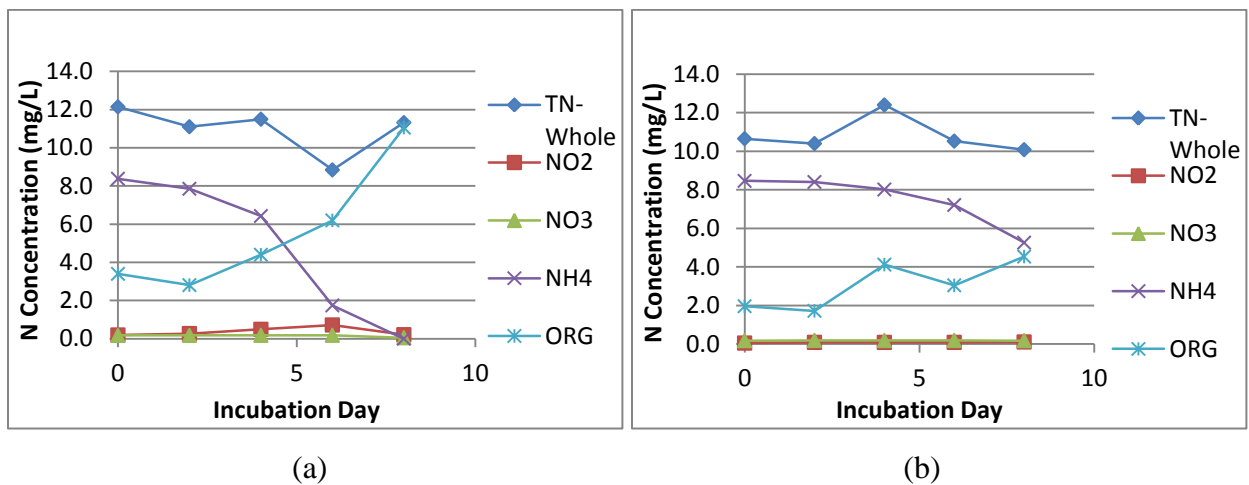
Compared to the effluent water samples, the river water contained a relatively low concentration of total nitrogen. Nitrogen from the effluents is the main nitrogen source in the incubations. Since the AM effluent contains higher total nitrogen level, the AM effluent incubation bottles contained a higher nitrogen concentration than SF bottles at the starting point.

The whole and soluble fraction of the AM and SF effluent samples were incubated separately with river water. Filtration was performed for effluent samples because this could possibly represent facilities that installed tertiary treatment processes. To understand the impacts of tertiary processes on the receiving river water, the soluble part of effluent was incubated with river water separately.

June, 2010 incubation set

This incubation was conducted during June, 2010. Both AM whole and filtered effluent incubation sets generally shared the same change pattern of change in nitrogen species as shown in Figure 6. The whole total nitrogen concentration was relatively constant compared to inorganic nitrogen in AM incubations. Ammonium, which was the major inorganic nitrogen in the AM effluent, decreased right after the incubation started. Unlike the whole effluent incubation, the filtered AM effluent incubation showed lower decrease rate than the whole one in the change of ammonium concentration. Organic nitrogen concentration increased in both AM incubation bottles. The soluble fraction of AM effluent incubation had 4.5mg/L organic nitrogen while the whole AM effluent incubation reached up to 11.1mg/L at the end of the incubation.

The AM incubation results suggest that the particulate fraction of the effluent also contributed to the reaction with river water in terms of ammonium utilization although the ammonium utilization rate is not as high as the whole AM effluent incubation rate. This utilization of inorganic nitrogen in laboratory incubation is also observed in previous research works (Bronk, D A, et al., 2010; Glibert et al., 1995). In addition, it is noted that filtration would benefit the receiving water because the generation of organic nitrogen in the soluble AM effluent incubation bottle is less than the whole AM effluent incubation bottle in this incubation set.



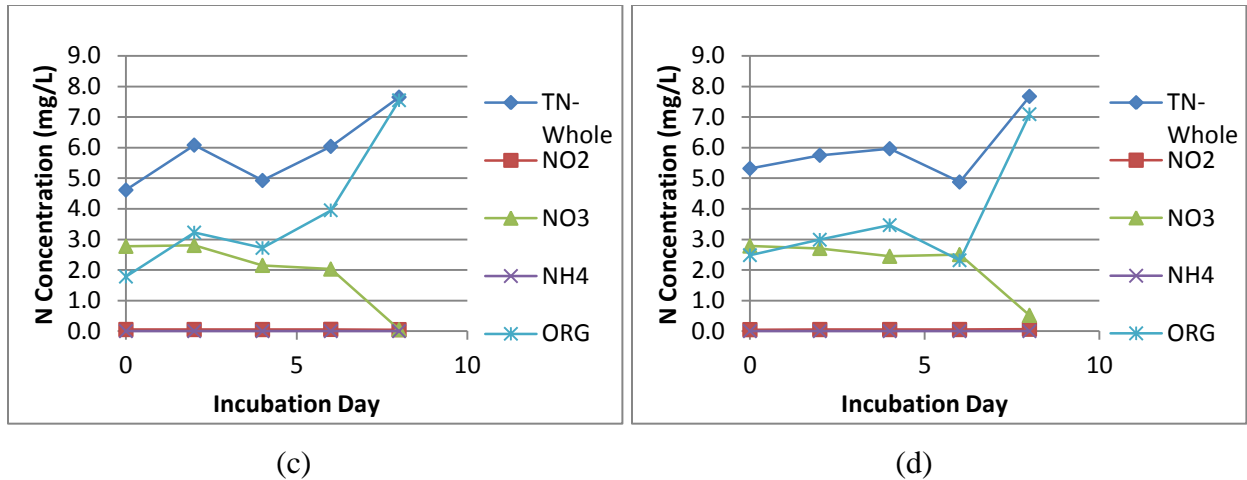


Figure 6: The change of nitrogen species concentrations in river water incubation over incubation time (a) AM effluent incubated with river water; (b) Filtered AM effluent incubated with river water; (c) SF effluent incubated with river water; (d) Filtered SF effluent incubated with river water (Jun, 2010)

The SF effluent incubation showed an increase of organic nitrogen concentration from the start of the incubation. Nitrate concentrations declined after Day 7 during the incubation along with a sharp increase in organic nitrogen, protein concentrations, TSS and VSS (Figure 7). Both of the whole and soluble SF incubation reached the same level of whole TN (soluble and particulate nitrogen) and organic nitrogen concentration at the end of the incubation. The *Chlorophyll a* results (Appendix A) also showed that the AM incubations reached the highest *Chlorophyll a* concentration at the end of incubations. Dissolved organic carbon was measure to monitor the change of carbon during the incubation. The correlation between protein generation and the change of organic carbon requires more incubation results before conclusions can be made.

In this river incubation set, one major difference between the whole and filtered effluent incubation was the amount of newly generated organic nitrogen. Obviously, the filtration lowered the generation of new organic nitrogen only for AM effluent incubation. The increase of organic nitrogen in the SF effluent incubation reached to similar level at the end of the incubations. Based on the results, filtration is likely to benefit the river water body in terms of organic nitrogen generation for the effluent from conventional treatment process. In addition, it should be noted that both of the SF effluent incubation sets had higher organic nitrogen

generation than the soluble AM effluent incubation set even if they started with less nitrogen concentration at the beginning.

Compared to the AM effluent incubations, organic nitrogen concentration in the SF effluent incubation remained not stable in the early period of the incubation. The organic nitrogen in the SF incubations increased all through the incubation with a faster rate in the late period of the bioassay. Several other studies were found on effluent organic nitrogen indicated that the growth of certain algae and bacteria were related to specific nitrogen source provided (Bronk, D, et al., 2007; Pehlivanoglu and Sedlak, 2004). In this incubation set, the different organic nitrogen production patterns were probably related to the microbes in the river, and the way they utilized the nitrogen species available in the incubation bottles. The bioavailability of effluent nitrogen in the SF incubation is greater than that in the AM effluent.

Besides the effluent nitrogen species concentration, the change of protein concentration was also monitored throughout the incubation. The AM whole effluent incubation revealed the highest production of soluble protein as shown in Figure 7. The soluble protein concentrations increased in the AM whole effluent incubation bottle and the rest bottles had similar soluble protein concentrations. The soluble protein concentrations again demonstrated that the filtration would lower the generation of organic nitrogen since protein is the largest group of the identifiable effluent organic nitrogen. The stable and low protein concentration in the kill control bottle (data not shown) served as evidence that the biotic reaction occurred inside the bottles was the leading factor contributing to the change of nitrogen species in the incubation. Effluent filtration reduced the nitrogen impacts on the receiving water body and resulted in comparatively low generation of protein, which was also confirmed by overall TSS and VSS generation.

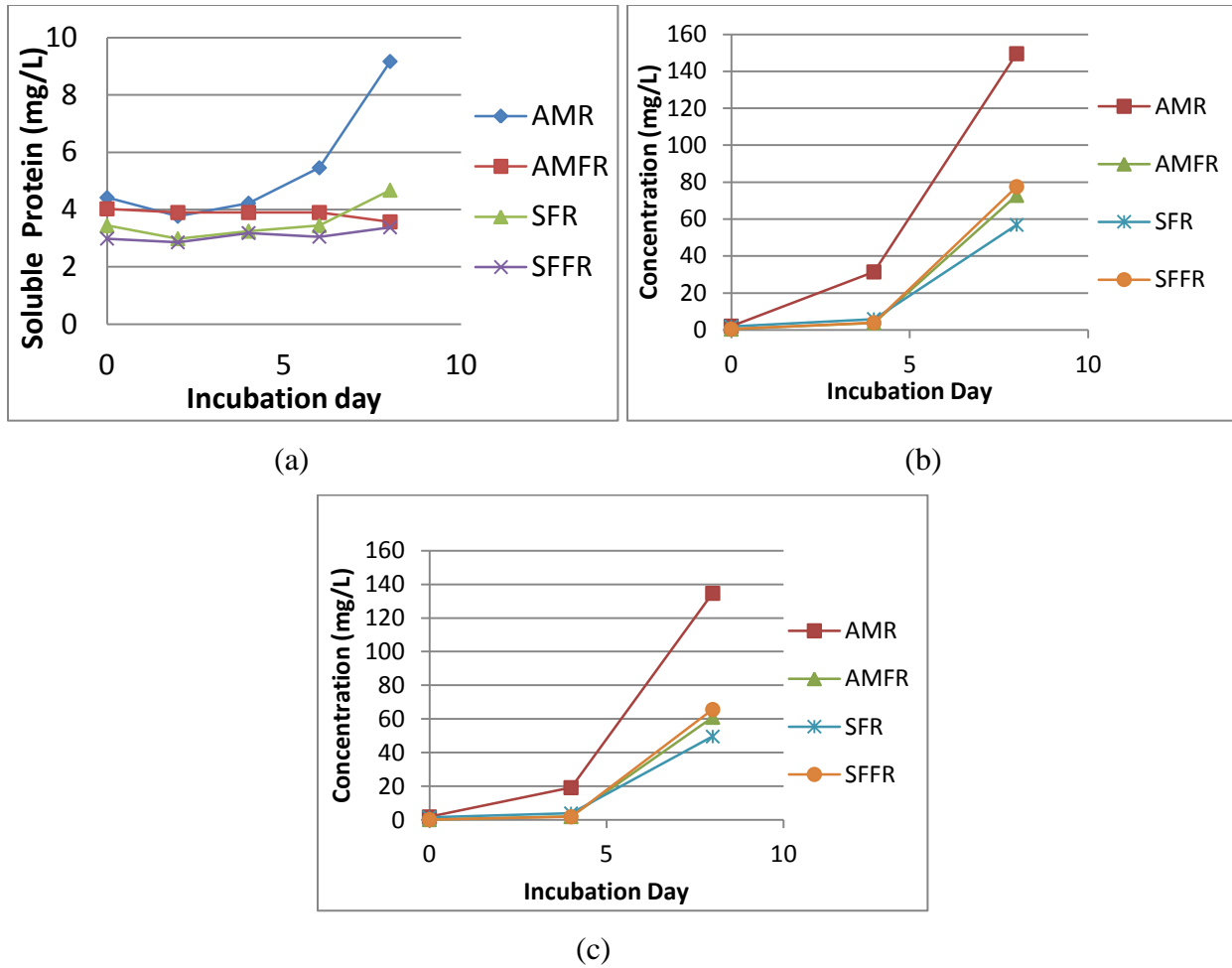


Figure 7: The TSS, VSS and protein concentration of river water incubation over time (a) Soluble protein concentration; (b) TSS; (c) VSS. (Jun, 2010)

It is important to note that the SF effluent contained much lower nitrogen concentration than the AM effluent at the starting point of incubation. However, both the SF whole and soluble fractions contributed to the similar level of solids generation as the AM filtered effluent. The SF effluent incubation could achieve the same level of suspended solids as the AM soluble fraction effluent even the nitrogen level is low. This convinced the point that the potential of SF organic nitrogen is higher than the AM effluent organic nitrogen in terms of biomass generation during the incubation with river water.

For the soluble protein, only the AM whole effluent presented a sharp increase in the late incubation period. Other incubations did not show a similar production of soluble proteins. This

could be explained by not only the higher total nitrogen concentration in the AM effluent also but the materials present in the particulate fraction of the effluent contributed to large generation of protein. The newly generated protein in the AM whole effluent incubation bottle was related to the high yield of biomass in the bottle. The growth of biomass would alter the composition of organic nitrogen in the bioassay, which is also found in other effluent organic nitrogen study (Joergensen et al., 1999).

December, 2010 Incubation Set

In this incubation set, Figure 8 shows a decrease of soluble nitrogen at the end of incubation. The total soluble nitrogen concentrations in the both of the SF effluent incubations decreased to around 1mg/L while the AM total soluble nitrogen concentration dropped down to around 6mg/L in the end. The soluble organic nitrogen in the AM effluent incubation bottles was totally consumed while the whole and soluble fraction of the SF effluent incubation bottles remained 0.53 and 0.38 mg/L, respectively.

As shown in Figure 8, the decrease of inorganic nitrogen suggests that it was utilized by the algae and bacteria at the beginning of the incubation (Bronk, D A, et al., 2010; Urgan-Demirtas, et al., 2008). The soluble fraction of AM and SF effluent incubations shared the same pattern of nitrogen concentration change over the incubation; however, both of them were not as dynamic as for their whole fraction effluent incubation. Without the particulate fraction of the effluent, the overall interactions between effluents and river water were again not as vigorous as for the whole effluent incubation. The yield of biomass in the AM soluble fraction effluent incubation was less than the whole effluent incubation (Figure 9). Filtration for the effluent before incubation reduced the amount of nitrogen available for the microbial uptake, which resulted in the differences of biomass growth between whole and filtered effluent incubations. These results are in accordance with the results found in June, 2010 incubation sets.

In addition, the soluble organic nitrogen decreased quickly in SF incubation bottles compared to that in the AM bottles in the early period. And both of the AM and SF incubations showed that the inorganic nitrogen species were not depleted as fast as expected. A possible reason for this is that some inorganic nitrogen was from the conversion of organic nitrogen. The newly converted inorganic nitrogen inhibits the fast consumption of inorganic nitrogen. Photochemical release of labile nitrogen was found in other bioassay studies (Bronk, D, et al., 2007; Bronk, D A, et al., 2010; Minor, et al., 2006; Vähätalo and Järvinen, 2007).

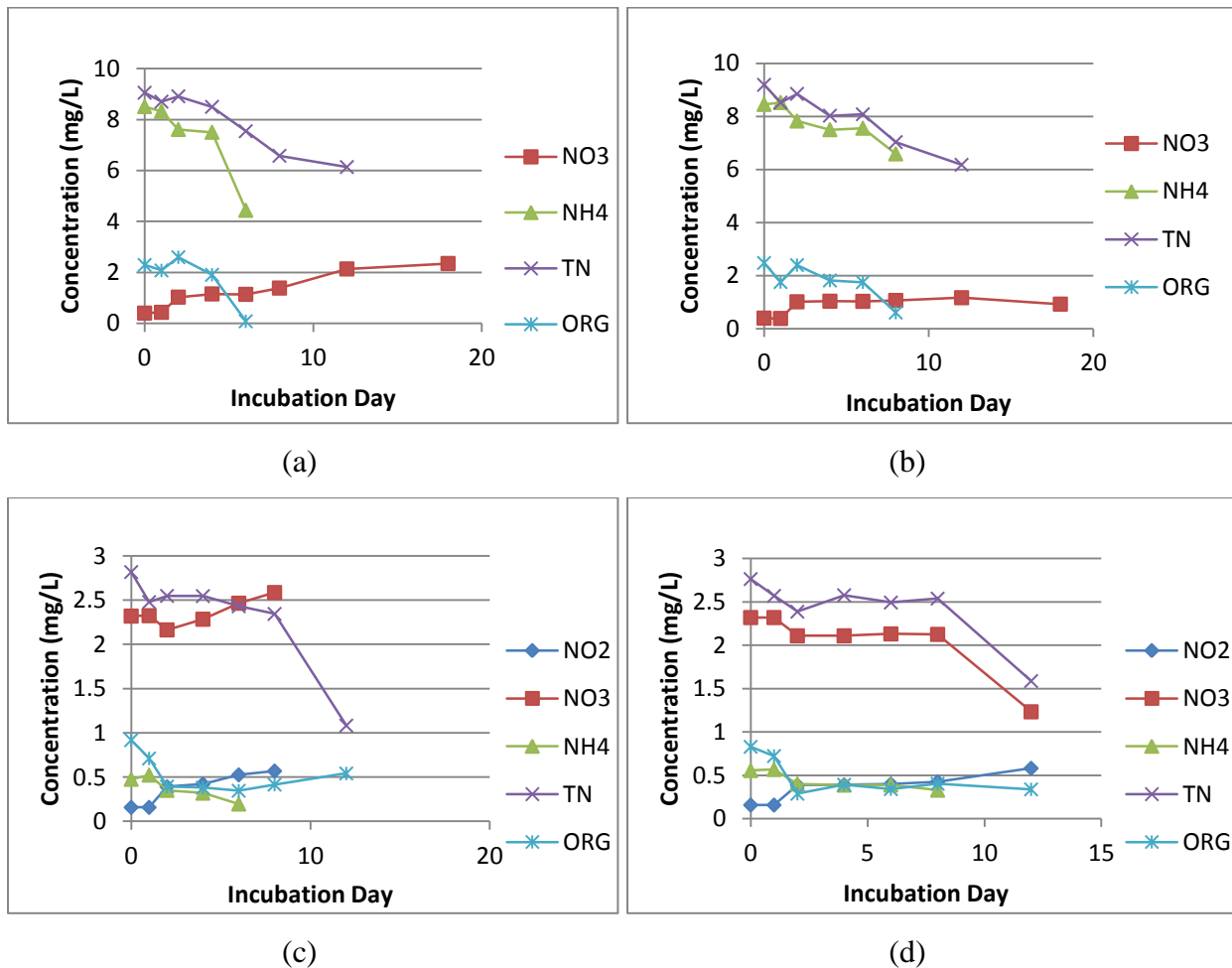


Figure 8: The change of soluble nitrogen species concentrations in river water incubation over incubation time (a) AM effluent incubated with river water; (b) Filtered AM effluent incubated with river water; (c) SF effluent incubated with river water; (d) Filtered SF effluent incubated with river water (Dec, 2010)

In our study, the incubation was exposed to natural sunlight and followed a natural dark/light cycle. The photochemical reaction might have altered the liability of organic nitrogen. The newly converted labile organic nitrogen could have assisted the growth of biomass. The soluble organic nitrogen in the AM effluent incubation was degraded until the late period. At the same time, the TSS and VSS concentration inside the bottles showed a sharp increase. The SF incubation bottles showed the consumption of organic nitrogen in the early period. This utilization of organic nitrogen could be attributed to the photochemical reaction occurred inside the bottles (Wiegner, et al., 2006). Also, the organic nitrogen remaining in the SF incubation bottles reached steady values in the late period because the newly generated biomass could have yielded a certain amount of soluble organic nitrogen with their metabolism. Another reason might be, as discussed before, the protein in the SF effluent was diverse. The diverse protein was strongly correlated with the complexity of organic nitrogen composition. It was likely that some of the organic nitrogen in the SF effluent was not biodegradable even after a long exposure to the receiving water body.

Since the results presented for this particular time of year are limited, it is still hard to identify the source of the recalcitrant organic nitrogen toward the end of the SF incubation. Whether this group of recalcitrant organic nitrogen was from the original SF effluent nitrogen or newly generated nitrogen during the incubation is not certain.

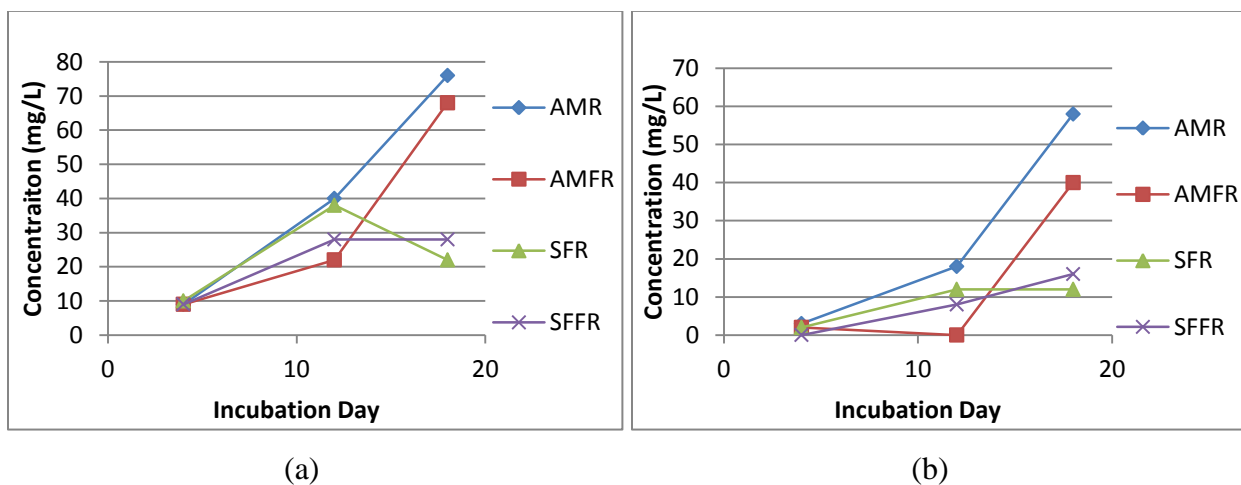


Figure 9: TSS and VSS of the river water incubation over time (a) TSS; (b) VSS (Dec, 2010)

The TSS and VSS data shown in Figure 9 reveal that a large amount of biomass was generated at the end of incubation. For both of the AM and SF effluent incubations, the VSS started from around 2-3 mg/L. At the end of incubation, AM and SF incubation reached around 80 and 50 mg/L, respectively. The AM whole and filter effluent incubation bottles had more solids than the SF bottles. The suspended solid in this incubation is closely related to the amount of organic nitrogen. A similar organic nitrogen study also showed the increase in biomass and decrease in total nitrogen and soluble organic nitrogen was strongly related to DON bioavailability and cell assimilation (Urgun-Demirtas et al., 2008).

Filtration again lowered the impact of AM effluent on river water while similar result was not shown in SF effluent incubation. The SF whole and filtered effluents ended up with similar TSS and VSS generation. Filtration for the SF did not affect the final biomass yield in the end of the incubation.

March, 2011 incubation set

Figure 10 shows the results for the March, 2011 incubations of AM, SF and WL effluents. In the early period of the incubation, the inorganic nitrogen species available in the bottles were consumed rapidly. The inorganic nitrogen species in the SF and WL incubation bottles was almost consumed at the end of incubation, most of the soluble nitrogen remained was organic nitrogen. Different from other incubation bottles, the AM effluent incubation bottle showed an increase of nitrate in the late period of incubation. The concentration of nitrate increased from almost 0 to 2.0 mg/L. SF and WL incubation data suggested that organic nitrogen concentrations remained the same level during this early period of incubation, which was different from AM effluent incubation.

For the SF and WL incubation bottle, the ammonium and nitrate were utilized by the microbes in the river water; other studies also concluded that the inorganic nitrogen was available to most of the microbial in the aquatic system (Bronk, D A, et al., 2010; Glibert, et al., 1995). The organic nitrogen concentration in these two incubation bottles did not change a lot. However, a decrease

of organic nitrogen in the AM effluent incubation bottles was observed in the early period of the incubation. This decrease of organic nitrogen inside the AM incubation bottle might, shown in Figure 10, imply that the AM effluent contained certain amount of biodegradable soluble organic nitrogen. Also, some of the soluble organic nitrogen substances were potentially converted to soluble labile form and metabolized by the algae and bacteria in the river water (Bronk, D A, et al., 2010).

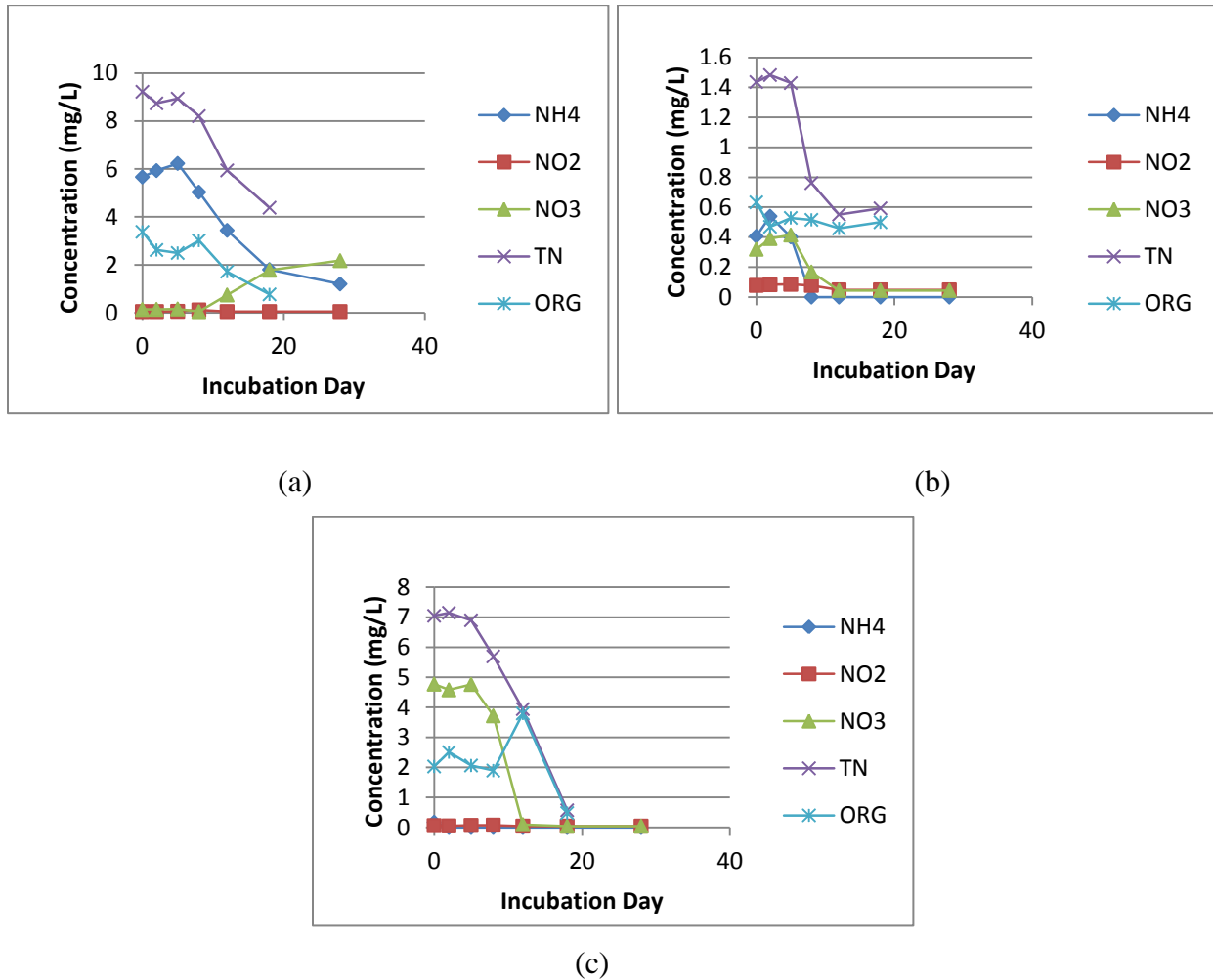


Figure 10: The change of soluble nitrogen species concentrations in river water incubation over time. (a) AM effluent incubated with river water; (b) SF effluent incubated with river water; (c) WL effluent incubated with river water (Mar, 2011)

At the end of the incubation, the SF effluent incubation again had a certain amount of organic nitrogen in the bottles. On the contrary, in AM effluent set, both ammonia and organic nitrogen got degraded while nitrate increased. This change in nitrate concentration was likely caused by

the nitrification that occurred inside the bottle. A bioassay study using known *Selenastrum Capricornutum* to investigate the impacts of bacteria on organic uptake also emphasized the assistance of bacterial activity (Pehlivanoglu and Sedlak, 2004).

The decay of organic nitrogen was strongly supported by the protease activity. As Figure 11 shows, the AM effluent incubation had the most dynamic activity in the later incubation period in terms of protease. The enzymatic data imply that the biodegraded organic nitrogen is related to the change of protease.

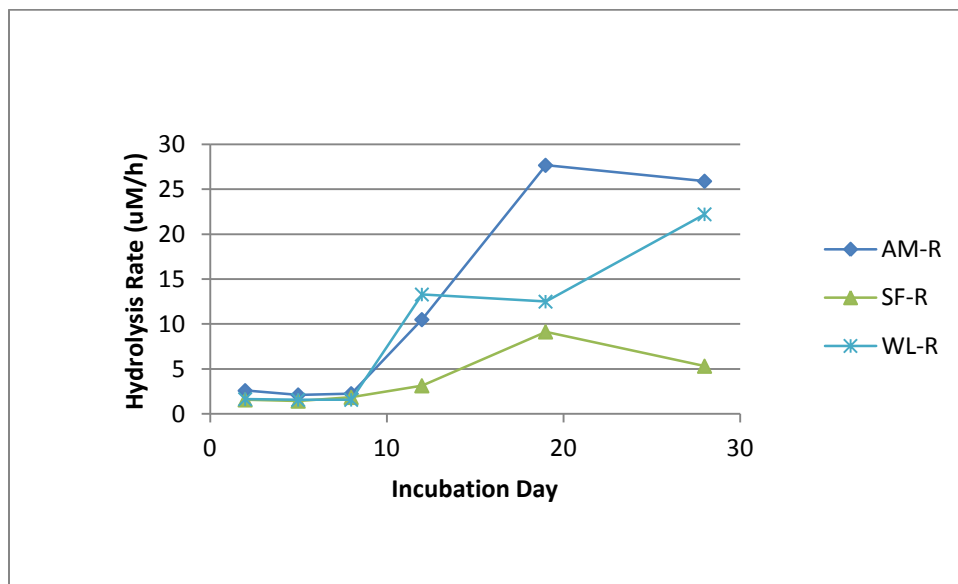


Figure 11: The protease activity of river water incubation samples over time (Mar, 2011)

The hydrolysis rate of protease in the Amherst effluent incubation set reached up to 27.7 $\mu\text{M/h}$ while the rate for the SF incubation just slightly increased during the whole incubation. For all the effluent incubation, the hydrolysis rate started at a similar level. The hydrolysis rate in the SF incubation bottle was below 10 $\mu\text{M/h}$ while the WL effluent incubation protease activity reached 22.2 $\mu\text{M/h}$ at the end of the incubation.

In AM effluent incubation, the protease hydrolysis rate was the highest. The vigorous protease activity suggested the abundance of protein present in the AM incubation bottles.

Several other studies showed the relationship between enzyme activities and bacterial generation (Chappell and Goulder, 1994; Mulholland and Lee, 2009; Mulholland et al., 2003). The enzymatic hydrolysis increases the supply of low-molecular-weight moieties available for microbial uptake. Aminopeptidase activity was strongly correlated with bacterial production in these studies. In addition, the study of algal growth on organic compounds as nitrogen sources shows that the component of DON is direct or indirect nitrogen sources for the plankton growth. The different algal species could utilize these nitrogen sources with varying capabilities so that the nitrogen substrates may stimulate the development of domain algal species.

The protease activity in this incubation set demonstrates the enzyme presence during the incubation. The large amount of enzyme shown in the incubation could have boosted the assimilation of organic nitrogen by generating more low-molecular-weight substrate, and then increase the growth of biomass. Thus, the enzymes could potentially serve as a pollutant in the real environment. Continuous research should be conducted to analyze the change of enzymatic activity during the incubation. The real impact of the enzyme activity should be monitored after the exposure to the receiving water body.

It is also noted that the SF effluent had a larger variety of proteins than the AM effluent (Westgate and Park, 2010). The diverse proteins might also indicate the complexity of the organic nitrogen in the SF effluent. The remaining organic nitrogen at the end of the SF incubation demonstrates that some of the organic nitrogen could not be thoroughly consumed by the microbes in the river water (Figure 10). However, the decrease of soluble organic nitrogen in all the incubation bottles shows some bioavailability of effluent organic nitrogen in the river water incubation. All the changes in the soluble organic nitrogen during the incubation reveal the fact that not all the organic nitrogen in CAS or BNR treatment effluent is totally non-bioavailable.

The WL effluent was supposed to have lowest total nitrogen concentration due to its MLE process. Large amount of inorganic nitrogen should be removed by the advanced treatment process. However, the spring runoff caused high influent to the facility, which caused partially

nitrified and denitrified effluent. Thus, the inorganic nitrogen was relatively high for the March incubation set. At the end of the WL incubation, the protease hydrolysis rate was close to AM incubation, which suggests that the large amount of inorganic nitrogen in the WL effluent also contributed to the generation of protease. The WL incubation was included first time in the incubation set. The information we can get is far from enough. Intensive lab-controlled WL incubation should be continued and the further analysis is needed.

The three sets of river water incubations discussed above reveal several important findings. First, the river water samples had relatively low concentrations of nitrogen compared to the effluent. The major source of nitrogen in the incubation was from treated effluents. Secondly, microorganisms in the river water sample are effective in utilizing the inorganic nitrogen species prior to organic nitrogen, which are seen in Figure 6, 8 and 10. Thus, the real environment's response to the discharged effluent greatly depends on the nitrogen concentration level and composition of nitrogen in the effluents.

In addition, the composition of nitrogen species in the effluent varies between CAS and BNR treatment processes. CAS effluent usually contains higher total nitrogen concentration, most of which is inorganic nitrogen. The uptake of inorganic nitrogen is fast in the river water incubation. For the organic nitrogen fraction, however, not all the organic nitrogen species which remain in the wastewater treatment plant final effluent are inert. A certain amount of organic nitrogen could contribute to the yield of biomass in the incubation. The decrease of soluble organic nitrogen through the incubation shows the bioavailability of soluble organic nitrogen in the real environment. Microbial community in the river water could stimulate the yield of biomass by utilizing the effluent organic nitrogen as nitrogen sources. Photochemical reactions and bacterial activity, which are reported in other studies, could change the lability and bioavailability of organic nitrogen species in the effluent.

For the bioassay, another factor that should be taken into account is that the water quality varies with season and the cold temperature would result in a different composition of organic compounds in the river water. More research should be done to investigate the seasonal variability and its impact on the natural receiving water body (Minor, et al., 2006). Also, the

water quality of effluent is subject to the operation parameters in the plant. The temperature difference between winter and summer time might alter the composition of nitrogen species in the effluents (Badr, et al., 2008; Minor, et al., 2006).

4.2.3 Ocean Water Incubation

Ocean water incubation is another major part of this study. The public concern for nitrogen impacts on estuaries, especially the Long Island Sound, is increasing. Ocean environment is totally different from the freshwater environment. The salinity and the resident microbial community in the ocean are different from those in river. The recalcitrant organic matter that remains stable in the freshwater environment might be bioavailable in the ocean environment. Effluent Organic nitrogen could be utilized differently in ocean environment. The inert organic nitrogen in the river can be transported to the ocean and might be consumed by the microorganism in the ocean; therefore, the remaining organic nitrogen potentially stimulates the growth of biomass in the estuaries area.

The ocean water incubation in this study was set up to investigate the change of effluent nitrogen in the estuary area. The effluent was incubated with the ocean water in the lab and fate of nitrogen was monitored.

December, 2010 Incubation Set

Results for the ocean water incubation of Dec. 2010 are shown in Figure 12 below. The whole and soluble fractions of the AM and SF effluents were incubated with ocean water. The AM effluent with ocean water (AMO), soluble AM fraction with ocean water (AMFO), SF effluent with ocean water (SFO) and soluble SF fraction with ocean water (SFFO) incubations show the decrease of total soluble nitrogen toward the end of incubation. Some of the soluble nitrogen was utilized during the incubation. However, the specific nitrogen species that was consumed in the incubation was uncertain. Due to the high salinity of ocean water, the determination of inorganic

nitrogen was subjected to salt interference. The results are not available at this moment. Therefore, it was difficult to correctly measure the organic nitrogen.

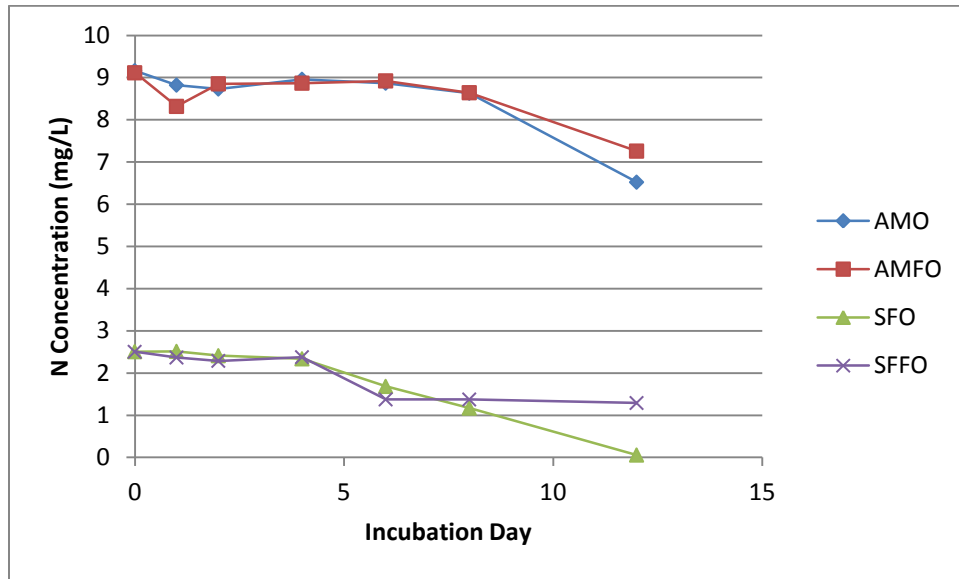


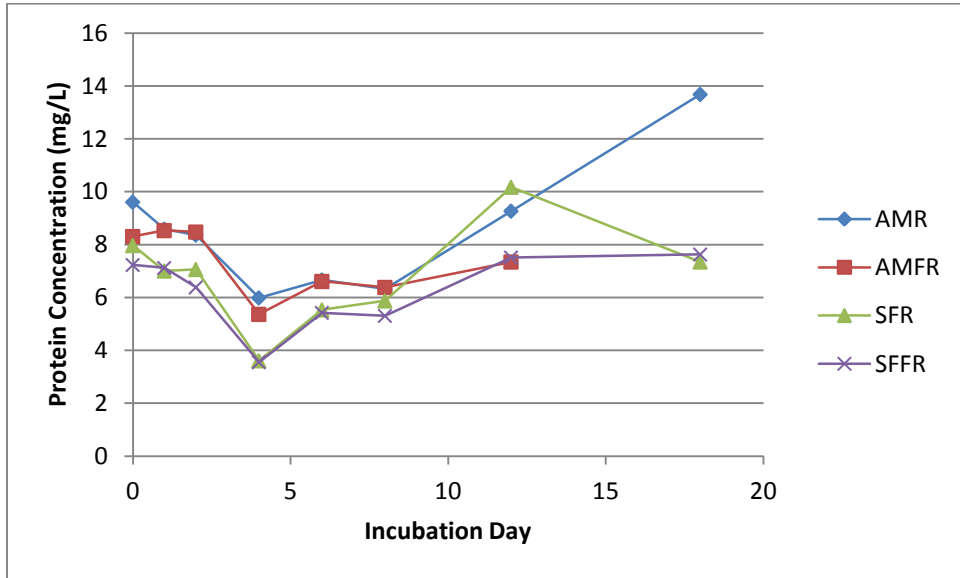
Figure 12: Total soluble nitrogen concentration of ocean water incubation over time (Dec, 2010)

However, other organic nitrogen related measurements provided useful information about the utilization of effluent organic nitrogen in the ocean incubation. Protein concentration, TSS, VSS and COD results indicated how the ocean responded to the effluent discharge.

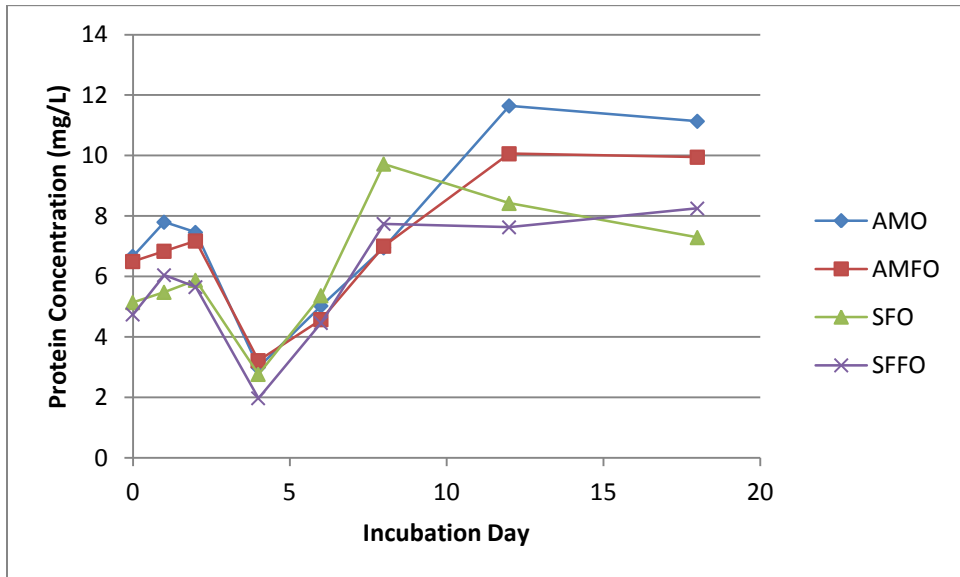
The Lowry Protein concentration changed differently between river and ocean water incubation according to the results in the Figure 13. In the first 1-2 days, all the AM effluent incubation reacted slowly while the SF effluent incubation showed a slight increase and then sharp decrease in the early period. After the degradation of some proteins in the effluent, all the AM and SF incubation bottles showed an increase of protein and reached even higher protein concentrations than those in the original effluents. In this ocean water incubation, all of the samples revealed a similar decay pattern in terms of protein concentration.

Day 4 was the day that all the incubations reached the lowest concentrations. The AMO, AMFO, SFO and SFFO incubation contained 2.99, 3.21, 2.76 and 1.97 mg/L protein respectively. In the

late period, the effluent nitrogen stimulated an increase of proteins and they were 3.89, 3.13, 3.52 and 4.19 times as those at the lowest points of the AMO, AMFO, SFO and SFFO.



(a)



(b)

Figure 13: The change of protein concentrations in river and ocean water incubation over time. (a) Protein in river water incubation; (b) Protein in ocean water incubation (Dec, 2010)

In the river water incubation, the protein increase rate was not lower than that in ocean incubation after the initial decay of original protein. The different response to the same effluent

in the ocean and river water could be caused by the microbial community in the receiving water. The resident microorganisms in the ocean water might utilize the effluent nitrogen in a different path way and this would result in different protein increase pattern. At the lowest protein concentration point, the ocean incubation showed lower concentrations in all the bottles than the river incubation bottles. The microbial community in the ocean water is more effective in utilizing the effluent nitrogen.

In addition, the filtration of SF effluent did not benefit the ocean water quality in terms of biomass production. The soluble fraction of the SF effluent led to a similar yield of proteins as for the whole effluent in the ocean water incubation. Another finding that should be noted is that the AM and SF effluent presented different potential in the ocean environment. The SF effluent incubation generated similar level of proteins with less starting total nitrogen concentration compared to the AM effluent incubation. The SF organic nitrogen played an important role in stimulating the protein production to a similar level as the AM incubation. After the inorganic nitrogen in the SF effluent was consumed, the effluent organic nitrogen was the main nitrogen source for protein production. Microbial community in the ocean water effectively utilized the effluent nitrogen for the biomass generation. Severe environment in the ocean enhanced the bacteria and algae capabilities of uptaking the available for their growth. Even a small amount of organic nitrogen could greatly fuel the growth of biomass in the ocean incubations. Therefore, the reduction of TN, especially the inorganic nitrogen, might not really solve the problem in the estuary area because some of the effluent organic nitrogen is bioavailable in the estuary. The potential of effluent organic nitrogen in stimulating environmental problem is also reported in other research (Bronk, D, et al., 2007). This research also implied the ocean environment could assist the biomass generation after the effluent discharged to the ocean receiving body.

March, 2011 Incubation Set

As shown in Figure 14, the total soluble nitrogen in all the Mar, 2011 incubations kept decreasing over the incubation period. Nitrogen could be transformed from the soluble phase to the particulate phase during the interaction with microbes. Either inorganic nitrogen or organic nitrogen was utilized by the microorganism community in the ocean water incubations. Because

of the high salinity in the ocean water, the determination of the inorganic nitrogen concentration would require a different method from Ion Chromatography analysis. Nitrite and ammonium concentrations were difficult to obtain by using this method. Sodium and chloride, which were substantial in the ocean water, inhibit the detection of the inorganic nitrogen species. The relationship between inorganic and organic nitrogen species was not as certain in this study as for the December incubation set.

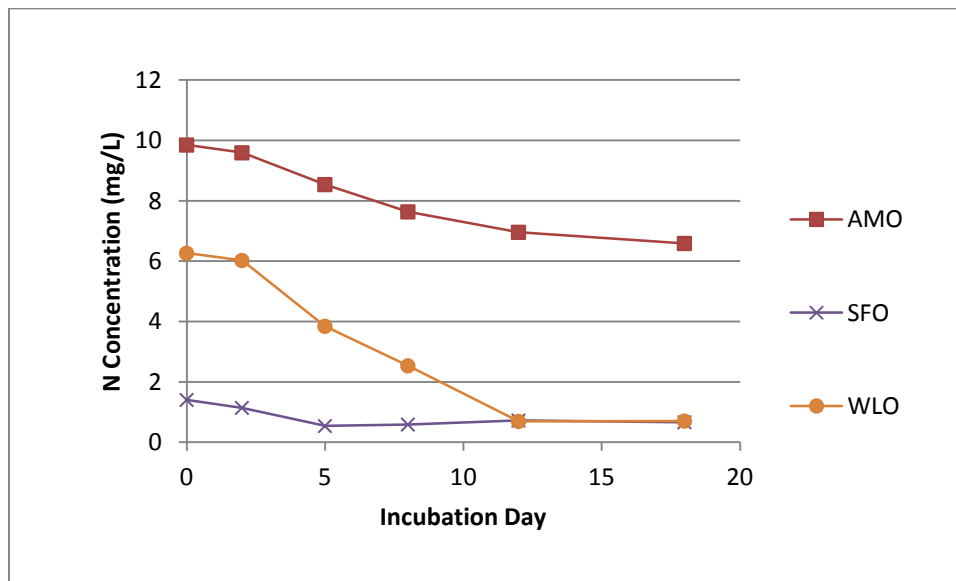


Figure 14: The total soluble nitrogen concentration of ocean water incubation samples over time (Mar, 2011)

The change in VSS, COD and protein concentrations during the laboratory incubation is shown in Figure 15. All of the AM, SF and WL effluents incubated with ocean water samples experienced the increases in biomass. However, the growth patterns were again not the same between different effluent incubations. The SF effluent responded faster in the early incubation period as shown in the VSS increase. The highest VSS increase rate during incubation was 23.3 mg/L VSS per day while the AM and WL were 20 and 12.6 mg/L VSS per day, respectively. These different rates are evidence that SF could trigger the most dynamic growth when it encountered the ocean water. The SF has the lowest total nitrogen concentration but showed a similar potential as AM effluents incubation. Interestingly, the WL and AM effluent incubation started with similar amounts of total nitrogen, but the WL effluent organic nitrogen percentage was higher than the AM one. The COD and VSS concentrations at the end of the AM incubation

were around 100 mg/L. Meanwhile, the WL effluent incubation ones were 171 and 148 mg/L, respectively.

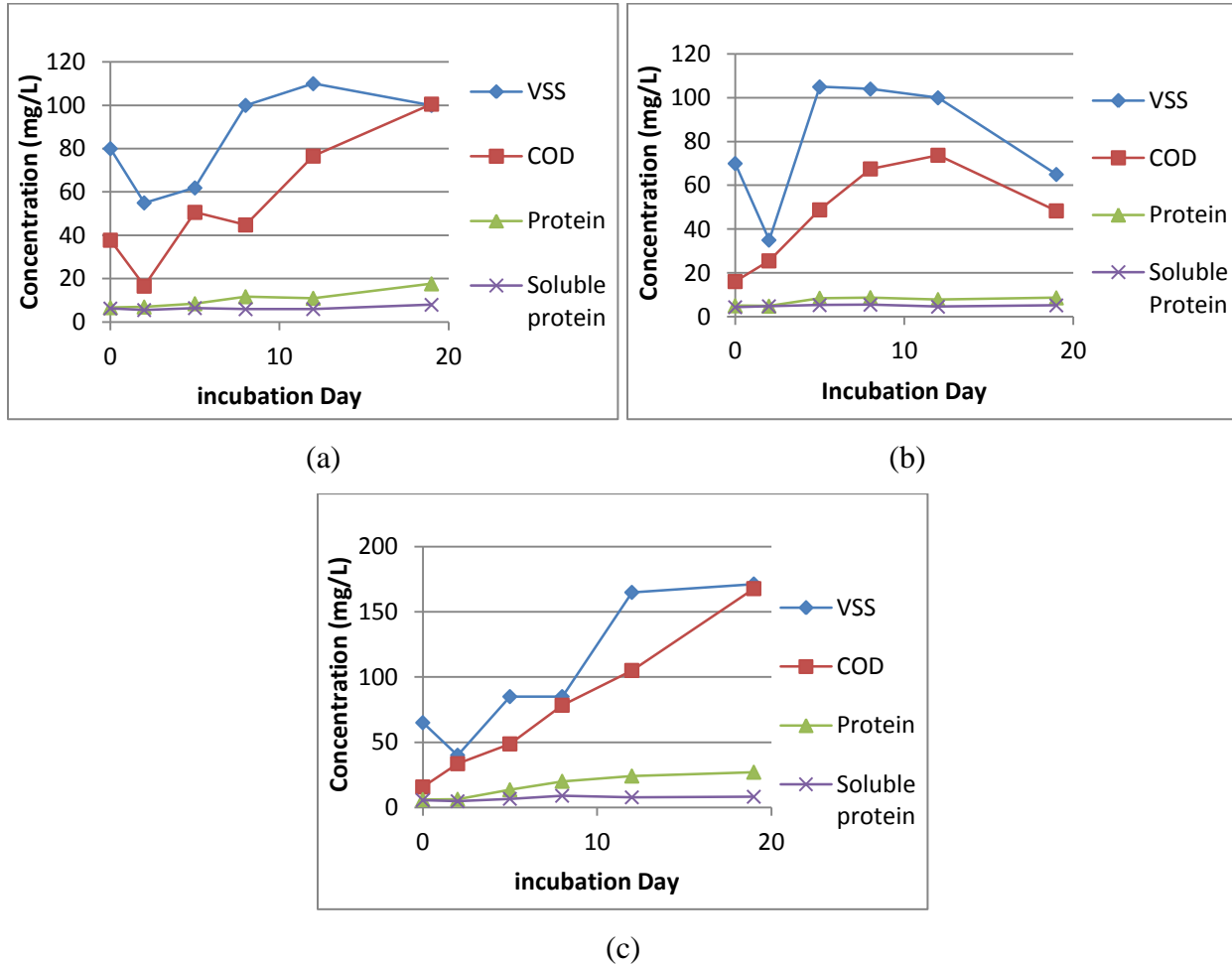


Figure 15: The change of VSS, COD and protein concentrations in ocean water incubation over time. (a) AM effluent with ocean water; (b) SF effluent with ocean water; (c) WL effluent with ocean water (Mar, 2011)

The organic nitrogen in the WL probably led to the high COD and VSS yield at the end of ocean water incubation. Again, this result suggested that the some of the effluent organic nitrogen could be biodegradable in the ocean environment. Similar notion was made by other research that the bioavailability of effluent organic nitrogen was related to the receiving water bodies it was discharged to (Bronk, D A, et al., 2010; Seitzinger and Sanders, 1997; Wiegner, et al., 2006). According to the yield of COD and VSS in this incubation set, the potential for effluent organic

nitrogen to stimulate the biomass production was much higher than for inorganic nitrogen since the WL organic nitrogen concentration accounted for 20% of the total nitrogen based on the effluent characterization results.

All three effluent incubations experienced low hydrolysis rates in the early period of incubation. However, protease became more active after 10 days of incubation. The WL effluent, which was subject to Modified Ludtzac Ettinger treatment, revealed a striking protease activity as shown in Figure 16. In the later period of incubation, the protease activity reached as high as 20 μ M/h. This rapid increase in protease hydrolysis rate showed the potential of advanced treatment process effluent compared to traditional treatment process effluent in terms of protein generation and decomposition.

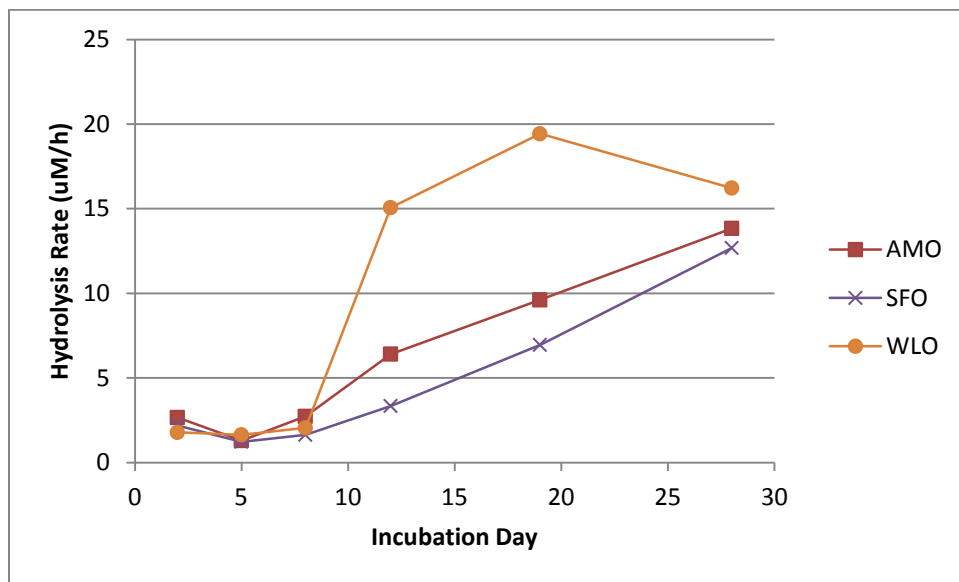


Figure 16: The protease activity of ocean water incubation samples over time (Mar, 2011)

Interestingly, the SF effluent incubation showed a similar protease activity as for the AM effluent over the whole incubation period even the incubation started from a low total nitrogen concentration compared to the AM effluent. These active enzymes were likely to assist the conversion of organic nitrogen for biomass growth. Some studies showed that the enzyme could be river pollutant and the small protein, such as peptide, fueled the growth of biomass (Chappell

and Goulter, 1994; Mulholland, et al., 2002; Mulholland and Lee, 2009). Leaving high concentration enzymes in the aquatic system would be problematic because they could trigger the growth of biomass and even a harmful algal bloom. The incubation results in this study reveal the generation of protease and the other enzymes were not monitored. The protease was only a group of enzyme present in the incubation. Further determination of other enzyme would show more information about the enzyme generation during the incubation.

Besides the resident microbial in the receiving water, the utilization of effluent nitrogen could be facilitated by the salinity. Bronk's effluent nitrogen research (Bronk, D A, et al., 2010) revealed the influence of salinity in the release of inorganic nitrogen in the estuary. The humic substances in the real environment adsorb the ammonium in the fresh water environment and release it in the estuaries due to the abundance of salt. The current data in this study was far from enough to show this release of ammonium during the ocean incubation. Whether the same phenomenon would show in the incubation was still uncertain.

Based on these two ocean incubation sets, the organic nitrogen was utilized in a different way in the saline environment. Compared to the river water incubation, BNR effluent was able to compete with the CAS effluent in terms of biomass generation. All of the WL and SF effluent incubations demonstrated a higher potential in stimulating the production of biomass than the AM effluent incubation. Protein and enzymatic activity, which are closely related to the organic nitrogen, show the difference in ways that treated effluent organic nitrogen was utilized. Also, the dynamic and strong interaction between BNR effluent and the ocean water served as evidence that the microbial community in the ocean were probably effective in uptaking nitrogen in the treated effluent from advanced treatment processes.

5. CONCLUSION

This study of effluent organic nitrogen reveals several important findings. They are of great importance and provide information about the receiving water response to the effluent discharges.

The evaluation of the effluent nitrogen species shows that BNR treatment produces more organic nitrogen than for the CAS treatment process. The composition of organic nitrogen in the BNR effluent is more complicated and diverse although the total nitrogen is usually low.

Based on size fractionation, a large amount of proteins with size smaller than 1kDa are found. What is the specific component in this group of small size protein is untouched. Further exploration of these small and soluble proteins in the effluent would greatly benefit the understanding of the effluent organic nitrogen.

Bioassays performed in this study demonstrate the response of receiving waters to which the effluent is discharged. River and ocean water react differently to the same effluent. River water incubation showed the preference of utilizing inorganic nitrogen prior to organic nitrogen in cell assimilation. Also, not all of the organic nitrogen in the final effluent was used in the river water. In the ocean water incubations, BNR effluent was able to compete with the CAS effluent in terms of biomass generation even their total nitrogen concentration was low. Organic nitrogen in BNR effluent also contributes to the biomass production. All of the BNR effluent incubations with river and ocean water suggest a higher potential in stimulating the production of biomass than for the CAS effluent incubations.

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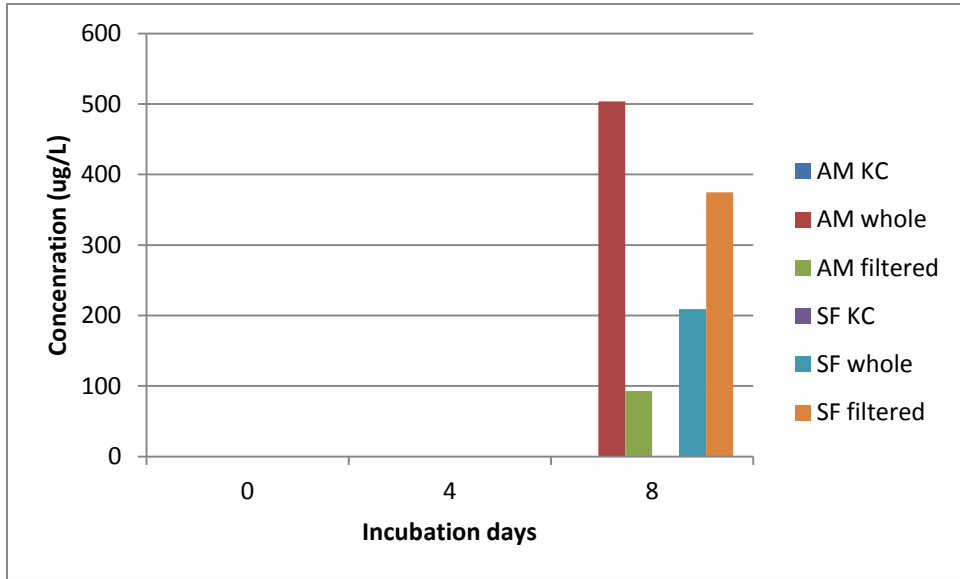
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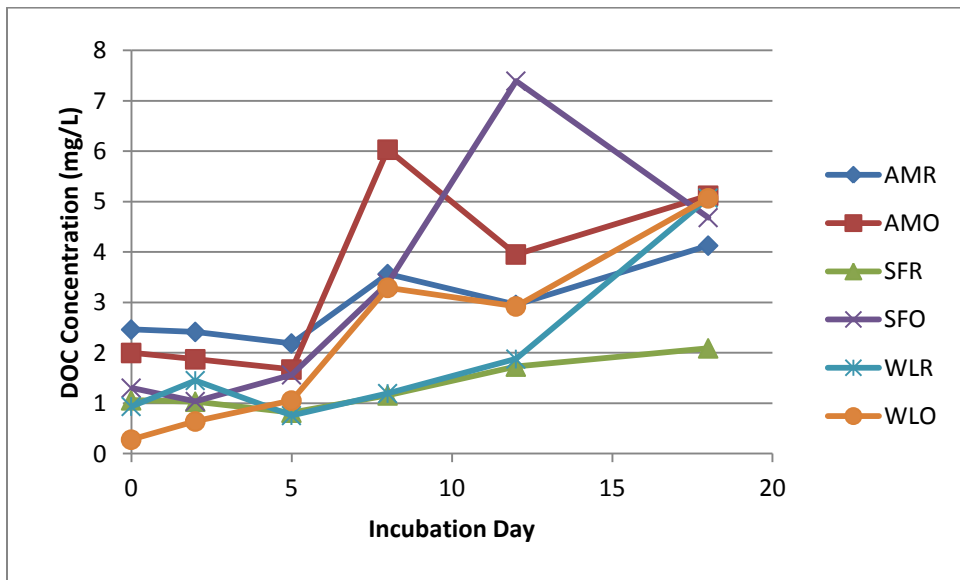
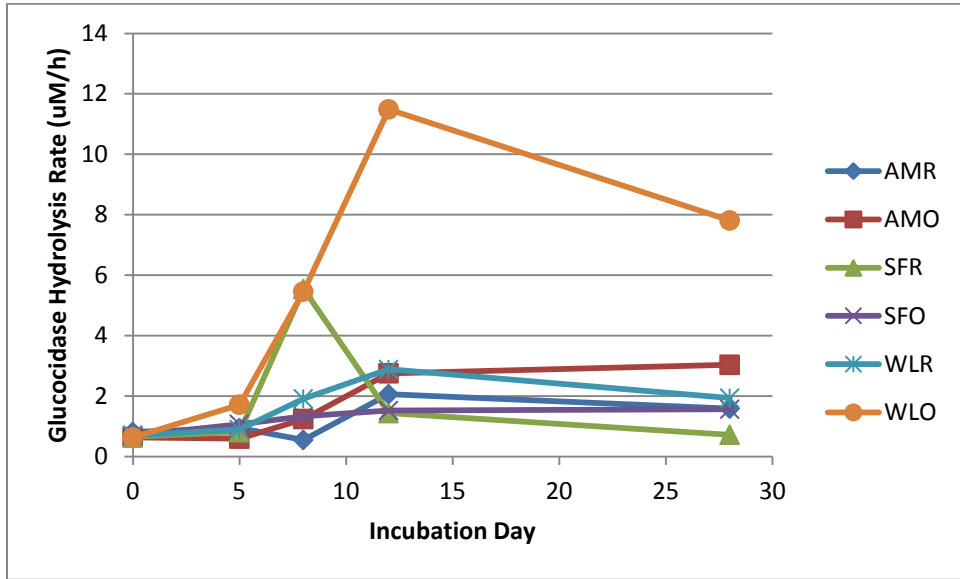
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7. APPENDICES

Appendix A: *Chlorophyll a* concentration of June, 2010 river incubation set samples



Appendix B: Glucocidase activity and the DOC of March, 2011 set samples



Appendix C: The data of Nitrogen species concentration in effluents and receiving waters.

The data of nitrogen species and phosphate concentrations in the effluents and receiving waters

Sample(N mg/L)	SF	AM	WL	Ocean	River
Nitrite	0.15	0.17	0.11	0.09	0.00
Nitrate	0.68	0.18	1.91	0.26	0.28
Ammonium	0.79	16.35	4.06	0.00	0.00
TN	2.23	16.97	8.44	0.31	0.32
Organic N	0.62	0.26	2.36	-0.04	0.04
Org N/TN	0.28	0.02	0.28	-0.14	0.12
Phosphate	0.34	0.24	0.10	0.18	0.07

The data of protein concentrations in the effluents and receiving waters

Protein(mg/L)	Whole	0.45	1kDa
River	2.40	2.29	1.18
AM	13.15	11.82	7.51
BT	6.60	4.85	3.73
SF	6.50	5.65	3.41

The data of protein concentrations in the effluents for zymogram gel running

ASP	ABS	ABS	Mean	Con (mg/L)	ABS	Mean	Con (mg/L)
AM	0.063	0.062	0.0625	29.86	0.019	0.054375	21.21
BT	0.063	0.064	0.0635	30.92	0.016	0.059375	26.53
SF	0.066	0.066	0.066	33.58	0.019	0.05875	25.86

The data of protein concentrations in the effluents for SDS-PAGE gel running

Lane		Lowry	Modified Lowry
		Protein (mg/L)	Protein (µg/mL)
1	AM-1	268.96	120.5
2	AM-2	150.06	94.9
3	SF-1	272.35	159.0
4	SF-2	118.82	95.1

Appendix D: The data of nitrogen species concentration and other parameters in all incubations (1L effluent was incubated with 1L river water in the lab controlled conditions).

Jun, 2010 river incubation set.

Raw samples

Sample(N mg/L)	SF	AM	River
Nitrite	0.16	0.15	0.00
Nitrate	0.68	0.18	0.26
Ammonium	0.77	16.29	0.00
TN	3.23	10.01	0.34

TN	C, mg/L N	D0	D2	D4	D6	D8
N	AM KC	8.8	10.9	9.9	10.2	12.8
	AM	12.1	11.1	11.5	8.8	11.3
	AM F	10.6	10.4	12.4	10.5	10.1
	SF KC	7.8	7.7	6.9	5.8	6.7
	SF	4.6	6.1	4.9	6.0	7.7
	SF F	5.3	5.7	6.0	4.9	7.7

NH4	C, mg/L N	D0	D2	D4	D6	D8
N	AM KC	7.09	6.15	5.85	6.02	5.51
	AM	8.38	7.85	6.43	1.74	0.00
	AM F	8.46	8.40	8.01	7.21	5.26
	SF KC	0.00	0.00	0.00	0.00	0.00
	SF	0.00	0.00	0.00	0.00	0.00
	SF F	0.00	0.00	0.00	0.00	0.00

NO2	C, mg/L N	D0	D2	D4	D6	D8
N	AM KC	0.09	0.08	0.08	0.08	0.08
	AM	0.19	0.27	0.49	0.72	0.21
	AM F	0.05	0.09	0.08	0.09	0.11
	SF KC	0.05	0.05	0.05	0.05	0.05
	SF	0.05	0.05	0.05	0.06	0.05
	SF F	0.05	0.05	0.05	0.05	0.06

NO3	C, mg/L N	D0	D2	D4	D6	D8
N	AM KC	0.18	0.17	0.18	0.17	0.17
	AM	0.17	0.17	0.18	0.18	0.05
	AM F	0.18	0.19	0.18	0.19	0.18
	SF KC	2.86	2.87	2.86	2.91	2.82
	SF	2.77	2.80	2.15	2.03	0.05
	SF F	2.78	2.71	2.45	2.50	0.52

Organic-N	C, mg/L N	D0	D2	D4	D6	D8
N	AM KC	1.40	4.52	3.83	3.93	7.06
Whole	AM	3.40	2.81	4.40	6.20	11.06
	AM F	1.96	1.72	4.12	3.04	4.53
	SF KC	4.89	4.74	3.97	2.83	3.79
	SF	1.78	3.23	2.72	3.95	7.55
	SF F	2.48	2.99	3.47	2.32	7.09

Dec, 2010 River and ocean incubation sets (1L effluent was incubated with 1L river or ocean water in the lab controlled conditions)

Raw samples

Sample(N mg/L)	SF	AM	Ocean	River
Nitrite	0.14	0.17	0.09	0.00
Nitrate	/	0.18	0.26	0.28
Ammonium	0.81	12.41	0.00	0.00
TN	2.16	9.26	0.31	/

AM with River

Sample, mg/l	D0	D1	D2	D4	D6	D8	D12	D18
Nitrite	0.16	0.17	0.53	1.63	12.32	27.98	25.62	18.76
Nitrate	0.40	0.43	1.03	1.16	1.14	1.39	2.14	2.35
Ammonium	8.51	8.33	7.61	7.50	4.44	/	/	/
TN	9.05	8.70	8.91	8.50	7.54	6.58	6.14	/
ORG	2.29	2.08	2.60	1.91	0.08	/	/	/

AMF with River

Sample, mg/l	D0	D1	D2	D4	D6	D8	D12	D18
Nitrite	0.16	0.16	0.45	0.47	0.73	3.48	27.28	20.55
Nitrate	0.40	0.38	1.01	1.04	1.03	1.06	1.17	0.93
Ammonium	8.45	8.53	7.83	7.50	7.56	6.59	/	/
TN	9.19	8.52	8.85	8.03	8.08	7.03	6.18	/
ORG	2.48	1.76	2.40	1.82	1.75	0.61	/	/

SF with River

Sample, mg/l	D0	D1	D2	D4	D6	D8	D12	D18
Nitrite	0.16	0.16	0.39	0.42	0.53	0.57	/	/
Nitrate	2.32	2.33	2.16	2.29	2.47	2.59	/	/
Ammonium	0.48	0.52	0.35	0.32	0.20	/	/	/
TN	2.82	2.48	2.55	2.55	2.43	2.35	1.08	/
ORG	0.92	0.71	0.39	0.38	0.35	0.42	0.54	/

SFF with River

Sample, mg/l	D0	D1	D2	D4	D6	D8	D12	D18
Nitrite	0.16	0.16	0.39	0.39	0.40	0.43	0.58	/
Nitrate	2.32	2.32	2.11	2.11	2.13	2.13	1.23	/
Ammonium	0.56	0.57	0.40	0.39	0.39	0.33	/	/
TN	2.76	2.57	2.39	2.58	2.49	2.54	1.59	/
ORG	0.83	0.72	0.29	0.39	0.34	0.40	0.34	/

Protein Concentration (Lowry Method)

Sample, mg/l	D0	D1	D2	D4	D6	D8	D12	D18
AM-R	9.61	8.59	8.36	5.99	6.67	6.33	9.27	13.68
AMF-R	8.31	8.53	8.48	5.36	6.61	6.38	7.34	/
SF-R	7.97	7.01	7.06	3.61	5.53	5.87	10.17	7.34
SFF-R	7.23	7.12	6.38	3.55	5.42	5.31	7.51	7.63
AM-O	6.67	7.80	7.46	2.99	5.02	6.95	11.65	11.14
AMF-O	6.50	6.84	7.18	3.21	4.57	7.01	10.06	9.95
SF-O	5.14	5.48	5.87	2.76	5.36	9.72	8.42	7.29
SFF-O	4.74	6.04	5.65	1.97	4.46	7.74	7.63	8.25

Mar, 2011 River and ocean water incubation sets

Raw samples

Sample(N mg/L)	SF	AM	WL	Ocean	River
Nitrite	0.15	0.17	0.11	/	0.00
Nitrate	0.68	0.18	1.91	/	0.31
Ammonium	0.79	16.29	4.06/2.12	/	0.00
TN	1.30	8.03	8.43/10.87	/	0.30

AM with River

Concentration (N mg/L)	D0	D2	D5	D8	D12	D18	D28
NH4	5.662	5.930	6.228	5.033	3.435	1.799	1.198
NO2	0.048	0.048	0.057	0.116	0.048	0.048	0.048
NO3	0.136	0.142	0.157	0.045	0.740	1.771	2.172
TN	9.219	8.736	8.934	8.202	5.938	4.382	/
ORG	3.372	2.617	2.492	3.008	1.714	0.764	/

SF with River

Concentration (N mg/L)	D0	D2	D5	D8	D12	D18	D28
NH4	0.406	0.540	0.401	0.000	0.000	0.000	0.000
NO2	0.078	0.083	0.085	0.078	0.048	0.048	0.048
NO3	0.319	0.390	0.414	0.167	0.045	0.045	0.045
TN	1.436	1.482	1.429	0.761	0.551	0.592	/
ORG	0.633	0.469	0.528	0.516	0.458	0.500	/

WL with River

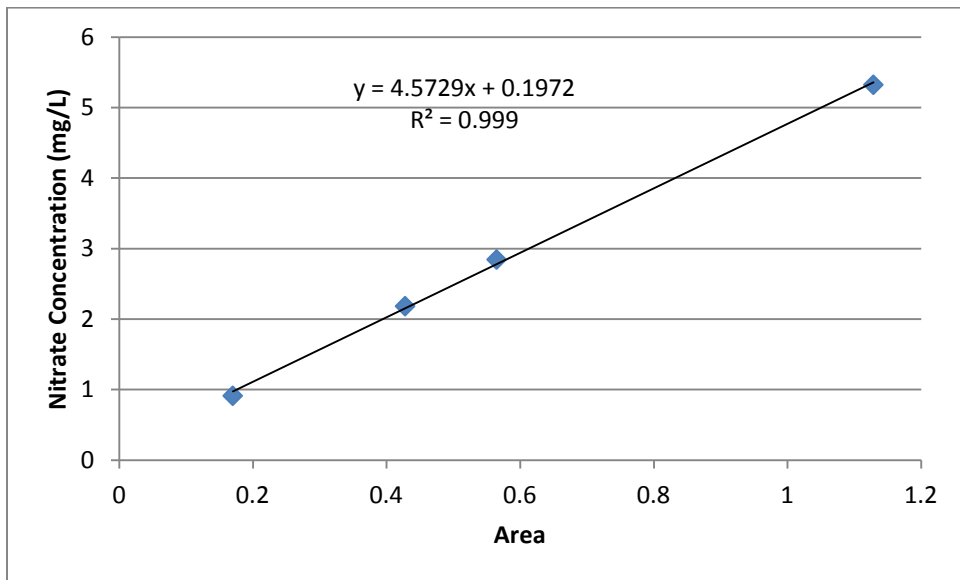
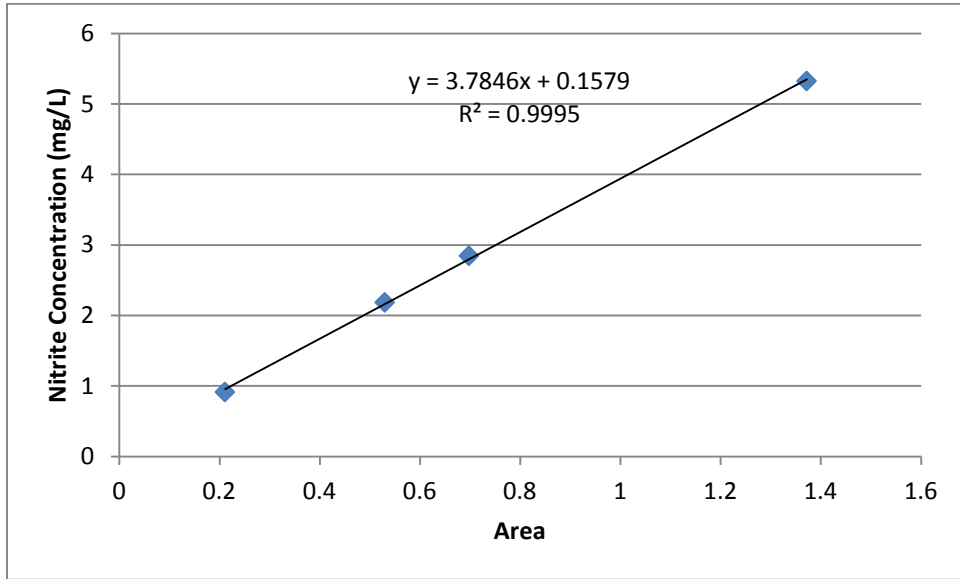
Concentration (N mg/L)	D0	D2	D5	D8	D12	D18	D28
NH4	0.177	0.000	0.000	0.000	0.000	0.000	0.000
NO2	0.064	0.055	0.071	0.080	0.048	0.048	0.048
NO3	4.773	4.584	4.763	3.718	0.097	0.045	0.045
TN	7.047	7.149	6.900	5.696	3.942	0.575	/
ORG	2.033	2.510	2.065	1.897	3.797	0.482	/

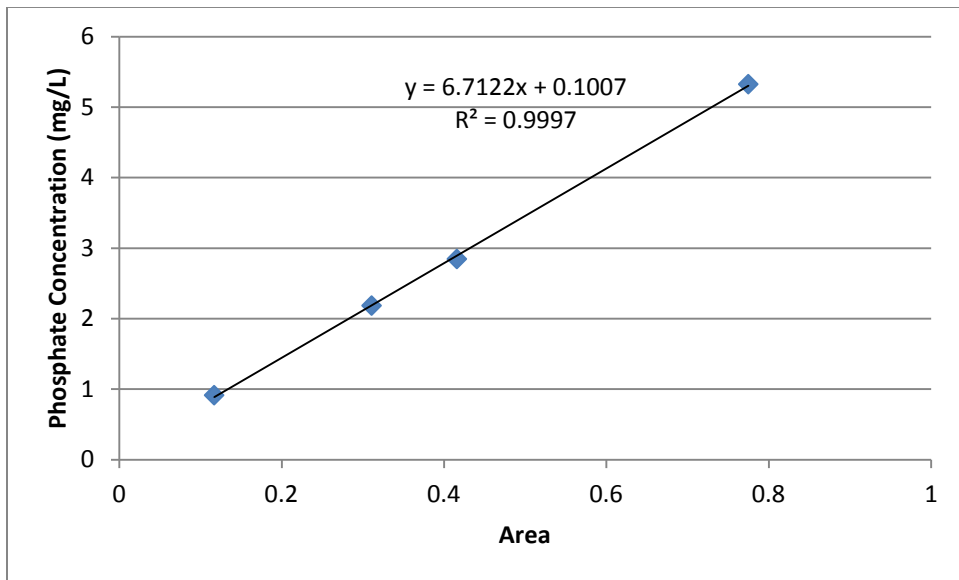
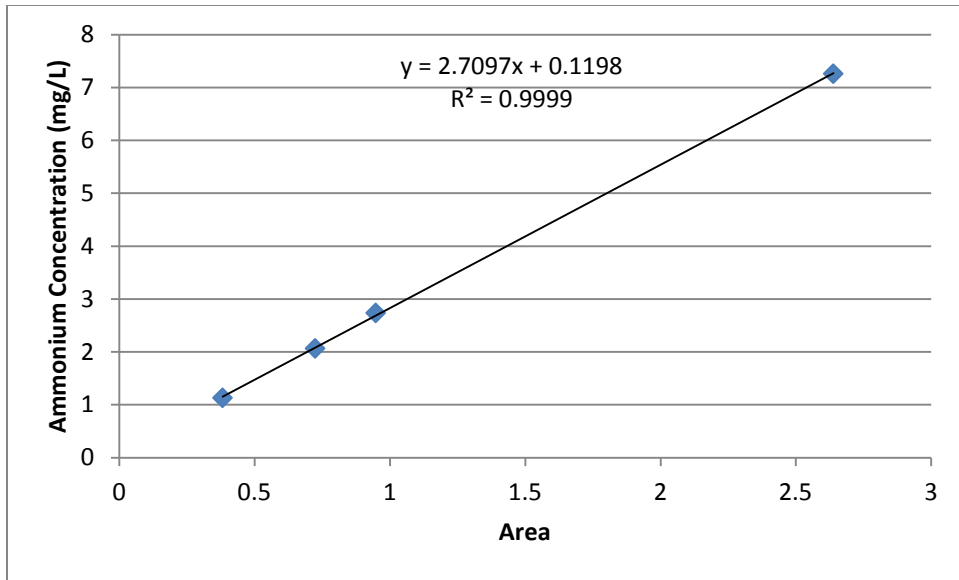
TSS							
Day	0	2	5	8	12	19	27
AM-R	0	15	9.090909	38.46154	66.66667	78.94737	95.45455
SF-R	0	0	0	33.33333	28.57143	42.85714	35
WL-R	0	10	0	35	57.14286	155	210.5263
Day (ocean)	0	2	5	8	12	19	28
AM-O	120	195	190.4762	245	240	245	340.9091
SF-O	205	115	220	216	200	150	209.0909
WL-O	170	110	200	210	290	328.5714	240.9091

VSS							
Day	0	2	5	8	12	19	27
AM-R	0	5	0	23.07692	57.14286	68.42105	77.27273
SF-R	0	0	0	23.80952	19.04762	33.33333	30
WL-R	0	5	0	20	42.85714	135	200
Day (ocean)	0	2	5	8	12	19	28
AM-O	80	55	61.90476	100	110	100	150
SF-O	70	35	105	104	100	65	81.81818
WL-O	65	40	85	85	165	171.4286	127.2727

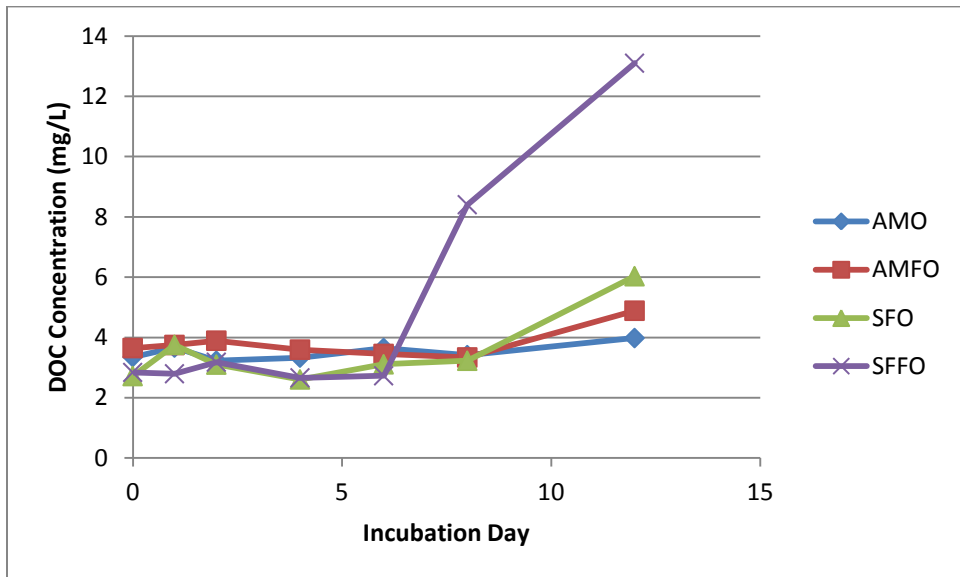
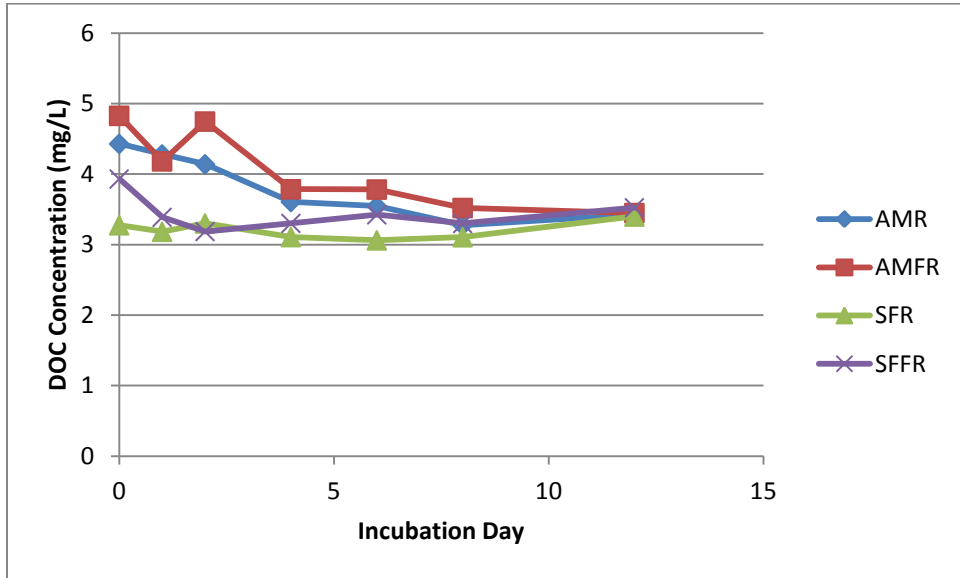
Protein	0	2	5	8	12	19
AM-R	9.382	9.552	9.099	14.475	17.926	23.924
SF-R	7.118	7.232	7.288	11.193	10.174	11.758
WL-R	8.080	7.741	8.080	13.682	13.852	26.414
AM-O	6.666	6.949	8.477	11.702	10.966	17.643
SF-O	5.025	4.855	8.477	8.759	7.854	8.646
WL-O	5.987	6.213	13.513	20.020	24.037	26.980
AM-R/F	8.477	8.477	7.854	8.024	7.628	9.495
SF-R/F	6.836	6.383	6.043	6.157	6.836	6.722
WL-R/F	7.288	7.005	6.836	7.005	6.949	11.136
AM-O/F	6.270	5.534	6.439	5.930	5.987	7.967
SF-O/F	4.346	4.798	5.308	5.478	4.572	5.195
WL-O/F	5.478	4.685	6.553	8.873	7.684	8.137

Appendix E: Calibration curves of nitrite, nitrate, ammonium and phosphate measured by Ion Chromatography





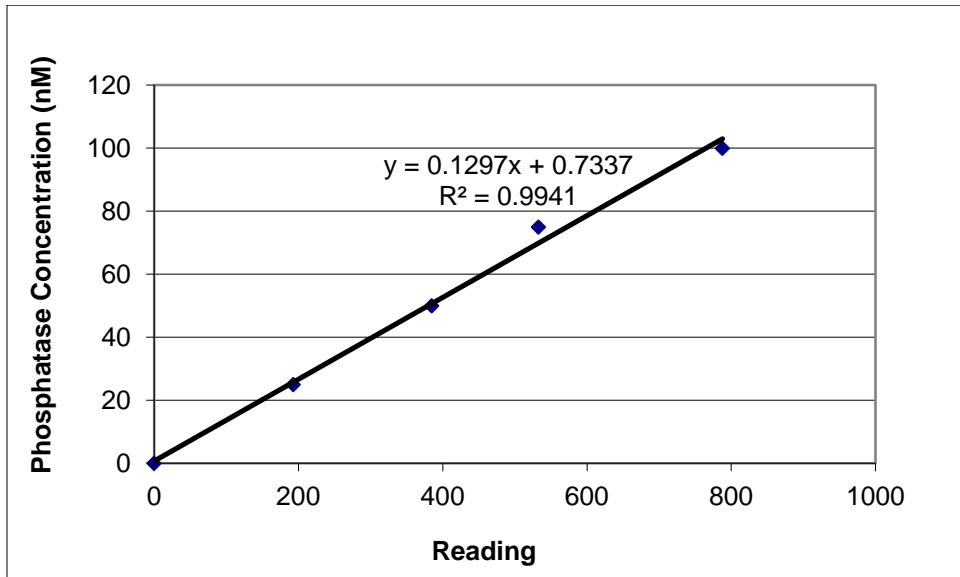
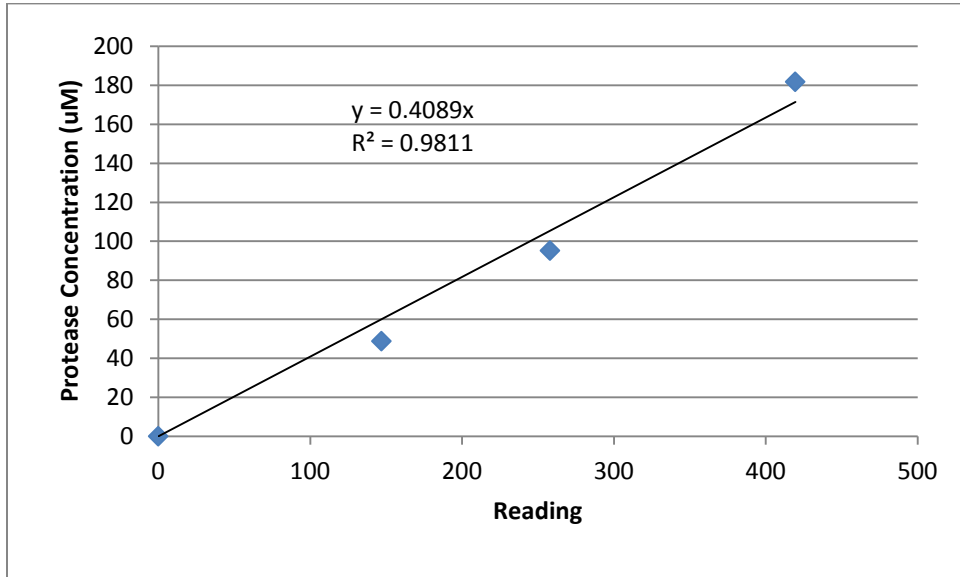
Appendix F: Dissolved organic carbon (DOC) concentration of river and ocean incubation in December, 2010 set



Appendix G: Enzyme Measurement Procedure

1. Make 5 mmol/L stock solution of L-leucine 7-amido-4-methylcoumarin and MUF phosphate
2. Combine stock solution with 2 mL of sample such that the substrate concentration in the solution is 200 $\mu\text{mol/L}$ (add 80 μL of a 5 mmol/L stock solution to 2 mL sample)
3. Incubate for 3 hours in darkness room temperature. Create control sample in the same way as described above. Using MilliQ water in place of the sample
4. Add 0.16 mL of pH 10 buffer to 2.5 mL of post-incubation sample
5. Read fluorescence activity at 455 nm, under excitation at 366 nm
6. Make standard solutions of 4-methylumbelliferone and 7-amino-4-methylcoumarin in 5 mmol/L

Appendix H: Calibration curves of protease and phosphatase measurement



Appendix I: Protease activity normalized by VSS (N-Rate) from the Mar, 2011 incubation set

Protease	2	5	8	12	19	28
AM-O	2.65785	1.29485	2.726	6.4061	9.60915	13.83445
SF-O	2.1808	1.2267	1.6356	3.33935	6.9513	12.6787
WL-O	1.778715	1.6356	2.0445	15.06115	19.42275	16.2197

VSS(ocean), mg/L	D2	D5	D8	D12	D19	D28
AM-O	55	61.90476	100	110	100	150
SF-O	35	105	104	100	65	81.81818
WL-O	40	85	85	165	171.4286	127.2727

N-Rate, umol/h-mg	2	5	8	12	19	28
AM-O	0.048	0.020	0.021	0.058	0.096	0.092
SF-O	0.062	0.011	0.015	0.033	0.106	0.155
WL-O	0.044	0.019	0.024	0.092	0.113	0.127