Long-term Carbon and Copper Impact on Nutrient Removal via Green Sorption Media in Dynamic Linear Ditch Environments

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LONG-TERM CARBON AND COPPER IMPACT ON NUTRIENT REMOVAL VIA GREEN SORPTION MEDIA IN DYNAMIC LINEAR DITCH ENVIRONMENTS

by

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ABSTRACT

Nutrient-laden stormwater runoff causes environmental and ecological impacts on receiving water bodies. Biosorption Activated Media (BAM) composed of the sand, tire crumb, and clay have been implemented in stormwater best management practices due to its ability to efficiently remove nutrients from stormwater runoff, such as in roadside linear ditches, via unique chemophysical and microbiological processes. In this study, a set of fixed-bed columns were set up to simulate some external forces in roadside linear ditches and examine how these external forces affect the performance of BAM. In our experiment, scenario 1 simulates the impact that animals such as tortoises, moles and ants produce conduits on the top layer of BAM. Scenario 2 simulates the presence of animals on BAM, together with external compaction. Finally, scenario 3 simulates external compaction such as traffic compaction alone. Furthermore, two baseline conditions were included to sustain the impact assessment of these three scenarios, respectively. They are the long-term presence of carbon in stormwater as carbon can be transported by stormwater runoff from neighboring crop fields, and the long-term presence of copper ions in stormwater as copper depositions can also be found because of electrical wiring, roofing, stormwater ponds disinfection and automobile brake pads in transportation networks. This systematic assessment encompasses some intertwined field complexity in real world systems driven by different hydraulic conditions, microbial ecology, Dissolved Organic Nitrogen (DON) reshape/removal, and long-term addition of carbon and copper (alone) on the effectiveness of total nitrogen removal. The removal efficiencies are substantially linked to varying microbial processes including mineralization, ammonification, nitrification, denitrification, and even dissimilatory nitrate reduction to ammonium, each of which is controlled by different dominant microbial species. The identification of DON compounds at the molecular level was done via a Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-IR-MS) whereas the quantitation of microbial species was done by using quantitative Polymerase Chain Reaction (qPCR). The results from the interactions between microbial ecology and DON decomposition were compared to the external forces and baseline conditions to obtain a holistic understanding of the removals efficiencies of total nitrogen. With the aid of qPCR and FT-IR-MS, this study concluded that the long-term presence of carbon is beneficial for nutrient removal whereas the long-term copper addition inhibits nutrient removal.

To my family and friend for all their support

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LIST OF ACRONYMS (or) ABBREVIATIONS

AMX	Annamox
AOA	Ammonia Oxidizing Archaea
AOB	Ammonia Oxidizing Bacteria
BAM	Biosorption Activated Media
BMPs	Best Management Practice
CC	Carbon case
COD	Chemical Oxygen Demand
Comammox	Complete Ammonia Oxidizers
DO	Dissolved Oxygen
DOM	Dissolved Organic Matter
DON	Dissolved Organic Nitrogen
FT-ICR_MS	Fourier-Transform Ion Cyclotron Resonance equipment
HRT	Hydraulic Retention Time
LC	Long-term copper case
LID	Low Impact Development
NC	Normal Case (also denoted non-copper case)

NOB	Nitrite Oxidizing Bacteria
NOX	Nitrate and Nitrite
ORP	Oxidation Reduction Potential
qPCR	Real-Time Polymerase Chain Reaction
SC	Short-term copper case
SPE	Solid-phase extraction
TN	Total Nitrogen
TP	Total Phosphate

CHAPTER 1: INTRODUCTION

1.1. Water Pollution

Climate change, ocean raise, air pollution, contamination of water bodies are some of many problems that the world is facing. The main contributor is the exponential population growth that begun with the industrial revolution. The bloom in population had increased the demand in daily use needs like water, food and energy. Hence, groundwater aquifers and different water bodies has seen affected by the intend of society to meet such demand. The use of fertilizer and pesticides in agriculture has risen significantly as result of the increase in food production. The chemicals utilized in fertilizer and pesticides are carried out by stormwater and end up in water bodies. Such phenomenon is denominated as nonpoint source pollution and is one of the main contributors of water pollution in the USA given their difficulty to identify its source and to regulate it. The nonpoint source pollution results in excess discharge of nutrients, metals, and contaminants that lead to eutrophication of lakes and river, algae bloom and death of aquatic animals. Other sources of nonpoint pollution are roadway runoff, acid rain, household's irrigation and roof runoff.

Best practice managements (BPMs), aim to mitigate the effects of nonpoint sources. Different governmental entities and engineering companies have integrated BMPs to current construction methods with the goal to preserve and remediate damaged ecosystem. BMPs include constructed wetland, infiltration basins, bioretention, green roof, porous pavement, grassed swale, rain barrel, sand filters, sorption media and others. Sorption media had been widely used and applied for the treatment of nutrient especially total nitrogen (TN) and total phosphorus (TP). Biosorption Activated media (denoted BAM, hereafter) has been studied for its efficiency to treat nitrogen and phosphorus. The efficiency on the treatment of TN and TP via BAM applications is summarized in Table 1 and thus, other media nutrient removal efficiencies have been included for comparison.

Table 1. Comparation between BAM and other adsorption media

Sorption Media	Components	TN Removal	Nitrate Removal	Nitrite Removal	NOx	Ammonia Removal	TP Removal	Phosphate	Reference
BAM	Sand (85.0% volume), tire crumb (10.0% volume), clay (5.0% volume)	42-51% 52-80% (groundwater) 62-70% 28-31%	-	-	72% 77-81%	(-127) - 14%	60% 62%	-	Hood, Chopra et al. (2013) Chang, Wanielista et al. (2018) Wen, Chang et al. (2018) Cormier and Duranceau (2019)
	Sawdust (15.0 %volume), tire crumb (15.0% volume), limestone (20.0% volume), sand (5.0% volume)	-	65-98%	-	-	64-100%	>99%		Hossain, Chang et al. (2010)
Iron and Aluminum Hydroxide coated Filter Media	Sand, Olivine, Aluminum chloride and ferric chloride	-	-	-	-	-	70-90%		Ayoub, Koopman et al. (2001)
IFGEM 1	Sand (96.2% volume), iron filing (3.8% volume)	-	85-90%	-	-	-	45-80%	-	Chang, Wen et al. (2018)
IFGEM 2	Sand (80.0% volume), tire crumb (10.0% volume), clay (5.0% volume), iron	-	61-92%	-	-	-	85%	-	Chang, Wen et al. (2018)

Sorption Media	Components	TN Removal	Nitrate Removal	Nitrite Removal	NOx	Ammonia Removal	TP Removal	Phosphate	Reference
	filing (5.0% volume)								
IFGEM 3	Sand (83.0 % volume), tire crumb (10.0% volume), clay (2.0% volume), iron filing (5.0% volume)	91-94%	-	-	-	95-98%	84-92%	-	Valencia, Chang et al. (2019)
Minnesota Filter	iron filings (5.0% weight), sand (95.0 %weight)	-	-	-	-	-	88.5%	-	Erickson, Gulliver et al. (2012)
Steel slag	Steel slag	84%	-	-	-	80%	74%	-	Lu, Zhang et al. (2016)
Dolochar	Waste in sponge iron industry	-	12-72%	-	-	-	-	59-100%	Rout, Dash et al. (2016)
SCL (Sandy Clay Loam)	Sandy clay loam	-	64-90%	93-94%	-	-	-	-	Güngör and Ünlü (2005)
LS (Loamy Sand	Loamy sand	-	93%	95%	-	-	-	-	Güngör and Ünlü (2005)
SL (Sandy Loam)	Sandy loam	-	45-73%	83-96%	-	-	-	-	Güngör and Ünlü (2005)

The further exploration of BAM for its implementation in the field is imperative to continuously treat polluted runoff and stormwater from urbanize areas and agriculture to mitigate its effects on the ecosystem.

1.2. Study Site

The effectiveness of Biosorption Activated Media (BAM) to treat TN in a linear ditch along a roadside was studied by Chang, Wen et al. (2019). The TN removal efficiency of BAM in a field scenario (Figure 1 (a-d)) was compared to the a TN removal efficiency obtained from a column study conducted in a laboratory scenario (Figure 1 (e)). The results indicated that BAM exhibits appropriate TN removal and that the data obtained in the lab scale experiment is comparable to the results in the field. However, a better performance of BAM was observed in the field. From the study done by Chang, Wen et al (2018), it was understood that the field conditions are complicated, and that external force impacts from traffic and animal activities need to be considered. The different field condition produced by different external forces affects hydraulic retention time (HRT) and oxygen availability that can influence the microbial ecology in BAM. Furthermore, its understanding is imperative for future real-world BAM applications.

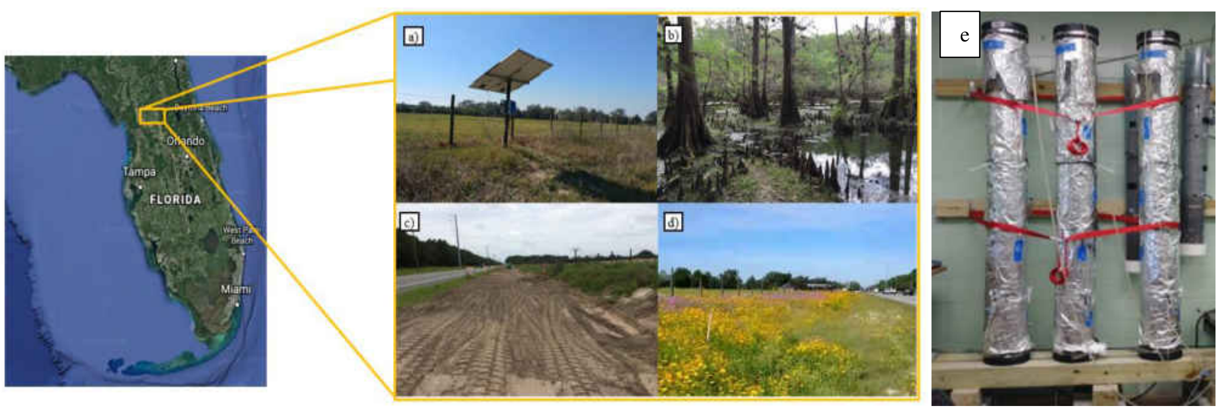

Figure 1. Photography of liner ditch study site were a & d) indicates the site during the operation of the test beds, b) Fanning springs before the application of the test beds, c) site after construction of the test bed and e) laboratory column study setup.

This study aims to explore the integrated effect of different field conditions and different influent conditions that can be encountered in the field (real-world applications). The most occurring external forces observed in the field is compaction and conduits created by traffic and animal activities respectively. To account the effect of such external forces three scenarios were mimic in a column study. Each column simulates a different scenario regarding traffic compaction, animal disturbance or a combination of both. Column 1 (scenario 1) simulates the activity of animals in Florida soil such as gopher tortoises, ants and other. Column 2 (scenario 2) replicates the presence of Floridian animals in the soil, in addition to compaction due to sligth traffic. While, column 3 (scenario 3) was compacted to imitate the presence of traffic on soils. Table 2 explains the different conditions simulated on each column (scenario).

Table 2. Field conditions simulated in each column

Scenario 1	**Scenario 2**	**Scenario 3**
• Column 1 • Simulates the impacts of animals on soils (gopher tortoises and ants) • Faster infiltration rate • A significant number of conduits of different sizes	• Column 2 • Simulates the impacts of animals on the soil, with slight compaction from small cars • Moderate infiltration rate • Conduits of smaller size	• Column 3 • Simulates compaction due to traffic • Slow infiltration rate • Fewer to no conduits

1.3. Study Framework

To obtain a holistic understanding of BAM the effect of the long-term presence of carbon and copper in the influent have been included in this study. Heavy metals naturally occur in nature in the form of copper, lead, cadmium and others. Thus, copper is found in the dissolved form as part of dissolved organic matter. Furthermore, high concentrations of copper have been found in runoff from urbanized areas. An study by Wen, Chang et al. (2018) found that copper in BAM can enhance the denitrification process as it promotes denitrifiers with copper dependence nitrous reductase (nosZ), which results in an enzymatic cascade effect that promotes denitrification due to more energy available for the reproduction of more denitrifiers. Hence, a bioactivity analysis indicated that other bacteria were depressed as a result of the presence of copper. Nonetheless, carbon can be found at high concentration in stormwater runoff and it is predominantly found in dissolved organic matter together with nitrogen and phosphorus. A similar study done by Chang, Wen et al. (2018) indicated that the presence of carbon can enhance the microbial population growth as well it can improve the removal of TN. However, these two studies did not consider the effect of the long-term presence of copper and carbon. Hence, it also ignores the effect that external forces have on the removal of total nitrogen. This information is essential for a more reliable implementation of BAM in the field in areas linear ditch along a roadside that receives transportation stormwater runoff and agricultural discharge.

CHAPTER 2: COPPER IMPACT ON ENZYMATIC CASCADE AND EXTRACELLULAR SEQUESTRATION VIA DISTINCTIVE PATHWAYS OF NITROGEN REMOVAL IN GREEN SORPTION MEDIA UNDER VARYING STORMWATER FIELD CONDITIONS

2.1. Introduction

The earth's nitrogen cycle has been largely impacted by anthropogenic activities since the industrial revolution, driven partially by the rapid increase in the human population and urbanization process (Buhaug and Urdal 2013, Seto, Parnell et al. 2013). This has resulted in more nitrogen consumption and random distribution (Jordan and Weller 1996, Smil 2002, Luo, Hu et al. 2018) in the last decades via various non-point sources such as stormwater runoff and agricultural discharge (Rawlins, Ferguson et al. 1998, Yang, Ma et al. 2004, Carle, Halpin et al. 2005, Grimm, Foster et al. 2008, Smith and Harlow 2011, Fixen, Brentrup et al. 2015). To reduce the nitrogen concentration in the stormwater runoff and control flood impact, best management practices (BMPs) have been adopted widely. However, many of them were refined later to meet nutrient removal requirements while maintaining flood mitigation and control (Ermilio 2005, Wanielista, Chang et al. 2011, Park, Kang et al. 2015). Sorption media are one of the most promising technologies given their ability to treat nutrients mainly via sorption and biological means. The sorption processes are effective and environmentally friendly and are dependent of the physical and chemical characteristics of the media. The inclusion of metals like iron-filing particles as a component of sorption media can interact to reduce nitrate via ion exchange mechanism that transforms nitrate to ammonia. In congruence the adsorption of ammonia from clay has been studied in Iron-Filing green environmental media (IFGEM) (Chang, Wen et al. 2019, Valencia, Chang et al. 2019).

This study focuses in Biosorption activated media (BAM) as a promising green sorption media that utilizes recycled materials in the media mixes to adapt to different landscapes for low impact development and is considered a new BMP for enhancing the effectiveness of nutrient removal in Florida and elsewhere in the United States. The study of its performance if applied in watershed systems are significant given that the leading causes of pollution of rivers and streams are nonpoint sources. Hence the implementation of sorption media in watershed systems can intersept the pollutants at its source (Kröger, Holland et al. 2008, Buchanan, Falbo et al. 2013). The characteristics of BAM facilitate and promote a suite of microbiological reactions in the nitrogen cycle. The essential steps of the nitrogen cycle: ammonification (Burger and Jackson 2003, Ryzhakov, Kukkonen et al. 2010), nitrification (Malhi and McGill 1982, Ruiz, Jeison et al. 2003, Di, Cameron et al. 2009), denitrification (Her and Huang 1995, Sun, Wu et al. 2017) and dissimilatory nitrate reduction to ammonia (DNRA) (Giblin, Tobias et al. 2013) are possitively impacted as BAM provides appropriate hydraulic control, moisturization, nutrient adsorption, and biofilm growth. In ammonification, organic nitrogen is converted into inorganic nitrogen, which serves as the food source for nitrifiers and denitrifiers in the microbial community. In nitrification, reduced nitrogen compounds are first transformed into nitrite by ammonia-oxidizing bacteria (AOB). Subsequently, nitrite is transformed into nitrate by nitrite oxidizing bacteria (NOB). Anaerobic ammonium oxidation is carried out by anaerobic ammonium oxidation bacteria (anammox). Two pathways of denitrification have been previously identified: the dissimilatory nitrate reduction to ammonia (DNRA) and common denitrification. The DNRA is carried out by denitrifying bacteria, the enzymes of which are coded by narG and nrfA gene sequences; DNRA is responsible for converting nitrate to nitrite and lastly into ammonia. The study of the DNRA is significant since it retains ammonia, which can hurt other components of the ecosystem. Lastly, in common denitrification nitrates are converted into nitrogen gas with the aid of denitrifying bacteria, the enzymes of which are coded in gene sequences narG, nirS, norB, and nosZ. However, the complexity of stormwater compositions and changing field conditions such as linear ditches at the roadsides are barriers to fully understanding the microbial ecology within the BAM mixes in depth, especially under the impacts of metallic molecules such as copper ions that are oftentimes present in stormwater runoff.

Copper is known as one of the most frequently used materials for stormwater disinfection (Borkow and Gabbay 2009) and it can commonly be found in stormwater runoff due to the use of algicides at concentrations ranging from 20 to 50 µg/L (Holtan-Hartwig, Bechmann et al. 2002, Wang, Shi et al. 2007, Ochoa-Herrera, León et al. 2011, Paus, Morgan et al. 2014). Copper is also involved in electron transfer and oxygen transport, as well as in redox reactions of multiple substrates (Dupont, Grass et al. 2011). In the N-cycle, copper can be helpful in reducing nitrous oxide (N_2O) emissions from agricultural fields as it is the cofactor of N_2O reductase (Felgate, Giannopoulos et al. 2012). However, the overdosage of copper can jeopardize the structure of a cell or enzyme protein surface and inhibit the microbial community (Thurman, Gerba et al. 1989). Through an investigation of the influences of copper on the N-cycle within BAM, our previous study confirmed that short-term copper addition might trigger enzymatic cascade effects in denitrifiers' population growth, initiated by enhancing the last step of denitrification (N_2O to N_2) (Wen, Chang et al. 2018). Yet the long-term copper impact on nitrogen removal in the N-cycle within BAM under different field conditions that could differ with short-term observations remains unclear due to the relatively unknown potential interactions between environmental forcing and physiological response in microbial species. Understanding the impacts of long-term copper addition is important given that many of the stormwater wet detention ponds across the world may have to face this condition. A linear ditch study is one of the closest references to actual field conditions. However, a study on nutrient removal via Biosorption-Activated Media at laboratory and field-scale indicated that the unawareness of external impacts from traffic and animal activity needs to be considered to better resemble field conditions (Chang, Wen et al. 2019). In this study various external forces, such as conduits created by animals, external compaction by construction and traffic impact, or the combination of both, were included as it may potentially change the hydraulic conditions for stormwater treatment processes, may compound the effect, resulting in unknown outcomes under the long-term presence of copper. With the long-term presence of copper in stormwater runoff, dissolved organic nitrogen (DON) occupies up to 80% of total nitrogen and is an essential nitrogen source supporting microbial processes (Berg, Glibert et al. 1997, Berman and Bronk 2003, Glibert, Heil et al. 2004). Understanding quantitative and qualitative changes of DON in BAM mixes provides unprecedented insight because it reflects the behavior and strategic changes of the entire microbial community facing the copper impact within varying field conditions. Identification of DON compounds at the molecular level requires advanced analytical techniques due to the immense polydispersity and compositional complexity of dissolved organic matter (DOM). Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-IR-MS) has been widely applied to address complex DOM in marine organics (Koch, Ludwichowski et al. 2008), surface water (Stenson, Marshall et al. 2003, Minor, Steinbring et al. 2012), stormwater (Zhang, Wang et al. 2016, Chang, Wen et al. 2018), biochar (Avneri-Katz, Young et al. 2017, Hagemann, Joseph et al. 2018), and wetlands (O'Donnell, Tfaily et al. 2016) in addition to numerous aqueous water and petroleum systems.(Rodgers and Marshall 2008, Headley, Peru et al. 2009, Smith, Rodgers et al. 2009) With unparalleled ultrahigh resolving power ($m/\Delta m_{50\%}$ > 2,700,000 at *m/z* 400) and mass accuracy (80-120 ppb) (Smith, Podgorski et al. 2018), FT-ICR-MS enables confident identification of tens of thousands of unique elemental compositions in DOM. To link the DON information with microbial denitrification activities in this study, a real-time polymerase chain reaction (RT-PCR), also known as quantitative PCR or qPCR, was employed to address microbial ecology studies, in which the fluorescent reporter signal strength is directly proportional to the number of amplified deoxyribonucleic acid (DNA) molecules (Hall, Hugenholtz et al. 2002, Harms, Layton et al. 2003). Thus, RT-PCR would provide quantitative information about the microbial species as another critical aspect for understanding the long-term copper impact. FT-ICR-MS in conjunction with RT-PCR is essential for deeper comprehension of the functionality of different DOM components between different microbial species, particularly the DON components of interest in stormwater treatment within BAM-based BMPs. Previous work has applied electrospray ionization FT-ICR-MS to identify biodegradable DON compounds at the molecular level in stormwater systems (Lusk and Toor 2016), and also determined the effectiveness of carbon for DON removal impacts in BAM (Chang, Wen et al. 2018).

The objectives of this column study are to (1) assess and compare the impact of short- and long-term copper addition on nitrogen removal under 3 different linear ditch field conditions that influence *in-situ* stormwater treatment; and (2) explore the long-term copper impact on DON concentration/composition changes. By linking the results from RT-PCR, FT-IR-MS, and nutrient removal, the novelty of this study lies in its enhanced realization of the relationship between the truth of enzymatic cascade effects triggered by copper impact and external forces in 3 different typical linear ditch field conditions in association with DON changes. Some scientific questions to be explored include: (1) What is the effect of the copper addition on nitrogen removal under various external forces or field conditions related to traffic compaction and animal disturbance? (2) How does copper affect the microbial population dynamics, metabolic rate, and cell conditions under short-term and long-term influence? (3) How do the enzymatic cascade effects perform differently between short-term and long-term copper influence? (4) How will the DON concentration and composition be removed/reshaped with respect to short- and long-term copper addition? We hypothesize that: (1) the short-term copper addition will inhibit the DON removal but enhance the denitrification process; (2) the microbial community will adapt to the long-term exposure to copper and start to recover its population; (3) the changes of DON concentration and composition may be restored after the adaption under the long-term exposure to copper; (4) the external forces or field conditions may trigger important impacts on copper influences.

2.2. Materials and Methods

2.2.1. BAM and the Impacts of External Forces

The composition of BAM is 85% sand, 10% tire crumb, and 5% clay by volume in this column study. To assess the effects of different external forces, three different scenarios (i.e., field conditions) have been identified as part of a column study and each scenario is represented by one column filled with a consistent BAM recipe given the external force(s) at the top layer as a boundary condition. Disturbances were applied to the top layer (30 cm) of the three scenarios over the three columns (Table 2): (1) Column 1 has external force driven by animal conduits resulting in a faster infiltration rate so the field condition has a significant number of conduits of different sizes. (2) Column 2 has external forces driven by routine traffic compaction and animal conduits with a moderate infiltration rate, so the field condition has small size conduits, and (3) Column 3 has external forces driven by traffic compaction only with a low infiltration rate so the field condition has few to no conduits.

2.2.2. Experimental setup

Three identical columns of 1.5 m in height and 15 cm in diameter were assembled in this study. On the side of each column, three sampling ports for water and media were installed in 30 cm intervals, as shown in Figure 2. To conclude the setup, all three columns were filled up to 1.2 m depth with BAM. All columns had been cultivated for 6 weeks with 10 mL/min of stormwater (collected from a campus pond) spiked with nitrate standard solution (item#: 1279249, HACH) and glucose (as carbon source) at a concentration of 5 mg/L N and 40 mg/L COD. The carbon source facilitated the cultivation processes.

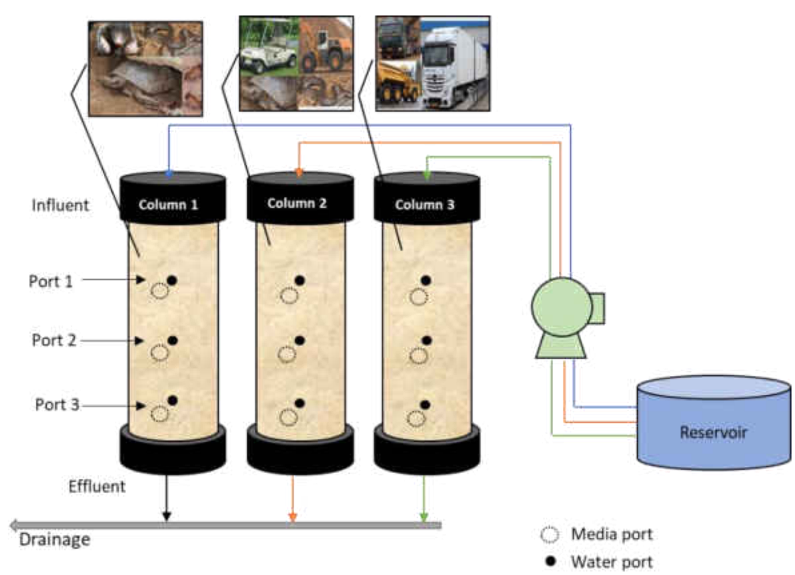

Figure 2. Column experimental setup

After cultivation, all columns were fed with stormwater spiked with nitrate alone (5 mg/L N) for one week to normalize the condition in each column (non-copper case). Then copper (Cat#: SC194100, Fisher Scientific) was spiked to the concentration of 25-50 µg/L for 7 days, after the previous addition of stormwater. To assess the short-term copper impacts, water and media samples were collected one day after copper addition. One more set of water and media samples were collected on the 7th day after copper addition for evaluating the long-term copper impacts. Periodically during the column study, the top layer of each column was disturbed to simulate the different field conditions explained in section 2.1. Additional water samples were collected and were preserved for further analysis, as explained in the following sections.

2.2.3. Water parameters analysis

Water samples (75 ml) were collected in triplicate form from the influent, port 1, port 2, port 3, and the effluent of each column at the end of the non-copper case (NC), short-term copper addition case (SC), and long-term copper addition (LC) case. Each sample was analyzed for dissolved oxygen, pH, and ORP right after the sample collection. Subsequently, samples were analyzed within 24 hours of collection at the University of Central Florida (UCF) laboratories for total nitrogen (TN), nitrate, nitrite, ammonia, and alkalinity with the methods specified in Table 3. The inlet, port 1 and outlet samples were collected (100 ml) and delivered to the Environmental Research and Design laboratories for copper concentration analyses. The analysis followed method SM-22 Sec3111B (Eaton, Clesceri et al. 2005).

Table 3. Water parameter, analysis method, and detection range information

Parameters	Analysis Method	Product Number	Detection range
Total Nitrogen	Hach Kit (10208)	TNT 826	1-16 mg/L N
Nitrate	Hach Kit (10206)	TNT 835	0.2-13.5 mg/L NO_3-N
Nitrite	Hach Kit (10237)	TNT839	0.001-0.6 mg/L NO_2-N
Ammonia	Hach Kit (10205)	TNT 830	0.015-2 mg/L NH_3-N
Alkalinity	Hach Kit (10239)	TNT 870	25-400 mg/L as $CaCO_3$
COD	Hach Kit (8000)	TNT 820	3-150 mg/L COD
DO	HACH HQ40D – IntelliCAL LDO101 LDO	N/A	0.1 – 20 mg/L
pH	Waterproof Double Junction pH Test® 30	N/A	0-14 pH
ORP	HACH HQ40D-IntelliCAL	N/A	-1999-1999 mV

2.2.4. Tracer study

To retrieve information regarding the impact of external forces on the internal hydraulics of BAM and to collect information on the HRT patterns on each column a tracer study with Rhodamine dye was applied. The procedure consisted of two stages, the injection of the dye and the collection and analysis of samples. In stage one, 5 mg of diluted Rhodamine dye was injected in the middle of the top section of each column. In stage two, water samples from the effluent were collected in 10-15-minute intervals and immediately analyzed for dye concentration with the aid of a fluorometer (AquaFlour model: 80000-010).

2.2.5. qPCR Analysis

Triplicate media samples (10 grams) were collected from the top of the column, port 1, port 2, and port 3 at the end of the NC, SC, and LC cases. All samples were stored at -80°C immediately after collection. To quantify the population of the microbial community in BAM, qPCR was used to analyze all media samples for a comprehensive microbial population dynamics study. The DNA extraction was performed via QIAGEN DNA extraction kit, following the instructions provided by the vendor. The qPCR setup and analysis were performed at UCF with the aid of the computer software StepOne. Plates with 48 wells were used; each well contained 5 µl of sample, 10 µl of PowerUp SYBRGreen Master Mix, 0.8 µl of forward and reverse primer, and 3.4 qPCR water. The primers and running method are summarized in Table 4.

Table 4. Primers information for qPCR analysis

Target Prokaryote	Target gene	Primer Name	Oligonucleotide Sequence	Running method	Reference
Ammonia Oxidizing Bacteria	amoA	amoA1F	GGGGTTTCTACTGGTGGT	2 min 50 º C and 95º C; 45 cycles [15 s at 95º C and 1 min at 62º C]	Rotthauwe et al. (1997)
		amoA-2R	CCCCTKGSAAAGCCTTCTTC		
Ammonia Oxidizing Archaea	amoA	Arch-amoA-F	CTGAYTGGGCYTGGACATC	2 min 50º C and 5 min for 95; 40 cycles [30s at 95º C, 45s at 56º C and 45s at 72º C]	Wuchter et al. (2006)
		Arch-amoA-R	TTCTTCTTTGTTGCCCAGTA		
Comammox	amoA	A378f	TGGTGGTGGTGGTCNAAYTAT	2 min 50º C and 5 min for 95; 40 cycles [30s at 95º C, 30s at 58º C and 30s at 72º C]	Xia et al. (2018)
		C616r	ATCATCCGRATGTACTCHGG		
Nitrite Oxidizing Bacteria	NOB	NSR1113F	CCTGCTTTCAGTTGCTACCG	2 min 50 º C and 95º C; 45 cycles [15 s at 95º C and 1 min at 62º C]	Dionisi et al.(2002)
		NSR1264	GTTTGCAGCGCTTTGTACCG		
Anaerobic ammonium oxidation	AMX	809-F	GCCGTAAACGATGGGCACT	2 min 50 º C and 95º C; 45 cycles [15 s at 95º C and 1 min at 62º C]	Tsushima et al. (2007)
		1066-R	AACGTCTCACGACACGAGCTG		
Denitrifying bacteria	nosZ	nosZ-F	CGYTGTTCMTCGACAGCCAG	2 min 94 °C; 35 cycles [30 s at 94°C; 40 s at 57 °C; and 40 s at 72 °C]	Kloos Karin et al. (2001)
		nosZ1622R	CGSACCTTSTTGCCSTYGCG		
Denitrifying bacteria	nirS	Cd3AF	GTSAACGTSAAGGARACSGG	2 min 50 °C and 10 min for 95 °C; 40 cycles [60 s at 95 °C; 60 s at 51 °C; and 60 s at 60°C]	Gaston Azziz et al.(2017)
		R3Cd	GASTTCGGRTGSGTCTTGA		
Dissimilarity nitrite reducing bacteria	nrfA	nrfA2F	CACGACAGCAAGACTGCCG	2 min 50 °C and 10 min for 95 °C;40 cycles [30 s at 95 °C; 60 s at 60 °C; 60 s at 72 °C]	Yin, Guoyu et al. (1998)
		nrfA2R	CCGGCACTTTCGAGCCC		

Furthermore, the change in cell volume was calculated based on equation 2, in which n_1 and r_1 corresponds to the number of microbial gene copies quantified by qPCR and the radius of the cell before copper addition, respectively. Besides, n_2 and r_2 corresponds to the number of microbial gene copies quantified by qPCR and the radius of the cell after copper addition, respectively. Equation 2 was developed based on equation 1 based on three assumptions. In the first assumption it is implied that the bacteria cell shape is spherical ($V_{cell}=4/3 \cdot \pi \cdot r^3$). The second assumption suggests that bacteria aim to occupy all the available living volume ($V_1= n_1 * V_{cell}$). In the third assumption a constant living volume for microbial growth was maintained before and after copper addition (V_1=V_2).

$$n_1 * \frac{4}{3} * \pi * r_1^3 = n_2 * \frac{4}{3} * \pi * r_2^3 \qquad (1)$$

Equation 1 was rearranged in terms of volume ratio resulting in the following equation:

$$\left(\frac{r_2}{r_1}\right)^3 = \frac{n_1}{n_2} \qquad (2)$$

Subsequently, the surface area to volume ratio (SA/V) change was theoretically calculated following equation 3 where the SA_1/V_1 ratio was calculated based on the assumption of a cell radius (r_1) of equation 1. Hence, to generate SA_2/V_2 the new cell radius was calculated based on the cell volume change ratio ($\left(\frac{r_2}{r_1}\right)^3$).

$$\Delta \frac{SA}{V} = \frac{(SA_2/V_2)-(SA_1/V_1)}{(SA_1/V_1)} \qquad (3)$$

2.2.6. DON analysis

Water samples (500 ml) were collected from the influent and effluent of each column at the end of the NC, SC, and LC cases. All water samples were filtered immediately after collection via a filtration kit and a GF/F glass filter of pore size 0.7 µm. Filtered samples were then stored at 4°C before performing the solid-phase extraction (SPE) according to the protocol developed by Dittmas et al (Dittmar, Koch et al. 2008). After SPE, all final samples were kept under -20 ºC until analysis. Sample analysis for DON was performed at the National High Magnetic Field Laboratory at the Florida State University (FSU) in Tallahassee, FL. DON extracts were analyzed at FSU with a custom-built FT-ICR-MS (Kaiser, Quinn et al. 2011) equipped with a 9.4 T horizontal 220 mm bore diameter superconducting solenoid magnet operated at room temperature and a modular ICR data station (Predator) (Blakney, Hendrickson et al. 2011) facilitated instrument control, data acquisition, and data analysis. Experimentally measured masses were converted from the International Union of Pure and Applied Chemistry mass scale to the Kendrick mass scale (Kendrick 1963) to identify homologous series for each heteroatom class (i.e., species with the same $C_cH_hN_nO_oS_s$ content, differing only by their degree of alkylation). For each elemental composition, $C_cH_hN_nO_oS_s$, the heteroatom class, type (double bond equivalents = number of rings plus double bonds involving carbon) and carbon number, *c*, were tabulated for subsequent generation of H:C ratio vs. carbon number images or van Krevelen diagrams in PetroOrg©. The full operation details of FT-ICR-MS can be viewed in an external link (https://nationalmaglab.org/user-facilities/icr) provided by the National High Magnetic Field Laboratory Ion Cyclotron Resonance Facility (ICR).

2.3. Results

2.3.1. Hydraulic patterns

The hydraulic retention time (HRT) of each scenario was analyzed by using a tracer study. The HRT variations for NC and LC cases are presented in Figure 3. The HRT patterns before and after the long-term copper addition can be realized through different scenarios with an understanding of multiple aspects. Scenario 1 showed the shortest HRT value in NC and LC cases, with HRT values of 20 and 60 minutes, respectively. On the other hand, the largest HRT value was retrieved from scenario 3 with 240 and 300 minutes in NC and LC cases, respectively. In scenario 2, the HRT in the case of NC was 140 minutes, and the value of HRT after copper addition (LC) exhibited an average value of 145 minutes.

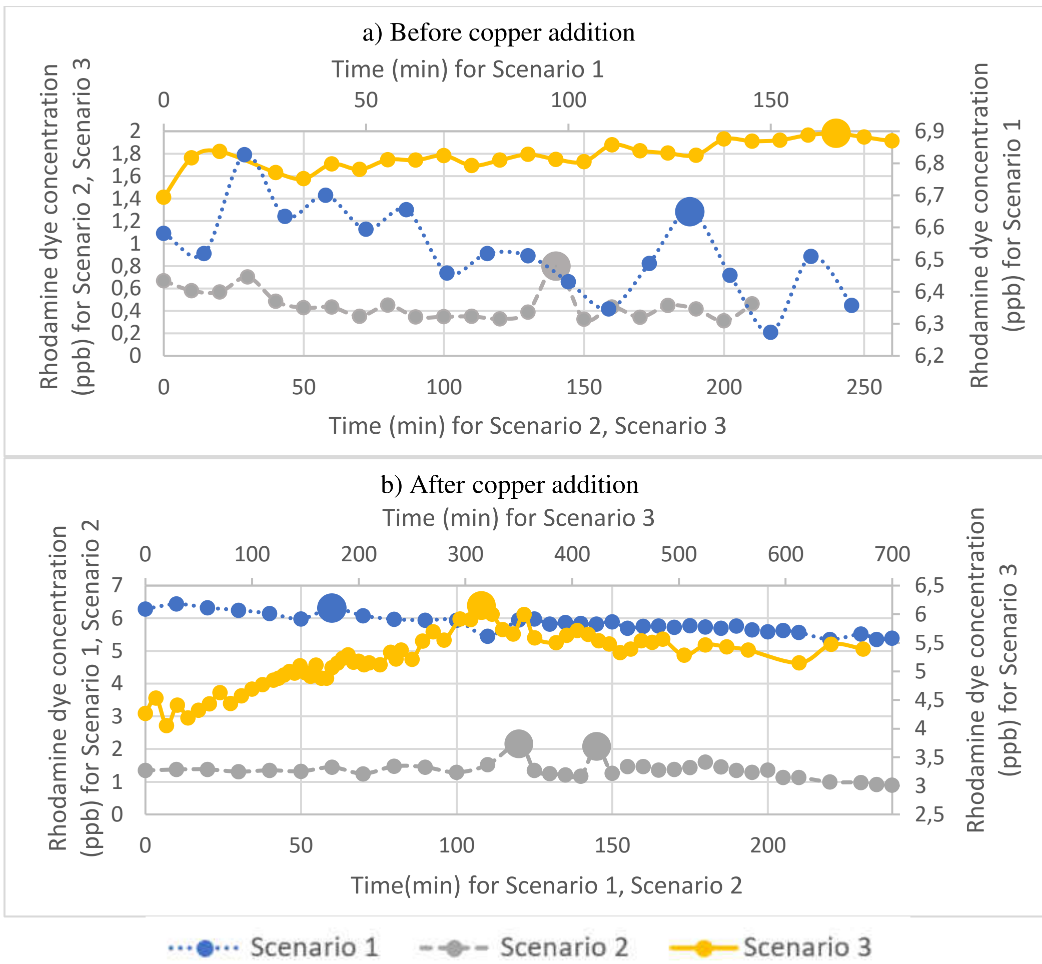

Figure 3. Tracer study results for scenario 1, 2 and 3 a) before copper addition and b) after copper addition.

2.3.2. Water parameters

The pH values fluctuated within the range from 7.4 and 8.1 throughout the study. The variations of ORP and alkalinity at different ports in the cases of SC and LC associated with scenarios 1, 2, and 3 are displayed in Figure 4.

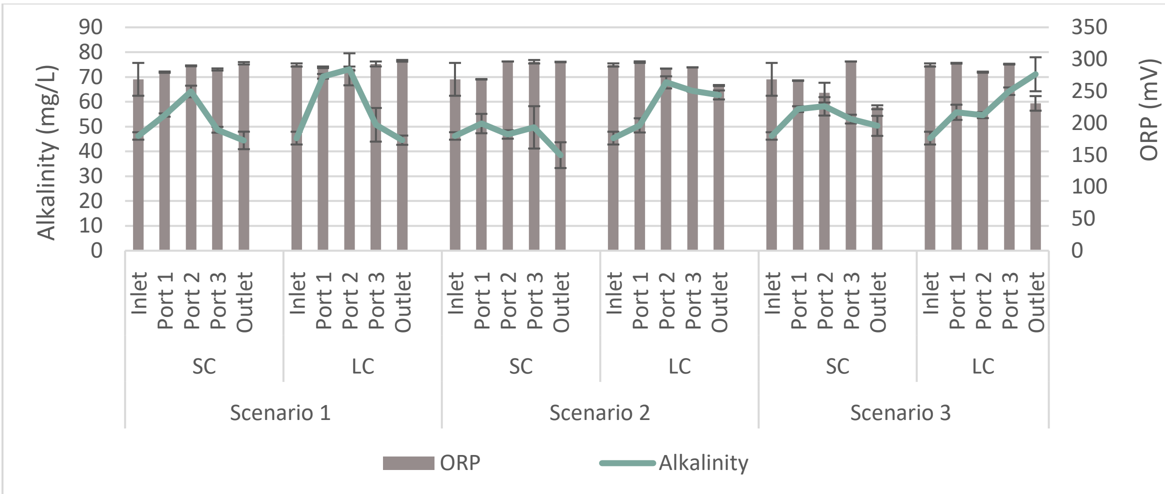

Figure 4. ORP and alkalinity values for short-term and long-term copper impact.

Table 5 lists the nutrient concentrations from the influent and the percent removal at each water sampling port relative to the influent. The overall total nitrogen (TN) removal in the case of NC was 13.7%, 41.5%, and 52.3% for scenarios 1, 2, and 3, respectively. A decrease of the overall TN removal in the case of SC for scenarios 1 and 2 of 12.8% and 36.7%, respectively, was observed. A slight increase of overall TN removal was observed in scenario 3 (54.5%). However, the long-term copper addition significantly decreased the overall TN removal of scenarios 1, 2, and 3 to 7.3%, 15.7%, and 34.0%, respectively. The decrease of denitrification accounts for the major loss of nutrient removal capacity, especially for scenarios 2 and 3, which dropped by 34-36% of NO_x removal. Figure 5 indicates the concentration and composition of each nitrogen species from the influent and effluent in each scenario and case. NO_x was found to be the most predominant TN component. Moreover, an increase in DON was found in the case of LC. The ammonia concentration increased at the effluent in the case of SC and decreased in the case of LC for scenarios 2 and 3. The overall copper percent removals in port 1 and effluent with respect to the influent condition are summarized in Figure 6. The removals within influent and port 1 ranged within 30% to 82% in scenario 1, 65% to 83% in scenario 2 and 73% to 88% in scenario 3. Such results indicate that the highest copper removal occured within the first 30 cm of the column (influent to port 1). A decrease in total copper removal efficiency was observed as time progressed. After day 7, the removal efficient in scenario 1, scenario 2 and scenario 3 decreased from 84% to 70%, 83% to 80 % and 95 to 82%, respectively. The results implied that copper removal efficiency was negative impacted by the long-term addition of copper.

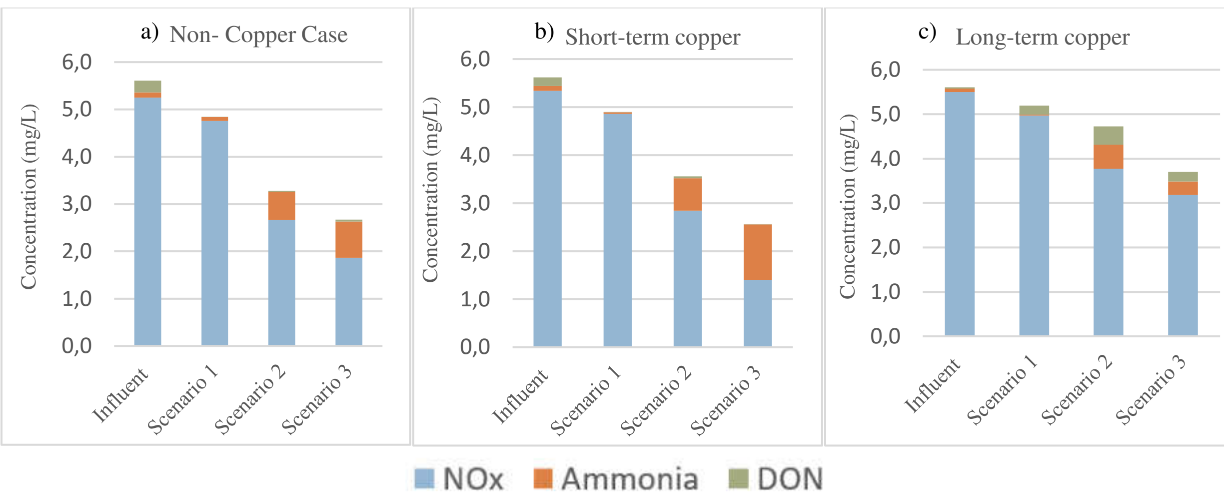

Figure 5. Total nitrogen (TN) concentration in mg/L and composition for influent and effluent for scenarios 1, 2 and 3 for NC (a), SC (b) and LC case (c)

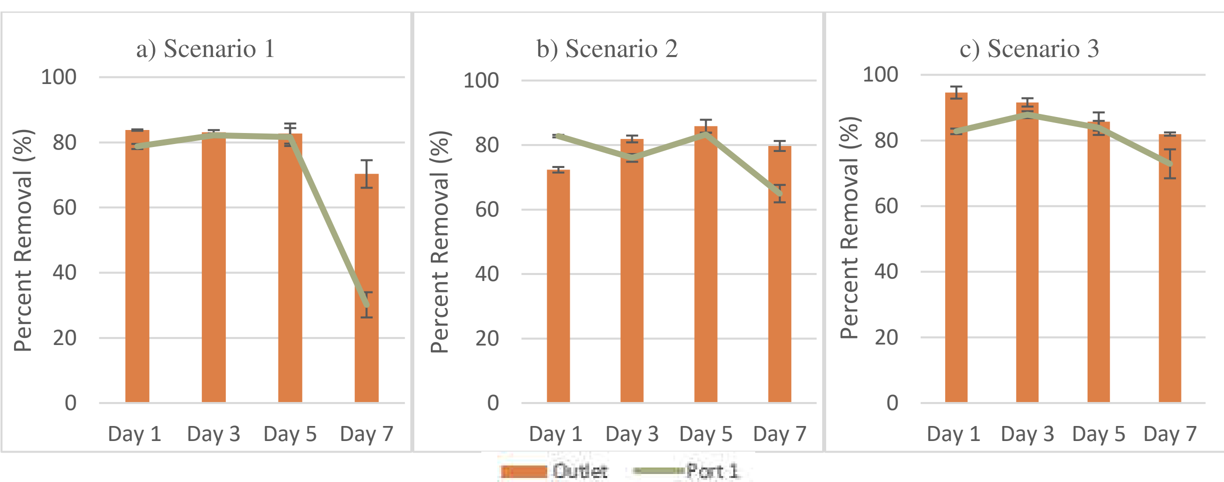

Figure 6. Copper removal in time series for scenario 1 (a), scenario 2 (b), and scenario 3 (c)

Table 5. Summary of water nutrient influent concentrations in mg/l and percent removal values at each water sampling port with respect to the influent for normal case, short-term copper and long-term copper impact

		Non-Copper Case (NC)				Short-term Copper Addition (SC)				Long -term Copper Addition (LC)			
Scenario		NOx	Ammonia	TN	COD	NOx	Ammonia	TN	COD	NOx	Ammonia	TN	COD
1	**In (mg/L)**	**5.25**	**0.11**	**5.61**	**17.83**	**5.34**	**0.10**	**5.62**	**18.53**	**5.50**	**0.08**	**5.61**	**19.30**
	P1 (%)	-1.3	-193.6	-0.3		8.7	-52.6	10.1		4.0	-70.1	-0.2	
	P2 (%)	-14.8	15.4	-7.7		-1.3	29.2	0.9		6.6	60.3	-0.9	
	P3 (%)	5.7	24.7	8.6		0.0	45.4	†		7.5	64.3	3.5	
	Out (%)	9.5	26.8	13.7	24	9.0	65.6	12.8	-14	9.6	72.8	7.3	29
2	**In (mg/L)**	**5.25**	**0.11**	**5.61**	**17.83**	**5.34**	**0.10**	**5.62**	**18.53**	**5.50**	**0.08**	**5.61**	**19.30**
	P1 (%)	4.4	-518.9	-24.0		13.9	-279.4	8.1		12.7	-466.5	10.6	
	P2 (%)	-5.2	44.8	0.1		2.2	-75.6	3.3		9.6	-312.5	6.2	
	P3 (%)	3.2	-114.3	1.7		23.0	-611.7	13.3		19.2	-461.6	12.5	
	Out (%)	49.2	-446.0	41.5	34	46.7	-590.0	36.7	23	31.3	-623.2	15.7	-4
3	**In (mg/L)**	**5.25**	**0.11**	**5.61**	**17.83**	**5.34**	**0.10**	**5.62**	**18.53**	**5.50**	**0.08**	**5.61**	**19.30**
	P1 (%)	36.2	-456.1	34.6		47.6	-520.3	37.1		27.6	-758.0	21.1	
	P2 (%)	48.5	-432.0	40.3		45.9	-721.6	40.7		56.6	-963.8	41.8	
	P3 (%)	69.2	-709.8	29.1		59.2	-1092.4	41.2		65.9	-172.3	58.8	
	Out (%)	64.5	-602.1	52.4	-14	73.7	-1085.6	54.5	29	42.2	-305.8	34.0	-20

† Data point was lost due to an error in the testing procedure during the analysis of total nitrogen.

2.3.3. DON results

The variations of inlet and outlet DON composition in each scenario are presented via Van Krevelen diagrams in Figure 7. In the case of NC (Figure 7 a-c), the inlet and outlet DON compositions are essentially the same, and the outlet showed dense dots, which indicates the composition change is minor for all scenarios, except that some proteins and amino sugars were produced in scenarios 1 and 2. For the case of SC (Figure 7 d-f), there was no additional production of proteins and amino sugars in scenarios 1 and 2, and the outlet DON compositions showed much less density compared to those from the inlet. However, scenario 3 was less affected and more lignin, proteins, and amino sugars were detected from the outlet. For the case of LC (Figure 7 g-i), scenarios 1 and 3 showed similar outcomes with less dense effluent DON compositions compared to those from the influent. However, scenario 2 exhibited noticeable differences with an intensive production of lignin, proteins, and amino sugars. This implies the increase of DON species, which is also can be inferred from Figure 5 due to the increased DON concentration.

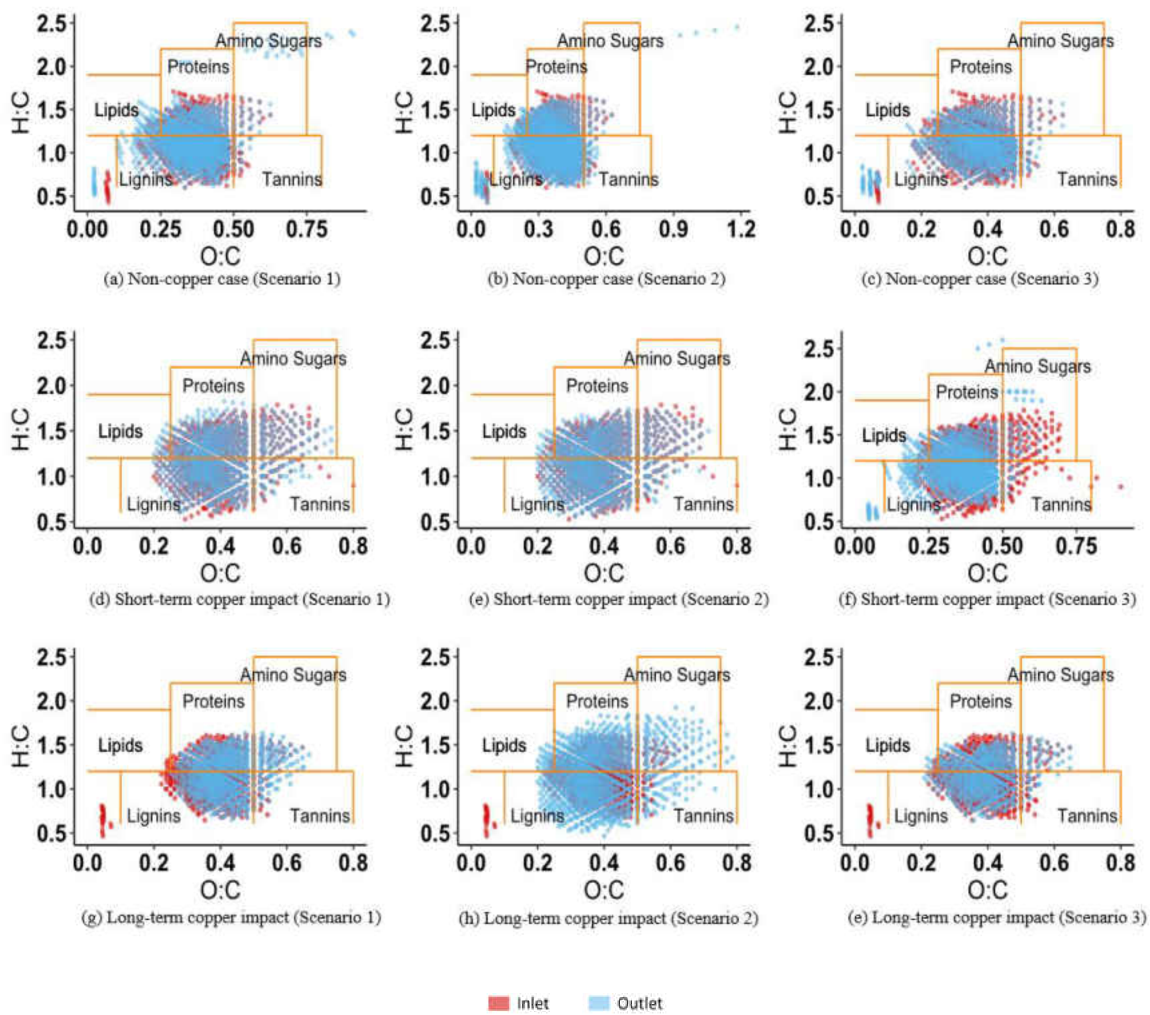

Figure 7. Van Krevelen diagram of DON composition for non copper case under scenarios 1 to 3 in (a) to (c), short-term copper impact case under scenarios 1 to 3 in (d) to (f), and long-term copper impact case under scenarios 1 to 3 in (g) to (i)

The peak number assigned for CHON classes from the inlet and outlet of each scenario over different cases are plotted in Figure 8. Note that even this cannot provide the exact concentration for each class, but the higher peak number indicates more molecules have been detected and potentially higher concentrations can be confirmed qualitatively. In the case of NC (Figure 8 a), similar CHON species distribution and peak numbers were found between the inlet and outlet samples across all scenarios. In the case of SC (Figure 8 b), both scenarios 1 and 2 showed identical influent and effluent CHON species distribution and peak numbers, which are also very similar to the inlet, while the scenario 3 effluent species exhibited fewer peak numbers with wider CHON distribution. In the case of LC (Figure 8 c), all scenarios showed similar CHON species distribution which includes only a part of the influent species, and scenario 3 showed substantially lower peak numbers than the other two scenarios, especially when compared with scenario 2, which showed the highest peak numbers.

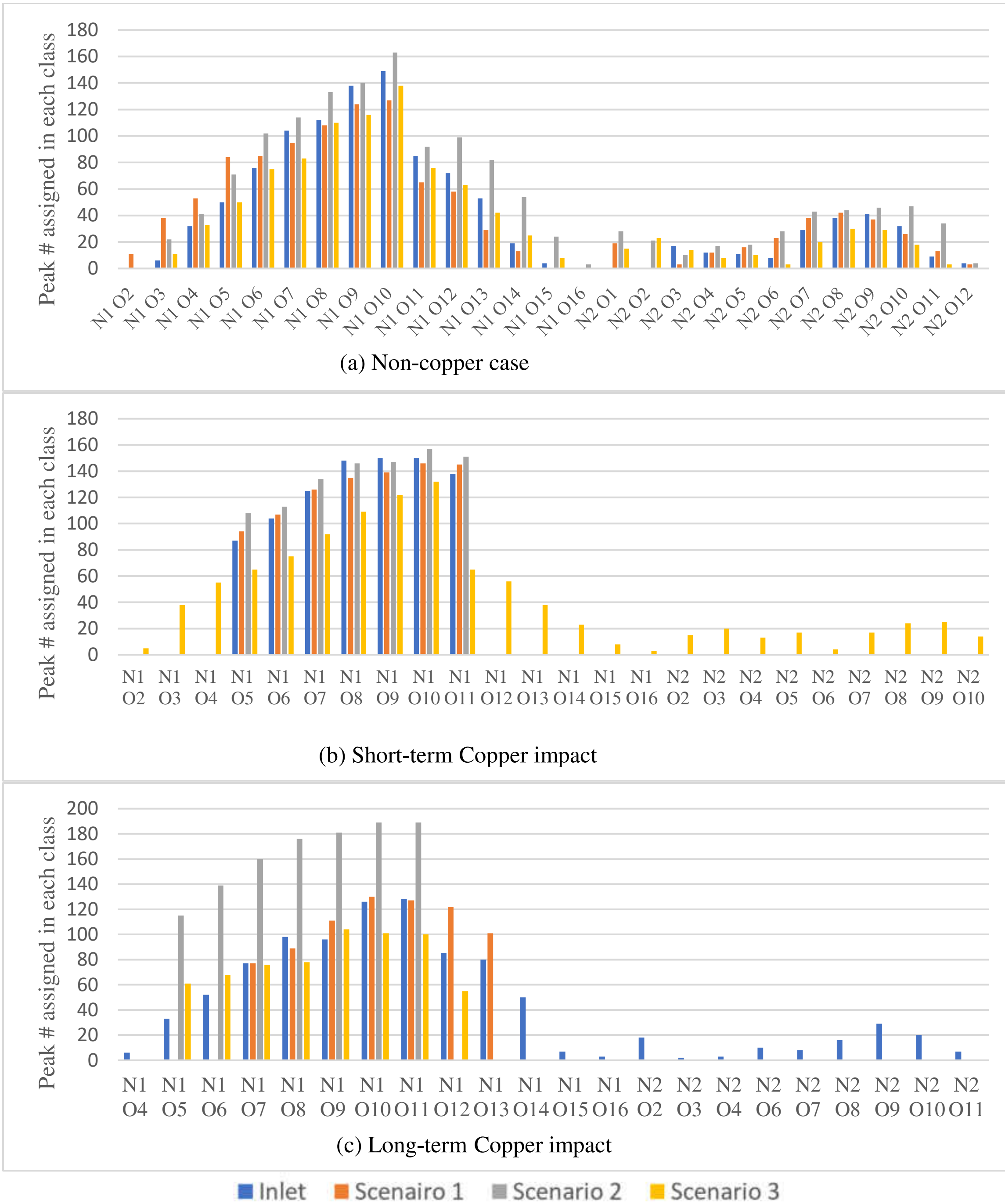

Figure 8. The peak number assigned in each CHON class for the non copper case under scenarios 1 to 3 in (a), short-term copper impact case under scenarios 1 to 3 in (b), and long-term copper impact case under scenarios 1 to 3 in (c)

2.3.4. Microbial Ecology

The information on the microbial population quantity retrieved from qPCR analysis indicated that the population density of AOB and annamox was below the detection limit in most of the cases and thus was not chosen for inclusion here. The NOB population density increased after the short-term addition of copper in comparison to the case of NC by an average per port of 18%, 37%, and 29% in scenarios 1, 2, and 3, respectively (Figure 9a). In the case of LC, the NOB population density per port decreased by an average of 10% and 6% in scenarios 1 and 3, respectively, in comparison to the case of NC (Figure 9b). Thus, in scenario 2 the population density of NOB slightly increased by an average per port of 5%. The population per port of DNRA bacteria increased in the case of SC by an average of 9%, 52%, and 16% in scenarios 1, 2, and 3, respectively, relative to the case of NC. Furthermore, the population per port in the case of NC increased by an average of 2%, 37%, and 14% for scenarios 1, 2, and 3, respectively.

Denitrifying bacteria are quite diverse thus the gene nirS, in charge of the second step of the denitrification pathway, can serve as an indicator of the population density, since it constitutes over 99% of the microbial population density. Figure 10 indicates the distribution of the denitrifiers population density at each port in the cases of NC, SC, and LC. The population density of denitrifiers is the most influential due to the short- and long-term presence of copper. In the case of SC, the average population per port increased by an average of 33%, 39%, and 61% relative to the case of NC in scenarios 1, 2 and 3, respectively. Furthermore, after the long-term copper addition, the population per port increased by an average of 92%, 83%, and 132% relative to the case of NC in scenarios 1, 2, and 3, respectively.

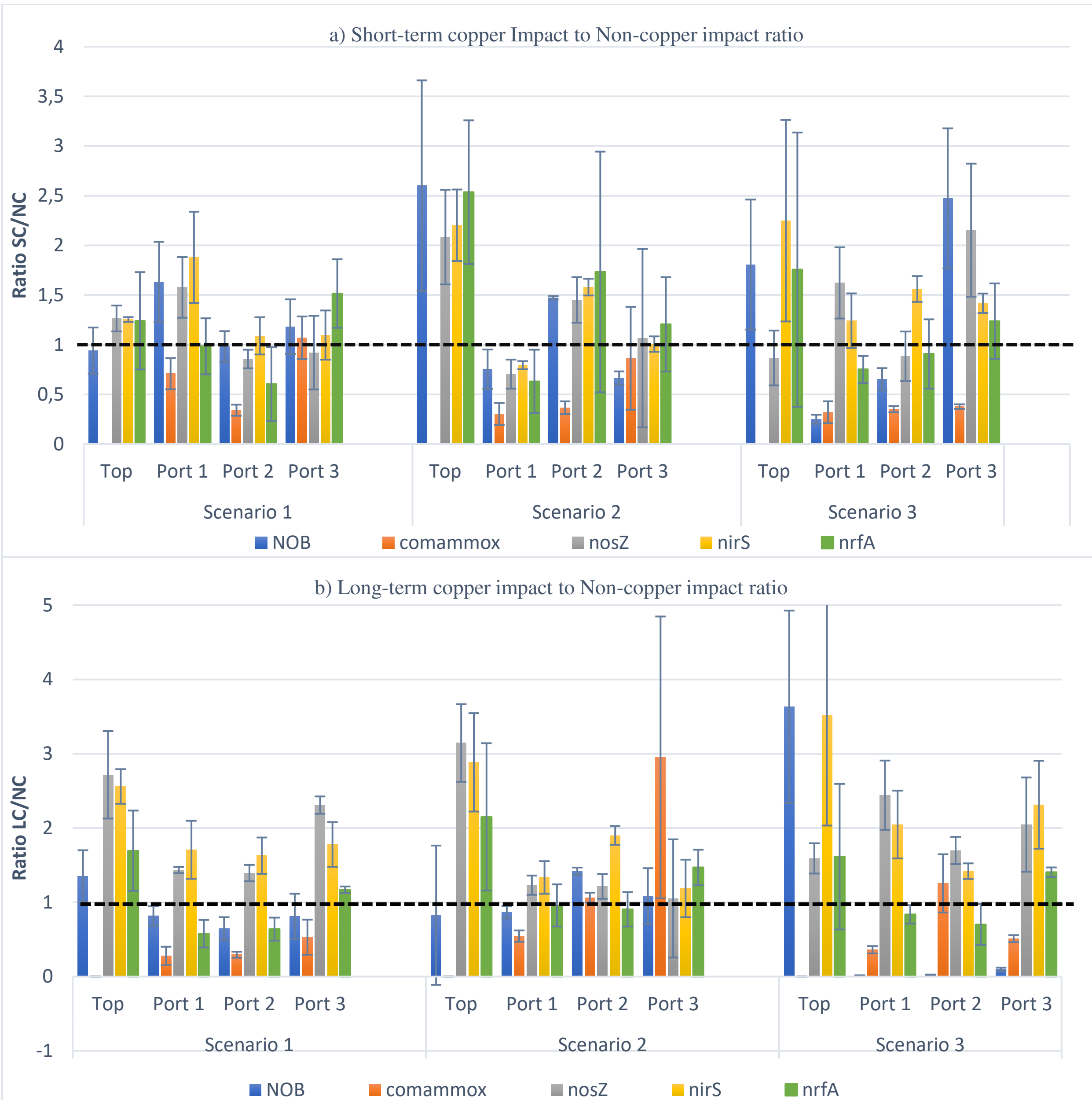

Figure 9. Microbial population ratio between Short term copper impact and non-copper case (a), and Long-term copper impact and non-copper case (b)

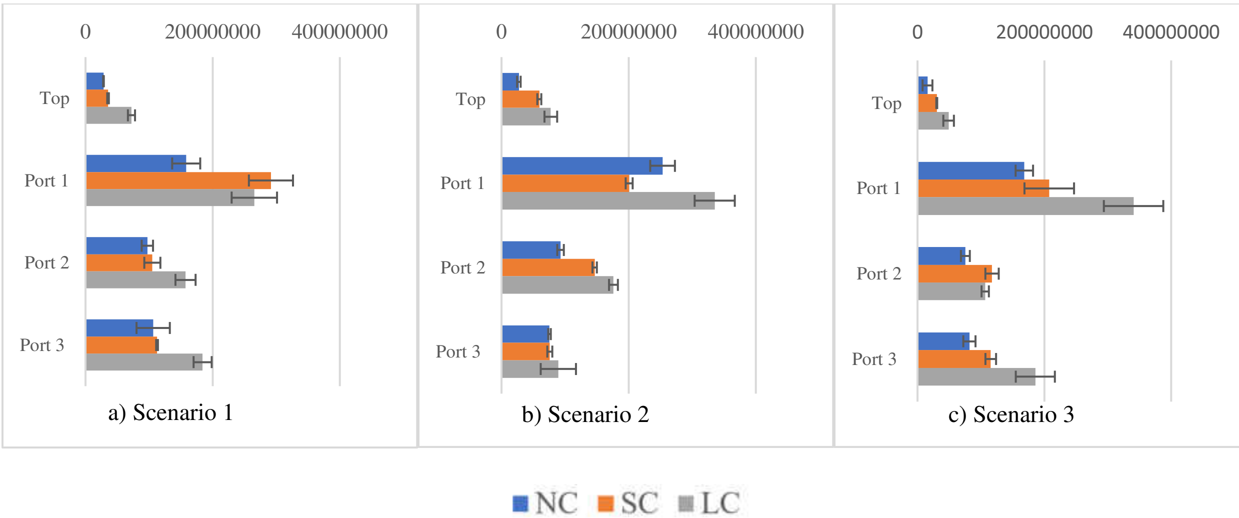

Figure 10. Denitrifiers (gene nirS) absolute quantity in copy/gram for non-copper case (NC), short-term copper impact (SC) case and long-term copper impact case (LC) under scenario 1 (a), scenario 2 (b) and scenario 3 (c)

2.4. Discussion

2.4.1. Short- and Long-Term Copper Impacts on Microbial Community and the DON Concentration and Composition

The population dynamics in microbial communities are directly connected to the DON concentration and composition changes. All microbial species significantly increased their population density in the case of SC when compared to the case of NC, as described in Figure 9. In addition, scenario 2 showed the highest microbial population and level of population increase among the three scenarios, mainly because scenario 2, with the consideration of both traffic compaction and animal disturbance, has the most suitable HRT for delivering enough nutrients, as well as the copper, in an appropriate time frame (Figure 3). For this reason, scenario 2 triggered the most intensive enzymatic cascade effects and resulted in a large growth of microbial population. However, even though copper is an essential metallic element for life, functioning as the cofactor of multiple enzymes due to its positive redox potential (Ladomersky and Petris 2015) such as the N_2O reductase for the last step of denitrification to convert the N_2O into N_2,(Magalhães, Machado et al. 2011, Wen, Chang et al. 2018) excessive exposure to copper may cause serious damage to the metabolic processes in bacteria (Dupont, Grass et al. 2011). This can be realized by looking at the DON concentration and composition changes from Figure 7 to Figure 8. The effluent DON concentration in the case of LC increased approximately 2.6 times when compared to the case of NC in scenarios 1 and 2. Although scenario 3 showed 15% improvement in DON removal, this may have been caused by other factors. In the Van Krevelen diagrams, the effluent DON composition in the case of SC showed significantly less density when compared to the case of NC in scenarios 1 and 2, but scenario 3 had no significant change. The slow filtration rate in scenario 3 postponed the copper toxicity from becoming fully effective in inhibition, while the other two scenarios had faster infiltration rates, allowing more copper to get into the media within a certain period of time. This elapse of copper toxicity in scenario 3 can also be observed in Figure 8 for the similar CHON classes distribution and the peak numbers in the case of SC when compared with the case of NC. This is because the copper impact on scenarios 1 and 2 was much more severe than it would be in scenario 3 due to the HRT differences. The continuous copper dosing in the case of LC pushes all scenarios to the same inhibitory outcome, as the nutrient removal was significantly decreased when compared to the case of SC, which showed nutrient removal nearly equivalent to the case of NC (Figure 5 and Table 5).

Minimal population densities of AOB and AOA were quantified, and the ammonia oxidizer comammox was observed at different ports in each scenario. Higher quantities of comammox were observed in scenario 3, followed by scenario 2, and scenario 1. These results can be associated with the affinity of comammox to environments with low DO concentration[31,71], given that scenario 3, designed to reflect compaction impact, fostered a more appropriate environment for commamox growth, which was phenomenal at port 3 (90 inches from the influent). The contrary was observed in scenario 1, in which the commamox population density turned out to be more evenly distributed over the three sampling ports due to the conduits that provided a more heterogenous flow regime for the delivery of DO throughout the column. Comammox population density decreased in all scenarios in the SC case compared to the NC case. In the LC case, however, the population density of comammox only increased in scenario 2, and this can be related back to it having the most suitable HRT.

NOB population density increased after the short-term copper impact, and population density decreased at most sample ports in the case of LC. The contribution of comammox in the first nitrification step converting ammonia to nitrite could have complemented the requirement of nitrite by NOB, and this clarifies why the low quantity of AOB and AOA was observed by qPCR in the system. However, the denitrifiers (nirS gene that accounts for over 99% in all detected species) continued to increase significantly (Figure 9 and Figure 10). This seems contradictory to the inhibited nutrient removal, but it works perfectly for the microbial community to endure the case of LC. Before the copper addition the bacteria tended to grow larger in size and lower in population density so they could consume, convert, and store more organics in their system without severe competition. With the persistent existence of copper, competition was no longer the first concern, so the bacteria unified their actions against the copper toxicity. They reproduced even more with smaller cell sizes that led to a much larger surface area (SA) to volume (V) ratio (SA/V)

(Figure 11a), through which the bacteria could slow down the copper diffusion from cell to cell. Figure 11a was theoretically determined based on the assumptions that microbial population intends to occupy all the space available, also that each cells area is spherical and a constant living space (or volume). Based on such assumptions it can be inferred that the increase in population size results in reduction of cell size thus increasing the SA/V ratio. Following the reproduction of smaller cell sizes, the microbial community also released more dissolved organic matter (DOM) into the solution (Figure 5). DOM, particularly the nitrogen bearing DON, was evaluated and confirmed to be able to bind with Cu(II), and the binding strength was inversely proportional to Cu:DON (Craven, Aiken et al. 2012). This so-called extracellular sequestration was one of the mechanisms that bacteria used to remediate the copper toxicity (Bondarczuk and Piotrowska-Seget 2013). Scenario 2 (conduits and compaction) showed the lowest Cu:DON ratio over both short- and long-term copper impacts (Figure 11b), because its suitable HRT provided nutrients in time for the growth of a larger and stronger microbial community which was able to react more quickly and thoroughly (lowest Cu:DON, largest increase and decrease in SA/V and cell volume) to the copper addition. The Van Krevelen diagrams (Figure 7 g-i) also confirm that scenario 2 had more potential to release organics as lignins, lipids, proteins, amino sugars, and tannins when compared with the other two scenarios. However, the inhibitory effects still proceeded as the copper removal dropped gradually (Figure 6) and the effluent CHON classes distribution tended to be similar in the case of LC across all scenarios. This implies that the microbial community was enduring under the sustained presence of copper but gradually lost its control and eventually vanished.

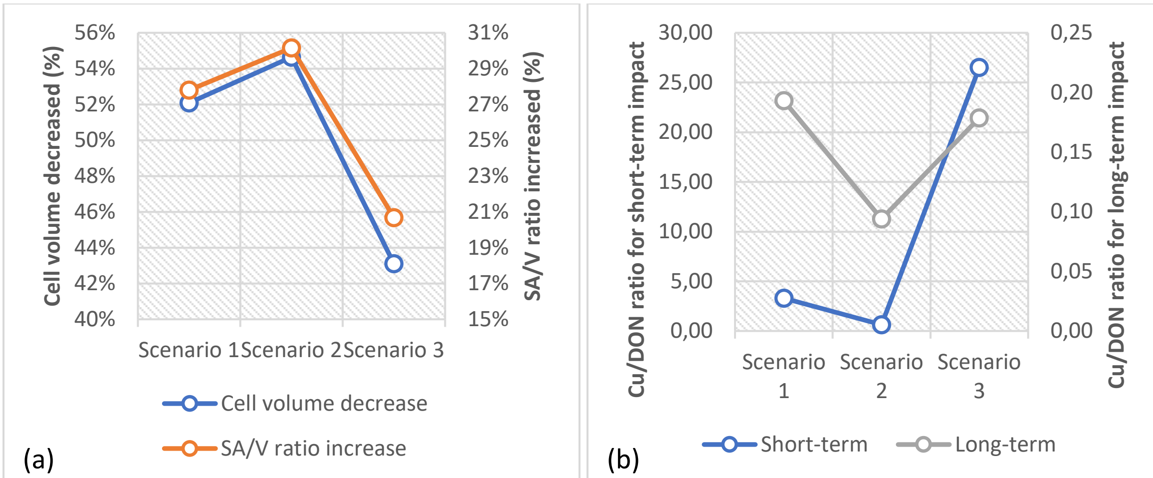

Figure 11. (a) The theoretical change of cell volume and SA/V ratio in percentage between NC and LC cases (assume all bacteria are in sphere) and (b) The inlet copper concentration to the effluent DON concentration ratio for short- and long-term copper impacts

2.4.2. Short- and Long-Term Copper Impacts on Nutrient Removal

As explained in the previous two sections, the changes in the microbial community in our study deeply affected the DON concentration and composition. These changes also influenced the nutrient removal in BAM. Before the copper addition, the nutrient removal performance was mainly affected by the different HRT in each scenario (Figure 5). Higher HRT usually resulted in more efficient nutrient removal since the nutrients could be thoroughly consumed by bacteria due to the longer contact time, which can be supported by the lower ORP value from scenario 3 (Figure 4). Especially for the removal of NO_x via denitrification, the longer HRT is beneficial for maintaining an anaerobic condition; more hydrogen ions can be consumed via denitrification and the alkalinity can increase more, as it did in scenario 3. The alkalinity values are higher in the case of LC than the case of NC (Figure 4), which is also because the increasing denitrifiers were able to consume more hydrogen ions. The copper impacts on nutrient removal were minimal in the case of SC when compared with the case of LC, but more ammonia was produced in the case of SC than the case of LC. Some researchers also observed that nitrification is more sensitive to copper toxicity than ammonification.(Kostov and Van Cleemput 2001) This is because free copper ions produce hydroxyl radicals via the Fenton and Haber-Weiss reaction,(Fridovich 2002) and due to the high standard reduction potential of the hydroxyl radical, it is able to cause oxidative damage to many types of macromolecules.(Yoshida, Furuta et al. 1993, Freinbichler, Colivicchi et al. 2011) Such damage is also diffusion-limited because of the short half-life of hydroxyl radicals (~10^{-9} s); hence the impact is restricted to the macromolecules within the immediate vicinity of copper. This means bacteria located at the surface of the biofilm (more likely nitrifiers: AOB and NOB) would be more vulnerable due to the faster diffusion rate and more available copper when compared to bacteria located at the bottom of the biofilm. In addition, very minimal AOB was found in the media for reducing ammonia in all scenarios, and hence more ammonia accumulated through ammonification in scenarios 1 and 2. The lack of dissolved oxygen due to compaction in scenario 3 is the reason it showed no ammonia increase. However, the presence of AOA in the system can support the minimal quantities of AOB found in the media, and the investigation of comammox can create a more complete study of the microbial population. The analysis of such bacteria was not performed; hence this can be considered as a limitation of this study to having a holistic view on the N-cycle. In the case of LC, even though the denitrifiers continued to grow in population, the denitrification could not follow up the population growth. The reason for this might be that nitrate and nitrite reductase are more sensitive to copper than the N_2O reductase.(Magalhães, Machado et al. 2011) This means the incomplete denitrification might be the major cause of the failure of nutrient removal in the end.

2.5. Conclusion

The short and long-term copper impact on nutrient removal and the changes of DON concentration and composition in BAM were systematically analyzed under different external forces that mimic the effects of conduits (Scenario 1), compaction (scenario 3), and the combination of both (scenario 2) in three different scenarios in linear ditch field conditions. Research findings indicate that longer HRT (compaction) resulted in better nutrient removal and a slower response to copper toxicity under a short-term copper addition, although all scenarios showed minimal fluctuations in nutrient removal and changes of DON concentration and composition due to a shorter contact time that restricted the copper toxicity. With continuous dosing of more copper, the microbial community responded by reproducing more smaller cell-sized bacteria through enzymatic cascade and extracellular sequestration and releasing more DON to minimize the copper toxicity impact via both inter- and extracellular approaches. Denitrifiers are the main contributors for denitrification, as nitrifiers are more sensitive to copper impact and receive more intensive copper diffusion at the top layers of biofilm. The short-term copper impact was minimal for all detectable increase in the population of bacteria species when compared to the long-term copper impact, during which only denitrifiers kept increasing. The BAM performance of nutrient and copper removal was negatively affected by the long-term copper addition. Nevertheless, scenario 2 showed stronger resistance due to its larger and stronger microbial community. Overall, the impact of copper on BAM performance largely varied due to field conditions (conduits, compaction, etc.) on both a short- and long-term basis. This paper clarifies the proper use of BAM to optimize stormwater treatment for nutrient removal under various impacts caused from external factor. Even though, such external factors are hard to control activities like mowing of the area can achieve the slight compaction while, the presence of animal are dependent on the locations. The understanding of the impact of external factors is important for a proper application of BAM in watershed systems to mitigate pollutant from non-point source to reach different water bodies (rivers, lakes, aquifers).

CHAPTER 3: FATE AND TRANSPORT PROCESSES OF NITROGEN IN BIOSORPTION ACTIVATED MEDIA FOR STORMWATER TREATMENT AT VARYING FIELD CONDITIONS OF A ROADSIDE LINEAR DITCH

3.1. Introduction

Rapid urbanization and agricultural production have resulted in environmental and ecological impact on receiving water bodies due to excess fertilization (Ghane, Ranaivoson et al. 2016, Burant, Selbig et al. 2018). Buchanan, Falbo et al. (2013) indicated that roadside ditch networks are ubiquitous in both rural and urban landscapes, contributing not only to hydromodification but also potentially to nonpoint-source pollution. Modeling the hydrologic effects of roadside ditch networks on receiving waters was developed for different watersheds associated with differing landscape conditions (Koivusalo, Ahti et al. 2008, Buchanan, Easton et al. 2013, Gupta, Rudra et al. 2018). Given that roadside linear ditch may intercept and shunt substantial quantities of stormwater runoff, the increased mobility of reactive nitrogen through the roadside ditch networks has substantially altered the nitrogen fate and transport processes among surface water and groundwater (Van Drecht, Bouwman et al. 2003).

The concerns of nitrogen and phosphorus pollution in surface and groundwater systems have triggered a necessity to develop various best management practices (BMPs) in the United States, which treat stormwater runoff at its source (i.e., low impact development) (Shutes, Revitt et al. 1997, Jang, Seo et al. 2005, Salamah 2014). Sorption media are a promising technology for the treatment of nutrients in stormwater runoff and agricultural discharge. Different “green” or “recycled” materials have been included in sorption media, such as iron-fillings, that attained nitrogen and phosphorus removal as high as 92% and 94% in stormwater treatment, respectively

(Valencia, Chang et al. 2019)). Besides, alkaline solid waste media have alike shown nitrogen and phosphorus removals of 58-70% and 82-97% in stormwater treatment, respectively (You, Zhang et al. 2019). Many studies have proven the cost-effectiveness of Biosorption activated media (BAM) in the removal of nitrogen due to the enhancement of the nitrification and denitrification processes in the nitrogen cycle at both the laboratory and field scales (O'Reilly, Wanielista et al. 2012, Xuan, Chang et al. 2013, Wanielista and Chang 2018). Biosorption activated media mix is regarded as one of the cost-effective BMPs at the field scale, which integrates hydrological, chemophysical, and microbial processes (O'Reilly, Wanielista et al. 2012). BAM mix is green sorption media that are composed of sand, tire crumb, and clay, in which tire crumb is a recycled material. The inclusion of tire crumb can help biofilm growth on the surface area and control the hydraulic flow pattern (i.e., hydraulic retention time, HRT) to some extent. The efficacy and efficiency of BAM for stormwater and groundwater co-treatment at a road side linear ditch was further confirmed (Chang, Wen et al. 2018, Wanielista and Chang 2018). However, some common physical, chemical, and biological impacts in the field such as external traffic compaction, long-term carbon availability, and animal disturbances may create various types of HRT as well as carbon and oxygen availability conditions especially at the top BAM layer of the linear ditch environment, which could impose unknown impacts on the nitrogen fate and transport processes in BAM-based in a linear ditch.

In the nitrogen cycle, dissolved organic nitrogen species (DONs) that are difficult to remove in wastewater treatment plants are often found in nature at a higher concentration than dissolved inorganic nitrogen species (DINs) (Berman and Bronk 2003). They can potentially provide carbon and nitrogen sources to microorganisms in the nitrogen cycle (Eppley and Peterson

1979). There are two unique nitrate respiration pathways in the nitrogen cycle which deserve further attention. Denitrification is considered the first denitrification pathway and the dissimilatory nitrate reduction to ammonia (DNRA) the second denitrification pathway (Song, Lisa et al. 2014) When converting NO_3^- to N_2 in the first pathway by using some well-known denitrifiers, NO_3^- can be concurrently converted to NH_4^+ via the second pathway; both pathways are anaerobic respiration processes (Tiedje 1988). The DNRA pathway is significant considering that ammonium is toxic to all vertebrates (Randall and Tsui 2002) and the nitrogen is kept within the system rather than going into the next stage of the nitrogen cycle (Giblin, Tobias et al. 2013). This situation can be compounded by the presence of the anaerobic ammonium oxidation bacteria (anammox or AMX) by which nitrite and ammonium ions are converted directly into diatomic nitrogen and water in an anaerobic environment. This pathway is in concert with the pathways in an aerobic environment in which ammonia oxidation is the first and rate-limiting step of nitrification after ammonification in the nitrogen cycle. Within this context, both ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) formalize the nitrification potential in the aerobic biofilm portion associated with DON variations. All of the interactions among AOA, AOB, anammox, DNRA, and denitrifiers impact the subsequent nitrification and denitrification pathways collectively or separately, resulting in differing composition shifts of DONs and therefore DONs may be selected as a series of representative indicators to observe the interactions among microbial species in BAM in due course.

This study aims to explore the profound impacts of differing linear ditch field conditions driven by traffic compaction, and animal disturbance under long-term carbon availability on the DON removal efficiency and associated microbial ecology in BAM, which affect total nitrogen concentration after treatment. In our experiment, three columns were prepared to reflect three scenarios of linear ditch field conditions, each reflecting varying HRT and oxygen/carbon availability. The fixed-bed column analysis was performed by following standard chemical analyses for understanding nitrogen cycle in BAM in harmony with *quantitative polymerase chain reaction* (*qPCR*) for microbial specie quantification, Fourier-transform ion cyclotron resonance equipment (FT-ICR-MS) for DON analysis, tracer analysis for HRT, and scanning electron microscopy (SEM) for biofilm growth monitoring.

Research questions to be answered include: (1) How do the three linear ditch field conditions with differing predominent microbial communities affect the ultimate total nitrogen removal? (2) How would the addition of carbon affect the structure and function of microbial ecology (competing, inhibitory, and complementary effects) among different species in each scenario? (3) What are the varying roles of DNRA and denitrifiers with or without the carbon addition under varying field conditions? (4) How will DON removal be affected by the microbial ecology in each scenario? (5) How would the composition changes of DON species be influenced by the microbial community in the three linear ditch field conditions?

3.2. Materials and Methods

3.2.1. Field Conditions and Biosorption Activated Media

In this study, a test site was selected for the construction of an innovative linear ditch BMP with variable depths (30 and 60 cm) of BAM layer on the top, for the co-treatment of stormwater and groundwater in Fanning Springs, Florida. The linear ditch located on the side of a state road, which receives runoff from the road as well as the farmland nearby. A solar powered pumping system continuously feeds the BAM layer with groundwater for treatment via infiltration, but it stops during storm events and the linear ditch is then switched for stormwater treatment only. It is noticeable that the linear ditch undergoes physical or mechanical impacts from the traffic compaction, chemical impact of carbon sources from the nearby farmland, and biological impact from potential animal activities (such as gopher tortoises, moles, and ants). BAM can remove nitrogen from stormwater and groundwater through integrated hydrological, chemophysical, and microbial processes. The fate and transport processes of interest are complicated by internal microbial processes including ammonification, nitrification, denitrification, and *DNRA*. The common presence of carbon sources in nature has had a significant impact on the nitrogen cycle, particularly in denitrification (Chang, Wen et al. 2018), and many studies have already confirmed the positive impact of carbon on the denitrification process since carbon can be used as an electron donor in denitrification reactions (Collins, Lawrence et al. 2010, Chang, Wen et al. 2018). However, each of the internal microbial processes is controlled by different microbial species, oxygen and carbon availability, and hydraulic retention and transport process in addition to some varying field conditions physically (e.g., traffic compaction), chemically (e.g., long-term carbon availability), and biologically (e.g., goffer turtle impact). Understanding these types of internal (hydraulic, chemophysical, and microbiological aspects) and external (physical, chemical, and biological impacts on linear ditch design is of critical importance. Such system design criteria are directly related to oxygen availability, carbon availability, and HRT for biofilm growth in BAM (Greig, Sear et al. 2007, He, Malfatti et al. 2015), thereby affecting ammonification, nitrification, denitrification, and *dissimilatory nitrate reduction to ammonium* (*DNRA*) processes separately or collectively at the field scale *per se* (Delwiche 1970, Tamm 2012). A mix of BAM was utilized for the treatment of stormwater runoff in this study, which is composed of 85% sand, 10% tire crumb, and 5% clay by volume. One major advantage of BAM is their ability to hold moisture contents when there is no rainfall, thus keeping aerobic bacteria and archaea active. The other major advantage is their capability to maintain appropriate HRT for nutrient removal when there is rainfall. Due to such advantages, BAM was utilized in the linear ditch field to test the application potential at a road side in Fanning Spring, Florida.

Three columns that individually simulate a different scenario regarding traffic compaction or animal disturbances were set up. The top layer of column 1 (scenario 1) was disturbed to create conduits similar to the ones created by animals in Florida soil such as gopher tortoises, ants and other. In column 2 (scenario 2) the top layer was altered to simulate the presence of Floridian animals in the soil, in addition to slight compaction due to golf carts or lawn mowers. The top layer of column 3 (scenario 3) was compacted to simulate the effect that traffic produces on the hydraulic conductivity of soils (Table 2).

3.2.2 Column Study

The three columns each of which has 1.4 m height with 15 cm-diameter were assembled identically to address the nitrogen fate and transport under those selected internal and external impacts. Three water ports were set up in each column in 30 cm intervals from the base of the column. Additionally, three media sample ports were placed beside each water port, as shown in Figure 2. All columns were filled up to 1.2 m depth with BAM. Furthermore, the top layer of each column was disturbed to characterize the effects of different external factor produced in the BAM, mainly affecting its hydraulic conductivity. For column 1, different conduits were created by poking the top layer with sticks of different sizes to simulate animal disturbances. Conduits on column 2 were created by poking the top layer with a stick of a single size multiple times and slight compaction. Column 3 was designed by compacting the soil a few times.

The column study procedure consisted of two cases: carbon case (CC) and no carbon (NC) case. During the carbon case (CC case) stormwater collected from a wet detention pond located at the main campus of the University of Central Florida was spiked with nitrate standard solution (item# is 1279249 from HACH) and glucose (as carbon source) to obtain a theoretical concentration of 5 mg/L N and 40 mg/L COD. Subsequently it was used to constantly feed each column at a rate of 10 mL/min via a peristaltic pump for 6 weeks in order to foster the biofilm growth. The no carbon case (NC case) lasted for one week, and in this step stormwater with spiked nitrate (5 mg/L N) alone was used to feed the columns at a constant rate of 10 mL/min.

Triplicate water and media samples were collected at the end of each stage. Water samples for nutrient analysis were collected from the following locations: the inlet, port 1, port 2, port 3, and the outlet. Supplementary water samples (500 ml) in triplicates were collected from the inlet and outlet of each column for DONs analysis. BAM media samples were collected from the top of each column, at media port 1, media port 2 and media port 3 (12 cm, 24 cm and 32 cm depth respectively) from each column.

3.2.3. Water parameter analysis

With the aid of Hach kits all water samples were analyzed accordingly for the parameters summarized in Table 3. Each analysis was performed within 24 hours of collection by following the vendor's instructions. Thus, pH, Oxidation-Reduction Potential (ORP) and Dissolved Oxygen (DO) were measured directly after collection.

3.2.4. qPCR Analysis

Real Time-PCR (RT-PCR) or quantitative PCR (qPCR) was used to quantify the microorganism population dynamics in BAM and to understand their holistic behavior under the absence and presence of carbon and oxygen at different field conditions physically, chemically, and biologically. All media samples were stored immediately after collection at -80° C until analysis. DNeasy PowerSoil, acquired from Qiagen, was utilized for the extraction of DNA from the media samples by following the instructions provided by the vendor. The qPCR analysis was performed by the Bioenvironmental Research Laboratory at the University of Central Florida, using PowerUp SYBRGreen Master Mix and computer software StepOne from Applied Biosystems. Primers and standards were acquired from ThermoFisher and GenScript; the primers' names, information, and the running method for all genes targeted are summarized in Table 4. Each well was composed of 10 μL of SybrGreen, 1.6 μL of primer (0.8 μL forward and 0.8 μL reverse), 5 μL of sample, and 3.4 μL of RT-PCR water.

3.2.5. Tracer study

A tracer study with fluorescent dye was applied to each column to retrieve information on its internal hydraulics, including velocity and water movement. Rhodamine dye was utilized in this study due to its inexpensive price to analyze, null toxicity, solubility in water, and its lack of influence on flow patterns (Wanielista and Chang 2018). The procedure consisted of injecting 5 mL of Rhodamine dye at the top of each column. Subsequently, water samples were collected in 15 minutes intervals from the effluent of each column and immediately analyzed by a fluorometer (AquaFlour model: 8000-010) (Chang, Xuan et al. 2012). Before proceeding with the tracer study effluent background concentrations were recorded to assure that there was no variability before dye injection. After injection of the dye, readings were recorded and a time versus dye concentration graph was plotted to retrieve the hydraulic conductivity information of each column. Thus, the HRT was measured in accordance with the time elapsed between the dye injection and the time when an increase in concentration (peak) was observed in the effluent. This increase in concentration signifies that the dye is leaving the column.

3.2.6. Scanning electron microscopy

Hydrated BAM samples were stored at -80° C until analysis. Before the analysis, samples were dehydrated by collocating them in an oven at 108° C for 24 hours. Subsequently, images of BAM samples from the top of each column were captured with a scanning electron microscopy (SEM) to obtain a visual observation of the media's surface characteristics, texture information, and biofilm growth conditions. Samples were analyzed by the Advanced Materials Processing and Analysis Center at the University of Central Florida using a JEOL JSM-6480 SEM.

3.2.7. DONs analysis

The decomposition of DONs is directly linked to ammonification, nitrification, denitrification, and DNRA under three field scenarios driven by either traffic compaction, animal disturbances, or both, affecting oxygen availability, carbon availability, and HRT in BAM. These changing environmental drivers may affect the abundance, composition, and activity of those microbial species through potential competing, inhibitory, and complementary effects in microbial ecology that are not yet well understood. First, AOA and AOB can thrive in an aerobic environment while competing for the same food source – ammonia driven by the ammonification process through the decomposition of DONs. Second, the simultaneous presence of AOB, nitrate oxidizing bacteria (denoted NOB hereafter), denitrifiers, and anammox could have unknown inhibitory effects among the microbial species. Third, while AOA and AOB are competing with each other in the aerobic portion of biofilm, anammox bacteria might smooth out the denitrification pathway by consuming NH_4^+ in the anaerobic portion of the biofilm. In microbial ecology, anammox and denitrifiers can thus work complementarily to complete the denitrification in an anaerobic environment.

Water samples (500 ml) were collected from the inlet and outlet location of each column for DONs analysis. Primarily, a solid-phase extraction (SPE) was applied to all water samples by following the protocol established by Dittmar, Koch et al. (2008). Subsequently, SPE samples were stored at 4° C until analysis. The analysis of DONs was performed by the National High Magnetic Field Laboratory at the Florida State University in Tallahassee, FL. This facility, funded by the National Science Foundation, counts with Fourier-transform ion cyclotron resonance equipment (FT-ICR-MS). FT-ICR-MS has allowed the retrieval of DONs information due to its high mass analysis, resolution, and accuracy, as well as its fast scan times and cation/anion capability.

3.3. Results

3.3.1. Hydraulic patterns

The evidence of how varying different external conditions and influent conditions affect hydraulic patterns is illustrated in Figure 12. Given the different influent conditions, each column has different background concentration. In scenario 1, two main conduits can be distinguished. For the NC case they appeared at minutes 20 and 130 after the dye injection, and for the CC case the peak occurred in minute 105 after the dye injection. In scenario 2, two main peaks can be distinguished at minutes 30 and 150 after the dye injection for the NC case, and at minute 190 for the CC case. In scenario 3, a peak can be observed 240 minutes after the dye injection for the NC case, and the peak appeared in approximately 268 min after the dye injection for the CC case. The results from our tracer study indicated that the CC case produced a slower hydraulic rate in comparison to the NC case. Alike, it can be observed that the HRT is shorter in scenario 1 and the longest in scenario 3.

Figure 12. Hydraulic patterns designed for: a) NC scenario 1, NC scenario 2, and NC scenario 3 (upper axis correspond to scenario 1, and thus bottom axis corresponds to scenario 2 and scenario 3) and b) CC Scenario 1, CC Scenario 2, CC Scenario 3 (bottom axis corresponds to scenario 1, scenario 2 and scenario 3 simultaneously).

3.3.2. Water quality analysis

The pH of the water samples throughout the experiment ranged between 7.3 and 7.9. Furthermore, the ORP values range between 150 to 300 mV. The alkalinity variation can be observed in Figure 13 and it indicates higher alkalinity concentration in the CC case than in the NC case. Total nitrogen removal was higher in the CC case in comparison to the NC case. Thus, the total nitrogen removal efficiencies vary in response to different field conditions, as simulated in this study. In the CC case, the total nitrogen removal of 89.1%, 86.4%, and 77.5% for scenario 1, scenario 2 and scenario 3, respectively, were observed. In the NC case, the total nitrogen removal observed was relatively lower in each scenario, at levels of 13.7%, 41.5% and 52.4% for scenario 1, scenario 2, and scenario 3, respectively. From Table 6 it can be observed that ammonia was generated at port 1 in all scenarios. Furthermore, ammonia with respect to the outlet was generated in all scenarios after carbon addition. However, before carbon addition, generation of ammonia with respect to the outlet was observed only in scenario 2 and scenario 3.

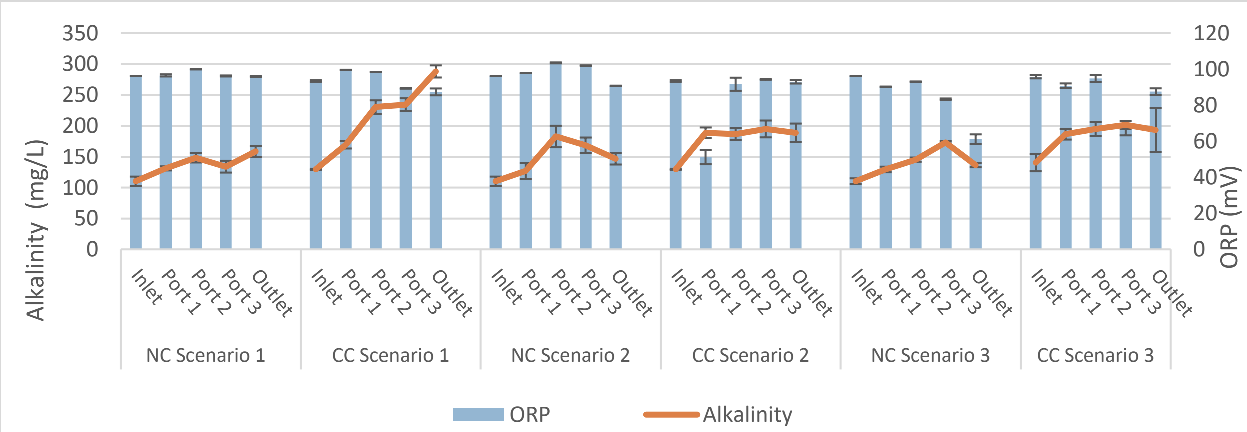

Figure 13. Alkalinity and ORP water parameter values

Table 6. Water parameter results, for each scenario for NC (No Carbon) case and CC (Carbon Addition) case

		No Carbon (NC)					**Carbon Addition (CC)**				
		NOx mg/L	Ammonia mg/L	TN mg/L	DO mg/L	COD mg/L	NOx mg/L	Ammonia mg/L	TN mg/L	DO mg/L	COD mg/L
Scenario 1	Inlet	5.253	0.109	5.610	7.480	17.833	5.302	0.017	5.425	6.137	24.700
	Port 1	5.320	0.321	5.627	7.560		1.096	0.133	1.370	6.680	
	Port 2	6.033	0.093	6.040	7.837		2.398	0.147	2.725	7.410	
	Port 3	4.953	0.082	5.127	8.657		0.702	0.207	1.130	8.963	
	Outlet	4.757	0.088	4.840	9.057	13.633	0.283	0.166	0.589	6.510	21.400
Scenario 2	Inlet	5.253	0.109	5.610	7.480	17.833	5.302	0.017	5.385	6.137	24.700
	Port 1	5.023	0.677	6.957	5.547		0.330	1.877	2.435	5.167	
	Port 2	5.527	0.060	5.607	5.860		0.436	0.114	0.821	6.570	
	Port 3	5.087	0.234	5.513	5.937		0.373	0.174	0.825	8.310	
	Outlet	2.667	0.597	3.280	6.813	11.697	0.237	0.140	0.731	5.727	19.567
Scenario 3	Inlet	5.253	0.109	5.610	7.480	17.833	5.090	0.015	5.910	6.550	24.700
	Port 1	3.353	0.608	3.667	6.540		0.659	0.291	1.230	6.493	
	Port 2	2.707	0.582	3.350	7.750		0.546	0.620	1.260	6.657	
	Port 3	1.620	0.885	3.977	3.790		0.391	2.540	3.380	4.133	
	Outlet	1.863	0.768	2.670	5.070	20.333	0.370	0.508	1.330	6.593	15.133

The composition changes of the influent and effluent conditions associated with the NC and CC cases are displayed in Figure 14 and Figure 15. Two reservoirs were utilized in the CC case, for this reason two graphs are presented for the influent condition. Figure 15-a represents the influent composition for scenario 1 and 2 and thus, Figure 15-b represents the influent composition for scenario 3. These figures indicate the effect that the carbon addition produces on the composition of nitrogen species in the effluent. In Figure 14 (the NC case), NO_x appears to be the main component in the effluent, and ammonia, which is the second most predominant component, appears at quite a different composition percent in each scenario, which could be driven by a more active nitrification process. Ammonia has been more dominant in the effluent of scenarios 2 and 3 (18.2% and 28.8%, respectively) relative to scenario 1, where there is lower nutrient removal (13.7%). In Figure 15 (the CC case), DONs and ammonia appear to be more abundant in the composition of the effluent, resulting in a sharp reduction in the abundance of NOx in the effluent driven by a more active denitrification process.

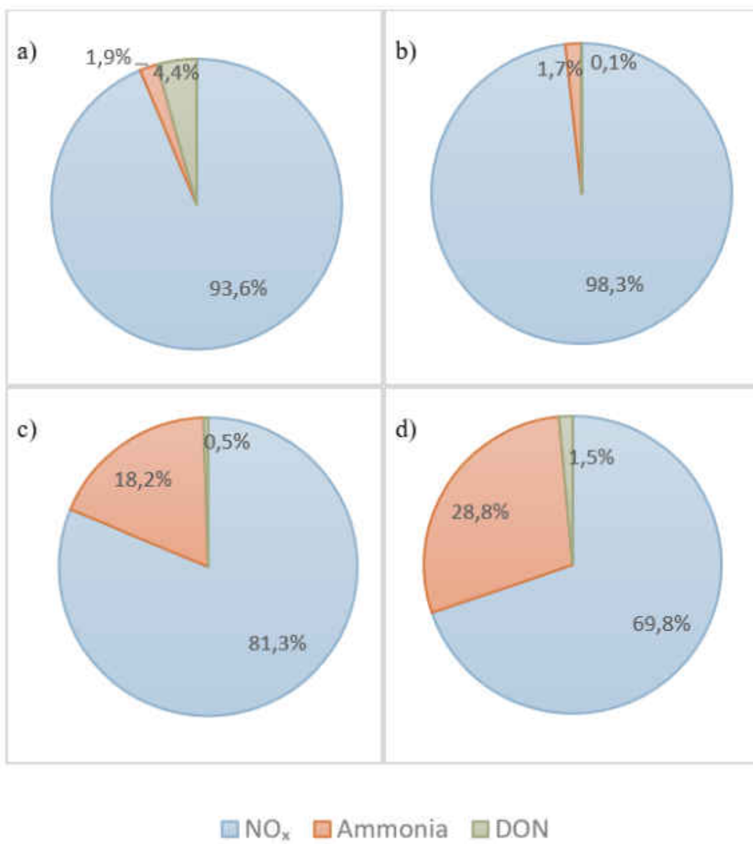

Figure 14. Percent chemical composition analysis of the influents and effluents over different scenarios for comparison: (a) NC case influent composition, (b) NC scenario 1 effluent, (c) NC scenario 2 effluent, (d) NC scenario 3 effluent

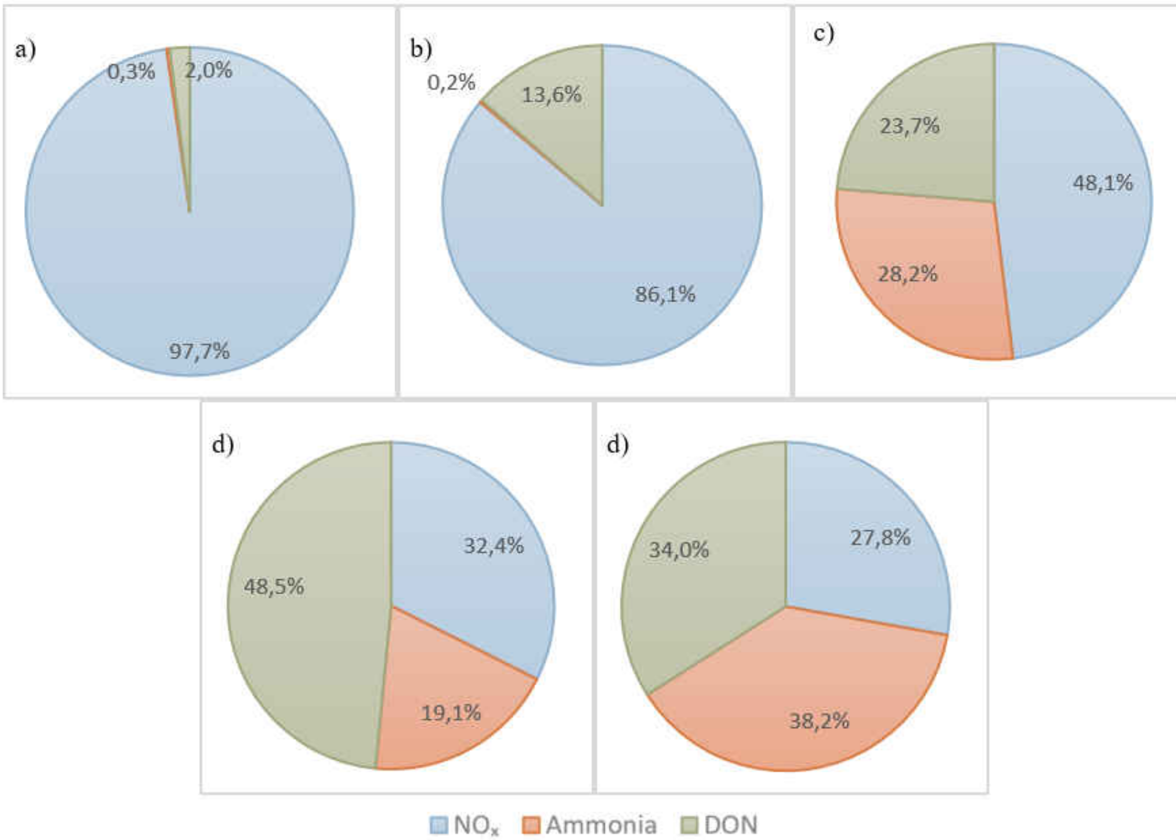

Figure 15. Percent chemical composition analysis of influents and effluents over different scenarios for comparison: (a) CC scenario 1 and scenario 2 influent composition, (b) CC scenario 3 influent composition (* a different reservoir was utilized to feed column 3) (c) CC scenario 1 effluent, (d) CC scenario 2 effluent, (e) CC scenario 3 effluent

3.3.3. DON analysis

Dissolved organic nitrogen (DON) concentrations were calculated by subtracting ammonia and NOx concentration from TN concentration (DON (mg/L) = TN (mg/L) - NH3 (mg/L) – NOx (mg/L)). Further the percent removal was calculated and is presented in Table 7. Higher DON removal was observed in the NC case. However, in the CC case DON removal was only observed in scenario 3, and thus DON recovery in scenario 1 and scenario 2.

Table 7. Dissolved Organic Nitrogen (DON) percent removal

	NC case (%)	CC case (%)
Scenario 1	92.83	-33.02
Scenario 2	88.56	-445.13
Scenario 3	62.88	44.26

The relative abundance of DONs was analyzed by utilizing FT-ICR-MS equipment (Figure 16). From these results, it can be observed that the presence of carbon (CC case) produces a more visible change in DON species composition in comparison to the counterpart. Scenario 1 of the NC case (Figure 16-d) indicated a minor increment on the relative abundance of species. In contrast to the NC case, no visible change in terms of relative abundance was observed in scenarios 2 and 3 (Figure 16-e and Figure 16-f). In the CC case of scenario 1 (Figure 16-a), two more DON species were targeted. In the CC case of scenario 2 (Figure 16-b), the DON species in the effluent were restructured and three new DON species appeared. Thus, the CC case of scenario 3 (Figure 16-c) showed the biggest change in terms of DON composition among all the scenarios, with the emergence of five new DON species in the effluent.

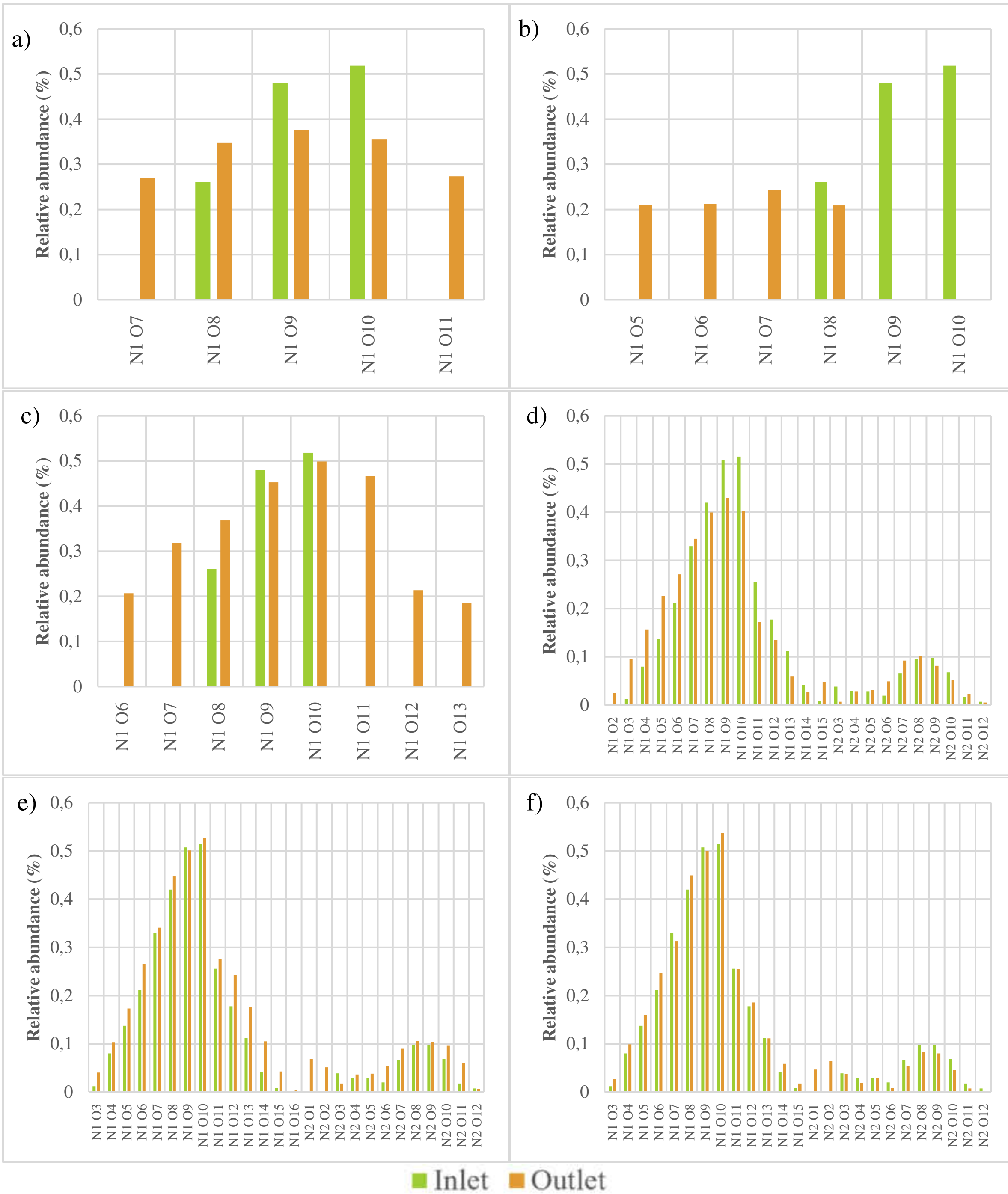

Figure 16. DON composition in the effluent associated with NC and CC cases:
(a) scenario 1 CC case, (b) scenario 2 CC case, (c) scenario 3 CC case, (d) scenario 1 NC case, (e) scenario 2 NC case, and (f) scenario 3 NC case

The composition change of the DON species can also be realized via the use of the van Krevlen diagrams in Figure 17. When compared to the NC case, the composition of outlet DON species with carbon addition is relatively different than that of the inlet DON species. Whereas more lipids and lignins were produced in scenario 2, a DON composition similar to the inlet apart from more tannins was produced in scenario 1. In scenario 3, more tannins, lignins, amino sugars and proteins were produced at the outlet. However, only scenario 3 NC case revealed slightly decreased density in the effluent.

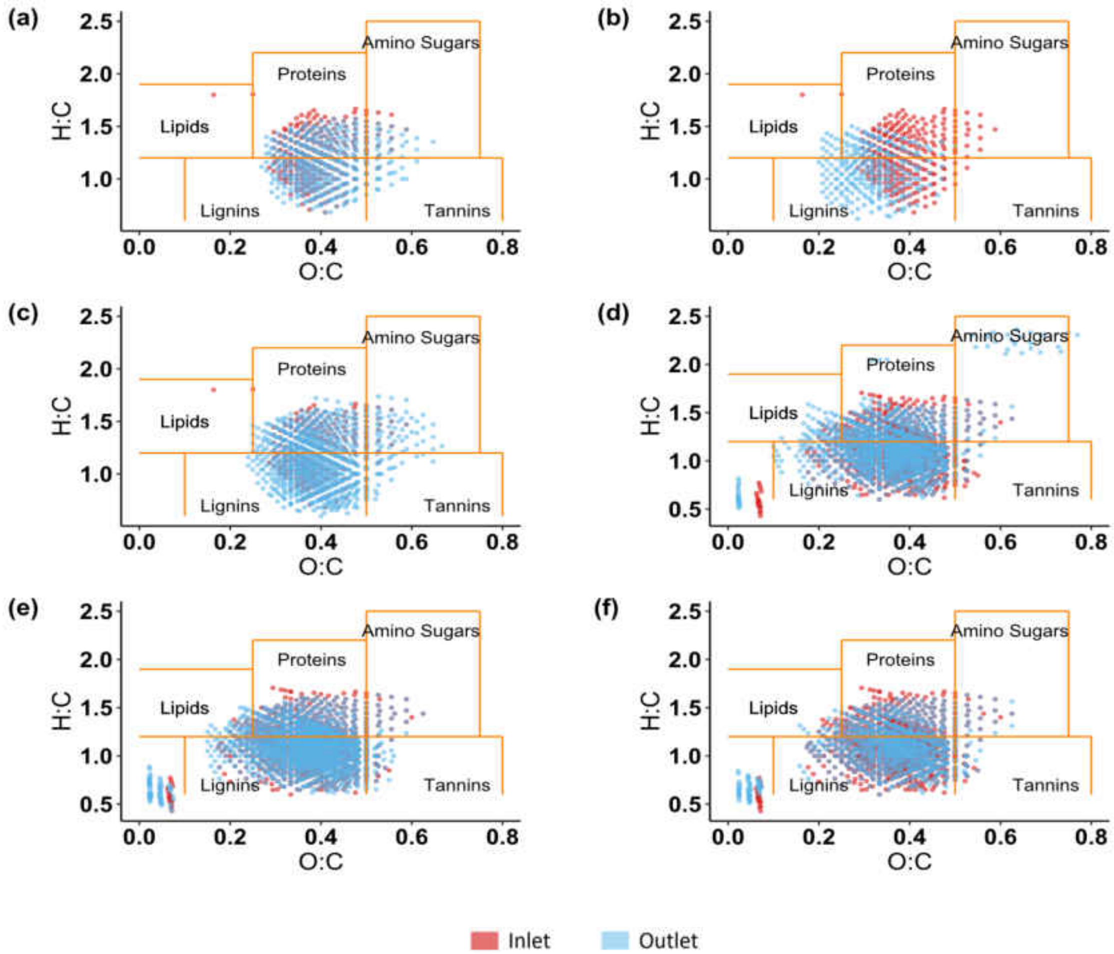

Figure 17. The van Krevlen diagrams derived from negative-ion electrospray ionization FT-ICR mass spectral analysis for all N-bearing formulas in the mass spectra of the inlet and outlet for (a) scenario 1 CC case, (b) scenario 2 CC case, (c) scenario 3 CC case, (d) scenario 1 NC case, (e) scenario 2 NC case, and (f) scenario 3 NC case

Likewise, the no carbon case showed higher overlapped percentage of DON species between the inlet and outlet with percentages of 96%, 88% and 92% for scenario 1, 2 and 3, respectively. However, much lower overlapped percentage were observed in carbon case and the values are highly variable across the three scenarios (60% for scenario 1, 17% for scenario 2 and 38% for scenario 3). The absolute DON concentrations from the effluent of the three scenarios were calculated by subtracting the average values of NOx and ammonia from TN regarding to carbon and non-carbon cases. Scenario 3 tends to show the highest DON concentration (0.04 μg/L before carbon addition and 0.45 μg/L after carbon addition) , followed by scenario 2 (0.0164 μg/L before carbon addition and 0.35 μg/L after carbon addition) and scenario 1 (0.00484 μg/L before carbon addition and 0.35 μg/L after carbon addition). The carbon addition significantly increased the DON concentration in all scenarios, but the trend stays the same.

3.3.4. qPCR analysis of microbial ecology

Nitrification is the process of transformation that oxidizes ammonia to nitrite and lastly nitrate. This process is carried out mainly by an autotrophic organism in an aerobic environment which follows equations 1-2 included in Figure 18. Equation 1 is carried out by ammonia oxidizers (AOA and AOB) with the gene amoA. The abundance of this species is highly dependent on the soil type and field conditions. The second step (equation 2) in nitrification is carried out by the nxrAB gene or nitrite oxidizing bacteria (NOB). Anaerobic ammonium oxidation is the process that transforms ammonium to nitrogen gas, following equation 7, with the aid of anaerobic AMX (Jetten, Wagner et al. 2001).

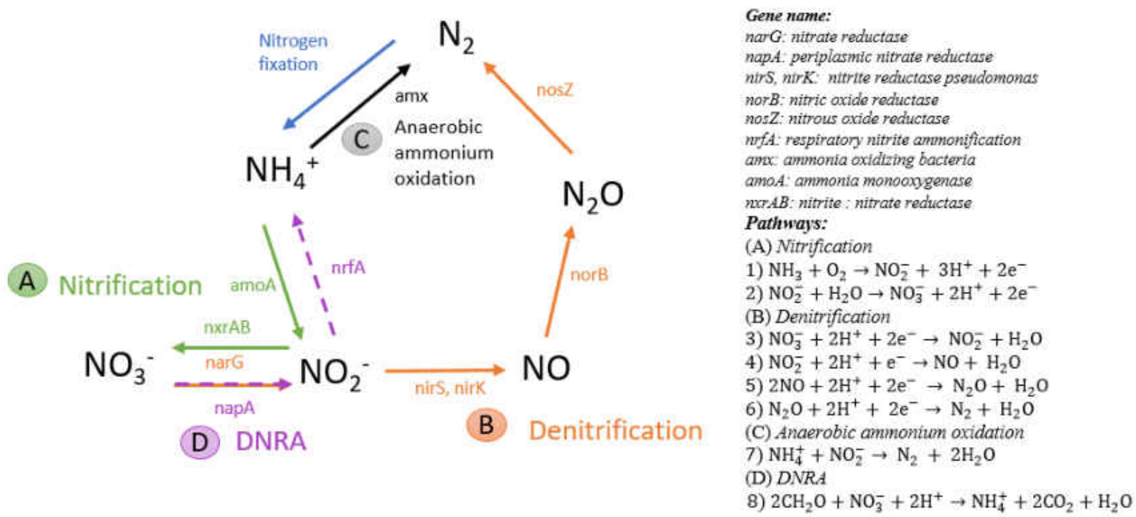

Figure 18. Graphical representation of the nitrogen cycle associated with the gene selection in our qPCR analysis

In denitrification, nitrate is converted into nitrogen gas with the aid of denitrifying bacteria with enzymes narG, nirS, norB and nosZ, following the Equations 3-6 included in Figure 18, respectively. The enzyme nirS in charge of the second step of the first denitrification pathway was quantified to obtain a profound understanding of the population dynamics on different field conditions. The enzyme nrfA was quantified to explain the abundance and presence of the DNRA pathway in each scenario. The DNRA pathway is regulated by oxygen and unaffected by ammonia availability following Eq. 8 in Figure 18. Instead, it produces ammonia that remains in the system (Robertson, Russell et al. 1996). This speculation can be confirmed by the generation of ammonia over different ports of the columns in this study.

The results obtained from the qPCR analysis indicate a negligible population level of AOA and AOB, where in most cases these values laid under detection limits. Thus, they are not included in the results and discussion. Yet the population density of NOB was found in higher quantities on port 1 and port 2; these ports are 30 and 60 cm in depth, respectively. The carbon addition produced an increment in NOB population at the top layer and port 3 with a decrease in population in the middle ports (ports 1 and 2) (Figure 19). The average population of anammox in each scenario was 3000 copies per gram per port in the NC case. The long-term addition of carbon positively impacted anammox population, increasing its population by an average of 76%, 71%, and 59% per port in each scenario, respectively. Denitrifiers were the most abundant bacteria in all scenarios in both CC and NC cases; they constitute above 99% of the total microbial population. Denitrifiers were also benefited by the long-term presence of carbon, since its population increased by an average per port of 27%, 76%, and 57% in each scenario, respectively. The second most predominant bacteria were DNRA (Figure 19). Its presence was more predominant in port 1, and the addition of carbon produced a decreased in population (Figure 20).

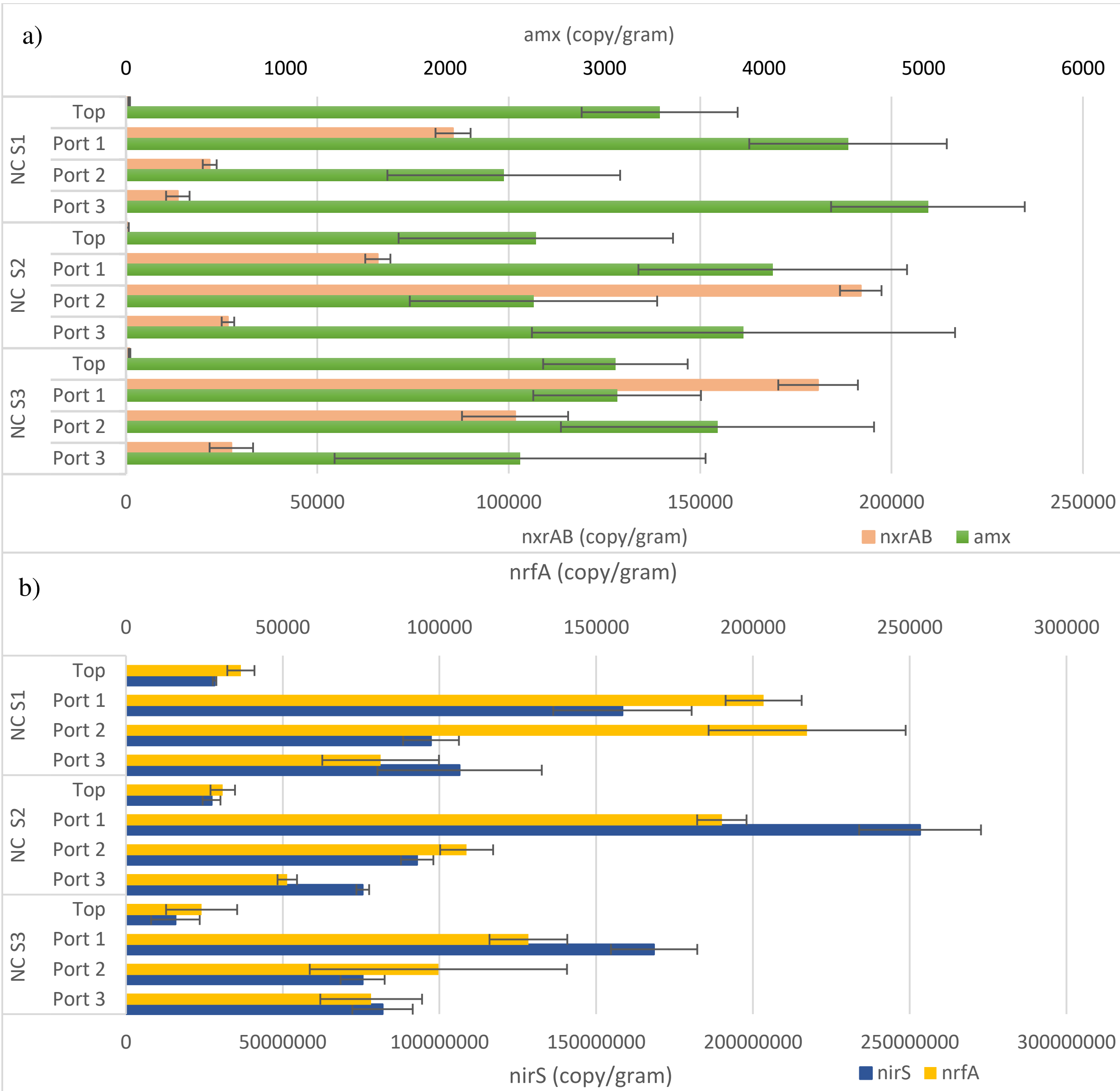

Figure 19. Population quantity for NC case in copy per gram for: a) annamox (AMX enzyme), NOB (nxrAB enzyme) and b) DNRA bacteria (nrfA enzyme), denitrifiers (nirS enzyme)

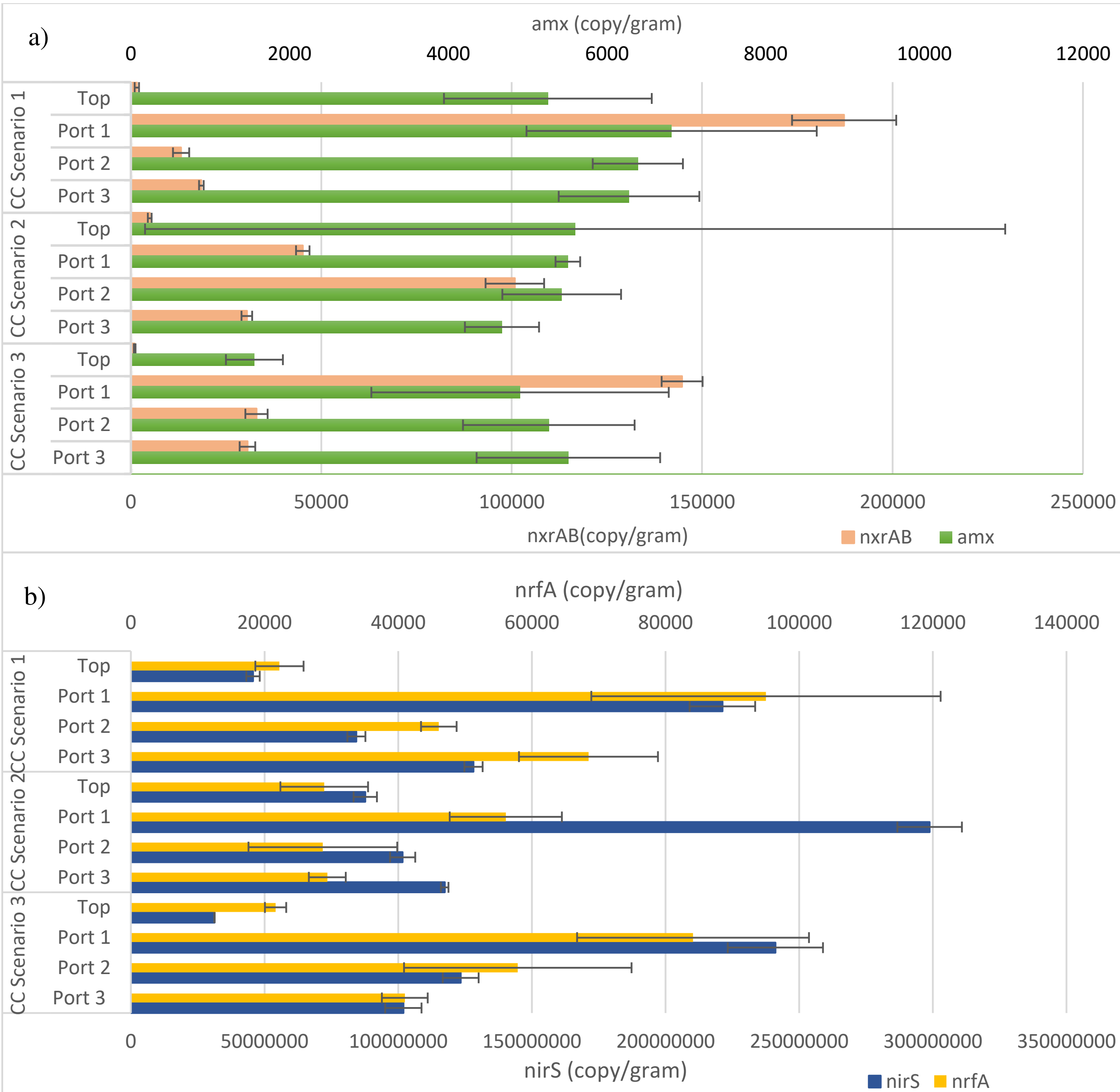

Figure 20. Population quantity for CC case in copy per gram for a) annamox (AMX enzyme), NOB (nxrAB enzyme) and b) DNRA bacteria (nrfA enzyme), denitrifiers (nirS enzyme)

Figure 21 indicates the composition of the microbial community with the exception of denitrifiers for all ports on all scenarios. The addition of carbon provokes an increase in NOB, resulting in a decrease of DNRA population. Furthermore, AMX composition increases after carbon addition, although it remains the second least abundant bacteria.

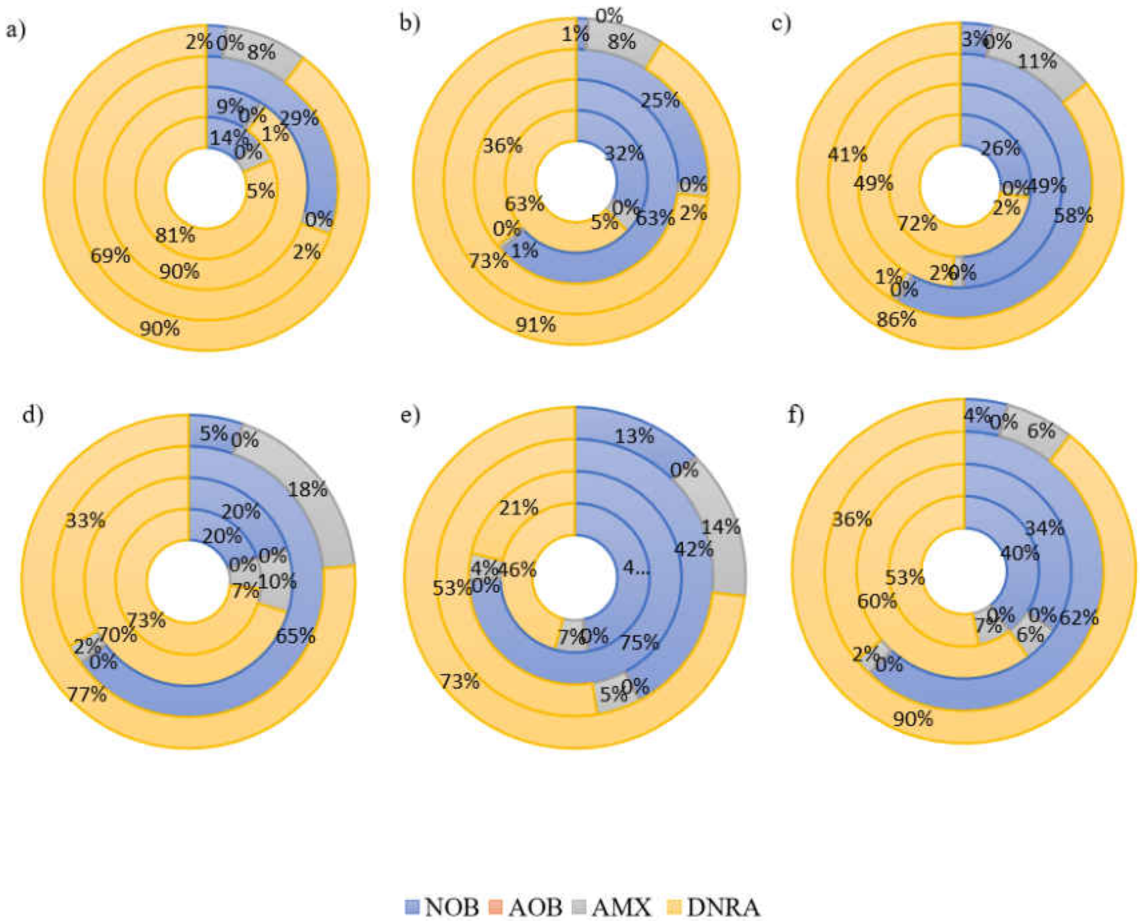

Figure 21. Microbial composition at each sampling port for a) NC scenario 1, b) NC scenario 2, c) NC scenario 3, d) CC scenario 1, e) CC scenario 2, f) CC scenario 3, where the outer circle corresponds to the top and the innermost circle corresponds to port 3; port 1 and port 2 lie in between.

Comparing each bacteria group to itself under carbon and non-carbon cases within three scenarios reveals how different impacting factors influence their living conditions. The population ratios of carbon to non-carbon case are shown in Figure 22, anammox and denitrifiers increased generally at various depths with typically values above 100%, which indicates the carbon addition enhanced their population density. The NOB population mostly increased only at the top layer, there was one enormously increased over 1200% in scenario 2 after carbon addition. Similar trend was observed for DNRA bacteria that its population density decreased with the carbon addition as well as the media depth.

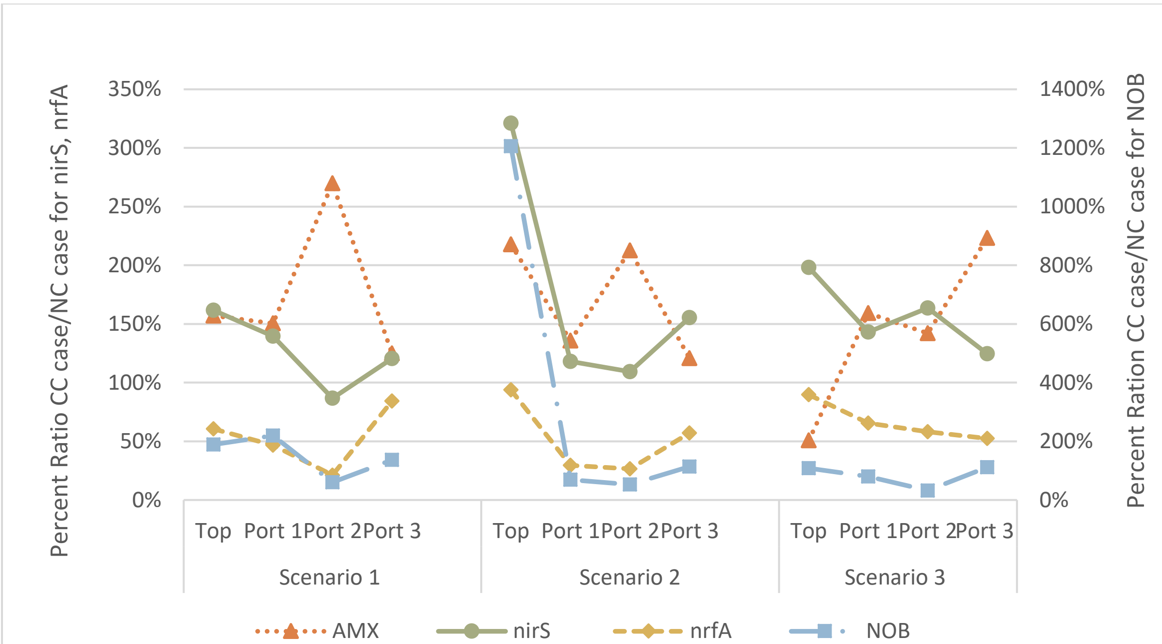

Figure 22. The bacteria population ratio of carbon addition to non-carbon cases in each scenario

3.3.5. SEM analysis

The SEM photos of biofilm before and after carbon addition are shown in Figure 23. The images obtained from the SEM Jeol exhibit the biofilm characteristics, media coverage with biofilm, and biofilm thickness of the media. The biofilms in each image are indicated by orange arrows and can be distinguished by their distinctive texture relative to their surroundings. Increased coverage of biofilm can be found in cases of carbon addition in all scenarios. This is the direct morphological evidence to support the existence of larger microbial communities under carbon influences, which were also confirmed by the qPCR results in the previous section. It is indicative that those enlarged biofilm communities occupy more porous space and slow down the flow speed in order to capture more nutrients as food. Hence, the enhanced microbial communities have more time to breakdown the complex DONs for use as electron donors in the denitrification process and may change the DON composition as well.

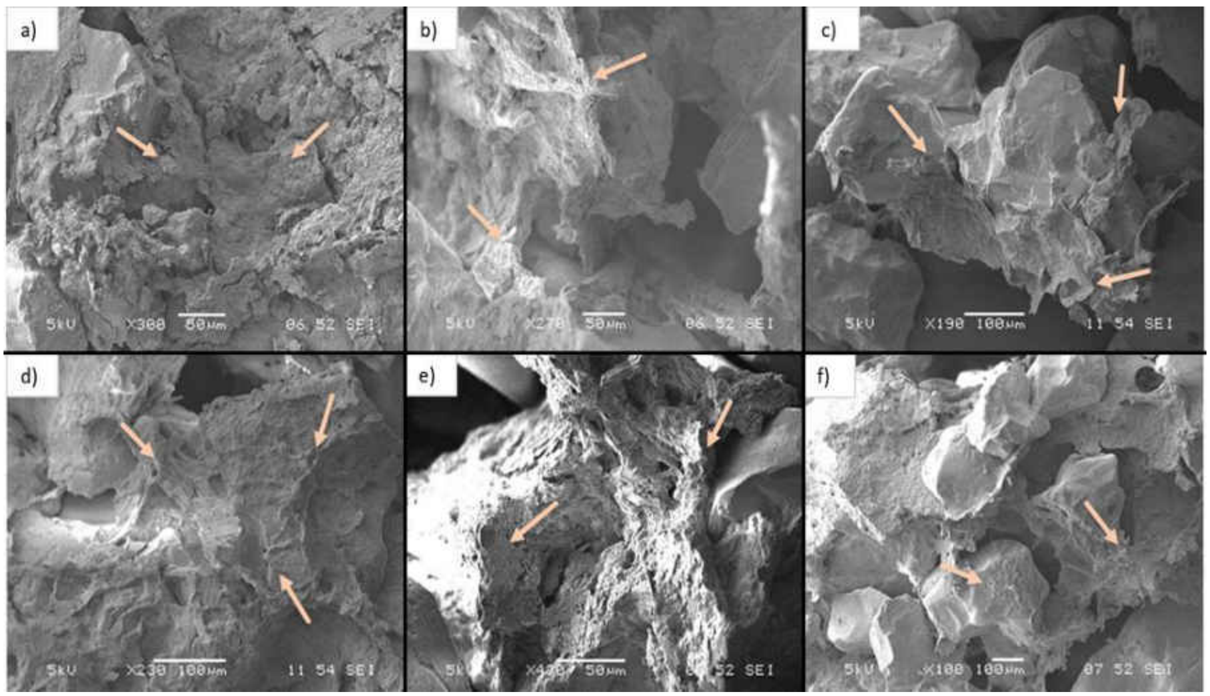

Figure 23. Biofilm images indicated by orange arrows from the top layer of a) scenario 1 NC case, b) scenario 2 NC case, c) scenario 3 NC case, d) scenario 1 CC case, e) scenario 2 CC case and f) scenario 3 CC case

3.4. Discussion

3.4.1 Microbial Ecology in Different Scenarios within a Carbon-Lean Environment

Before the carbon addition, scenario 1 showed the lowest NO_x (9.5 %) and TN (13.7%) removal, while scenario 3 showed the highest NO_x (64.5%) and TN (52.4%) removal (Table 6). The differences of HRT across the three scenarios are the main reason (HRT: scenario 3 > scenario 2 > scenario 1) to account for these differences (Figure 12). This is because the longer contact time allows the bacteria to consume more nitrogen via denitrification, which can partially answer research question 1. Interestingly, the largest bacteria population (mainly denitrifiers, occupying over 99% of the total population) showed in scenario 2 instead of scenario 3, which corresponds to the longest HRT (Figure 19-a and Figure 12-a).

Given the different field conditions, scenario 2 provided the most suitable hydraulic condition for microbial growth, whereas scenario 1 provided insufficient contact time for microorganisms to uptake the nutrient. At this moment, limited nitrogen was available in scenario 3 due to the low infiltration rate, which allowed microorganisms to scavenge nitrogen, leading to the best overall nitrogen removal. The overall second largest bacteria population was the DNRA species, as confirmed by the gene copy density of nrfA (Figure 19 and Figure 21), although at some location this was not the case. DNRA bacteria tended to have a positive relationship with denitrifiers at various depths in all scenarios before carbon addition (Figure 19). This phenomenon indicates a commensalism relationship between DNRA bacteria and denitrifiers, given that they share nitrite as an intermediate product, as it can be observed in Figure 18. Thus, both benefit by uptake nitrite for further reactions, which supports answering research questions 2 and 3. Moreover, the faster infiltration triggers more DNRA bacteria population in scenario 1 than the other two scenarios because DNRA bacteria can survive in an aerobic condition while the denitrifiers cannot (Hardison, Algar et al. 2015). This means DNRA bacteria are also in competition with denitrifiers and may try to avoid this competition by occupying a different ecological niche. Even though more DNRA bacteria existed in scenario 1, less ammonia was observed in effluent as the nitrifiers (especially NOB) benefitted from the presence of oxygen and consumed the ammonia and nitrite generated by DNRA bacteria (Table 6 and Figure 19).

However, less DNRA population existed in scenarios 2 and 3 but higher ammonia concentration was found in their effluents. This is because the nitrifiers gradually lost their capability to up-take the ammonia generated from DNRA when the oxygen availabiltiy was limited by the media compaction. Note that the negligible population of AOA and AOB were caused by the low DON and ammonia concentrations from the inlet water that was contaminated mainly by nitrate. Such a phenomenon is also driven by low DO, which can only be present at the top section outside the biofilm layers. This causes many nitrifiers to be autotrophic with much lower reproduction rates than heterotrophic denitirifiers.

The following discussion completes answering the research questions 1, 2, and 3. After carbon addition, the hydraulic condition changed substantially due to the expansion of biofilms that occupy more void space within the BAM media, diminishing the conduit effects (Figure 23). As a result, all three scenarios showed a significant increase in HRT; the same descending order of HRT remained in a sequence of scenarios 3, 2 and 1 (Figure 12-b). The slower infiltration provided longer contact time (HRT) for the bacteria to boost the nutrient removals (Table 6), especially for nitrate. This is because the denitrification is enhanced with less inhibition from oxygen, as the expanded biofilm further restricts the diffusion process of DO. Moreover, the denitrifiers also received more electrons from additional carbon for denitrification (Figure 20, Figure 23, and Table 6). The ratio of each group of bacteria population from carbon addition to no-carbon cases are shown in Figure 22. It is noticeable that anammox and denitrifiers benefit the most from the carbon addition, as stated before, because carbon addition restricts the DO diffusion and provides electrons that are favorable for denitrification reactions. Therefore, the NOB population only increases in the top section of scenario 2 based on the following two reasons. One is that limited oxygen depresses NOB in deeper depths in all scenarios. The other is that the most abundant denitrifier bacteria population, enhanced in scenario 2, allows NOB to receive more nitrite. However, the commensalism between DNRA and denitrifiers switched more into competition after carbon addition, and DNRA was eventually inhibited by the increased denitrifiers population. Even though some of the middle ports showed increased ammonia concentration due to the conduits that helped deliver enough water and nutrients for DNRA reactions, the overall ammonia generation was depressed in all three scenarios (Table 6). This is because the presence of DO is a key factor of ammonification, which is less available in carbon addition cases (Table 6), and the increased population of anammox also contributes to the decrease of ammonia concentration. Thus, the relationship between species, such as DNRA and denitrifiers, can be shifted from commensalism to competition or even inhibition. Overall, the changing structure and function of microbial ecology is dependent on the carbon and oxygen availability as well as the hydraulic conditions collectively.

3.4.2 Carbon Impact on DON Removal under Different Scenarios

The shift of the composition of DON species is mainly driven by some microbiological reactions. DON species can be utilized as electron donors in denitrification and DNRA processes, which resulted in increased critical biomass from the microbial reproduction activities. Furthermore, once the larger DON molecules could be broken down into smaller ones, the ammonification process supported converting them into ammonia as an important component in the nitrogen cycle. Before the carbon addition, it was indicative that all scenarios showed promising DON removal (Figure 14 and

Table 7). There is 4.4% of DON in the influent TN, while the DON percentages in effluent were 0.1%, 0.5%, and 1.5% in scenarios from 1 to 3. It shows efficient DON removal (92.83%, 88.56% and 62.88% in scenarios 1 - 3) because bacteria were eager to utilize DON as their carbon source in multiple biological reactions when there was no additional carbon. However, the carbon addition altered their behavior and survival strategy. They grew stronger and larger in population, with enhanced DON digesting capability that effectively converted the influent DON into metabolic waste and stopped later as DON was regarded as the precious electron source. As the population increased, the DON composition also increased to 23.7%, 48.5%, and 34.0% in scenarios from 1 to 3. Moreover, the DON removal decreased for all scenarios, particularly in scenario 1 and 2 (-33.02% and -445.13%), where the DON removal became negative (Figure 15 and

Table 7). However, this phenomenon is consistent with the microbial ecological changes as described in previous section, the scenario 2 shows the highest DON release potential as it has the strongest community to digest the DON and release more waste. The positive DON removal in scenario 3 after carbon addition, can be more associated with the overall TN removal thanks to the long HRT. Another reason is the influent DON composition is high as well (13.6%), which means the influent water quality can be another critical factor for DON removal. Overall, The reshape of DON species and result in more metabolic waste in the DON forming within the effluents, can be supported by having less CHON class due to the carbon addition impacts (Figure 16) and can lead to answering research question 5. Before carbon was added, tests showed 88-96% CHON classes in common between the influent and effluent DON composition. This means that the microbial community is not able to fully digest and restructure them. However, after carbon addition, the percentage of common CHON classes decreased to 60%, 17%, and 38% for scenarios from 1 to 3 (Figure 6). This implies that high-molecular-weight DON can finally be utilized effectively with the aid of carbon source and generate more similar and low-molecular-weight DON as the products from their boosted metabolism (McCallister, Bauer et al. 2005). Moreover, the van Krevlen diagram in Figure 17 shows the evolving directions of DON holistically. Before carbon was added, the effluent DON composition remained the same distribution as the influent DON composition with negligible density changes, which indicates limited biological consumption occurs in all scenarios. However, with the carbon addition, each scenario behaved differently. The DON composition was reshaped a little in scenario 1, causing it to be less obvious than the other two. However, scenario 1 at least showed that more tannins were produced, which could be from products of the plant's organics degradation (Kraus, Zasoski et al. 2004). In scenario 3, more amino sugars, lignins, and proteins were found from the effluent other than the tannins, which means the degraded organics have been converted into more bacteria cell structures (Van Veen and Kuikman 1990). This is probably due to scenario 3 having the longest HRT, which gives the microbial community enough contact time to do their job. This supports the positive DON removal in scenario 3 after carbon addition (Table 7). When it comes to the scenario 2 with the most abundant denitrifier bacteria population, the DON composition was changed more significantly than the other two scenarios. All tannins and amino sugars were consumed by the microbial community because of the efficient carbon utilization driven by the enhanced population of denitrifiers. The components left over are thus proteins, lipids, and lignins, which are the metabolic waste of the microbial community and are all easily biodegradable (Higuchi 1982, Liang and Jiang 2013). Again, scenario 2 showed the lowest DON CHON class in common between the influent and the effluent with the best DON conversion performance, which indicates that the most suitable environment for microbial growth is the condition with carbon addition under the combination of conduits and compaction effect, in which the active microbial community is the key to DON digestion. This is good evidence to showcase that carbon addition is beneficial for BAM to increase the biodegradability of the stormwater (Wanielista and Chang 2018). However, scenario 3 showed the highest effluent DON concentration in both cases with and without carbon addition. The first reason is that the slow infiltration limited the nitrogen feeding speed even with the help from additional carbon to fully unleash the digestion capability of the bacteria. This is especially true for the lower section of the column, which has lower DON digestion capability. The lower section of the column would potentially have more bacteria washed out as the slow infiltration causes insufficient nitrogen supply.

3.5. Conclusion

This study explores the possible impacts of carbon addition on the nitrogen removal efficiency of BAM with respect to different linear ditch field conditions, including conduits, compaction, and the combination of both. The impacts of carbon source on the structure and function of microbial ecology and the varying DON composition in these scenarios were also evaluated with respect to changing hydraulic characteristics and biofilm growth. Before carbon addition, the HRT was the key factor that determined the nitrogen removal, as more contact time resulted in higher nitrogen consumption by the microbial community in the case of scenario 3. Additional carbon stimulated the biofilm expansion and increased the HRT for all scenarios, as well as improving nitrogen removals. The carbon addition thereby resulted in the most abundant denitrifier bacteria in the case of scenario 2. Even though DNRA bacteria are the secondary most abundant bacteria, its population decreased after carbon addition, since more denitrifiers were competing with them. Finally, carbon addition shifted the relationship between denitrifiers and DNRA bacteria from complementary to competition, or even inhibition. The condition of microbial community is critical for DON decomposition and removal, too. The addition of carbon largely increased the bacteria population as well as their strength for digesting DON species with high-molecular-weight, but likewise boost them to release more metabolic waste DON and DIN in sequence. With the highest population density, scenario 2 showed the most promising DON digestion potential for converting more DON into lipids, lignins, and proteins when compared to the other two scenarios. Based on the results, we suggest creating artificial conduits after the compaction from construction and with reapplication periodically to ensure the microbial community stays active for more efficient nutrient removal. A carbon source can also be added depending on the quality of stormwater runoff and the desired level of nitrogen removal. Furthermore, given that external factors are hard to control, these results indicate promising outcomes for implementation at locations where compaction and disturbance by animals occurs naturally and carbon can be resulted as produced of waste produced by these animals.

CHAPTER 4: CONCLUSION

The short-term copper impact and the long-term copper and carbon impact on nutrient removal, microbial ecology and DON concentration and composition was analyzed under three different scenarios that mimic the external forces impact in BAM on a linear ditch field condition. The three scenarios mimiced in this study were animal conduit (scenario 1), traffic compaction (scenario 3) and animal conduits and traffic compaction together (scenario 2). This study concluded that the performance of BAM on nutrient removal was negatively impacted by copper addition, contrarily, carbon addition inhibits the removal of TN. This result was associated with the effect that copper and carbon have on the HRT of each scenario. In the normal case (no-carbon or copper assessment), scenario 3 exhibited higher nutrient removal given its longer HRT, the same trend was observed in the long-term copper addition. Although, after carbon addition scenario 1 displayed the longer HRT and thus, the higher nutrient removal. The HRT impact and the results of the TN removal can be associated with the microbial ecology behavior. Denitrifiers were the most abundant species in the system, and thus after copper addition, it was the only specie that increased in population. In the long-term copper addition, the microbial community responded by increasing its population size and decreasing in cell size (increase in SA/V, Figure 11(a)) to minimize copper toxicity via inter and extracellular approaches. Hence, in the short-term copper impact minimal fluctuation was observed in nutrient removal and DON concentration and composition. Moreover, carbon addition stimulated biofilm expansion and microbial population growth for most of the species. DNRA population in the carbon case decreased as denitrifiers increased showing a competitive behavior as the result of the growth of denitrifiers. Finally, better resistance to copper toxicity was observed in scenario 2 due to a more abundanct microbial community. While, more denitrifiers and a better potential for DON removal was observed in scenario 2 after long term carbon addition.

4.1. Future Work

The application of BAM in treating stormwater and wastewater has been explored and thus, its high efficiency supports the application in the field. Moreover, in previous studies presented on Table 1, BAM exhibit 60 to 62% TP removal mainly by adsorption. The in-situ regeneration of BAM and the nutrient (TP) recovery potential or soil amendment potential could be further explored. Given that the current phosphate resources are scarce, and mining of this nutrient causes negative effects to the ecosystem. Also, since one of the higher costs related to the application of sorption media is associated with the replacement of the media after exhaustion. The elimination of this step can significantly reduce the cost of this technology and reduce ecosystem impact produced by phosphate miming.

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