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Copper impact on enzymatic cascade and extracellular sequestration via distinctive pathways of nitrogen removal in green sorption media at varying stormwater field conditions

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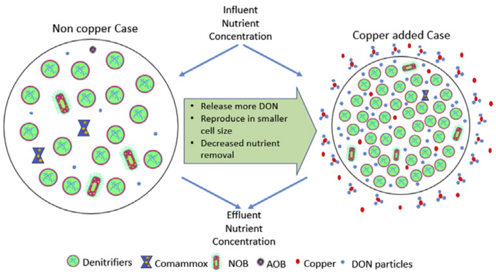
HIGHLIGHTS

- Dynamic field conditions in a linear ditch for stormwater collection and treatment need more investigation.
- Long-term presence of copper in stormwater reduces nutrient removal efficiency in biosorption activated media.
- Short-term presence of copper in stromwater runoff causes enzymatic cascade effect on microbial species.
- Long-term presence of copper in stromwater runoff results in extracellular sequestration when competing for nutrients.

GRAPHICAL ABSTRACT



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ABSTRACT

Nutrient removal efficiency in green sorption media such as biosorption activated media (BAM) for treating stormwater runoff can be heavily influenced either on a short- or long-term basis by varying field conditions of linear ditches due to the presence of copper in stormwater runoff. It is also 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). In the nitrogen cycle, two denitrification pathways, the dissimilatory nitrate reduction to ammonia and common denitrification, are deemed critical for such assessment. A fixed-bed column study was set up to mimic different linear ditch field conditions for BAM applications and measure the effect of short-and long-term copper addition on microbial dynamics given the varying decomposition of dissolved organic nitrogen (DON). The findings confirm that, as the denitrifiers (in the second pathway) were the dominant species, their population continued to grow and maintain small-sized cells for extracellular sequestration under long-term copper impact. Furthermore, the study indicated that the ammonia oxidizer comammox was found in higher quantities than ammonia oxidizing bacteria or archaea. An enormous amount of DON was released during this process to bind the copper ion and reduce its toxicity as the enzymatic cascade effect appeared. In addition, the long-term copper exposure posed salient inhibitory effects on the microbial community regardless of varying field

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conditions in BAM. Short-term copper toxicity exerted an important but varying role in the enzymatic cascade effect over different linear ditch field conditions in BAM.

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 the urbanization process (Buhaug and Urdal, 2013; Seto et al., 2013). This has resulted in more nitrogen consumption and random distribution (Luo et al., 2018; Jordan and Weller, 1996; Smil, 2002) in the last decades via various non-point sources such as stormwater runoff and agricultural discharge (Yang et al., 2004; Carle et al., 2005; Grimm et al., 2008; Rawlins et al., 1998; Smith and Harlow, 2011; Fixen et al., 2015). To reduce the nitrogen concentration in the stormwater runoff and control flood impact, best management practices (BMPs) have been widely adopted. Many of these practices were later refined to meet nutrient removal requirements while maintaining flood mitigation and control (Ermilio, 2005; Wanielista et al., 2011; Park et al., 2015). Sorption media are one of the most promising technologies given their ability to remove nutrients mainly via physicochemical and microbiological processes. The inclusion of metals like iron-filing particles as a component of sorption media can interact to reduce nitrate via an ion exchange mechanism that transforms nitrate to ammonia. The adsorption of ammonia through clay was studied in Iron-Filing green environmental media (IFGEM) (Chang et al., 2019a; Valencia et al., 2019).

This study focuses on 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. BAM is considered a part of new BMP for enhancing the effectiveness of nutrient removal in Florida and elsewhere in the United States. The implementation of such green sorption media (BAM) in watershed systems can intercept pollutants at their source, the effectiveness of which has been proven under varying field conditions to deal with nonpoint sources via the emerging BMP named linear ditch (Buchanan et al., 2013; Kröger et al., 2008). It is also 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). 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 et al., 2010), nitrification (Malhi and McGill, 1982; Ruiz et al., 2003; Di et al., 2009), denitrification (Her and Huang, 1995; Sun et al., 2017), and dissimilatory nitrate reduction to ammonia (DNRA) (Giblin et al., 2013), are positively impacted, as BAM provides appropriate hydraulic control, moisture content, 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, ammonia is converted to nitrate through two consecutive steps (NH_3 to NO_2^- and NO_2^- to NO_3^-). The reduced nitrogen compounds are first transformed into nitrite by ammonia-oxidizing bacteria (AOB). Subsequently, nitrite is transformed into nitrate by nitrite oxidizing bacteria (NOB). However, the discovery of ammonia-oxidizing archaea (AOA) (Könneke et al., 2005) and complete ammonia oxidizers (comammox) (Daims et al., 2015) suggests a more complex nitrification system. AOA contributes in the first step of nitrification by converting ammonia into nitrite, and it has been found able to survive in acidic soils and environments with limited nitrogen (He et al., 2012; Zhang et al., 2012). The higher affinity of comammox to low DO environments is associated with its cytochrome bd, which similarly to terminal oxidases, is theorized to have high oxygen affinity (Lawson and Lücker, 2018). Hence, the question of whether AOA or AOB is the higher contributor to the global nitrogen cycle remains debatable (Zhang et al., 2012). Since the discovery of comammox, much scientific research has focused on this bacteria, as it has the availability to complete both steps in nitrification. Theoretical and experimental observations have evidenced high growth yields and a low specific growth rate for comammox (Hu and He, 2017; Xia et al., 2018; Kits et al., 2017). The presence of comammox has been found in drinking water (Wang et al., 2017), waste water (Annavajhala et al., 2018) systems, and soils (Hu and He, 2017; Xia et al., 2018; Camejo et al., 2017). 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 DNRA is significant since DNRA retains ammonia, which can hurt other components of the ecosystem (Hall et al., 2002). 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, which are oftentimes present in stormwater runoff.

The refined copper used for electrical wiring, roofing, plumbing materials, cookware, stormwater pond disinfection, automobile brake pads, and agricultural products in the U.S results in the deposition of copper ions 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 (Wang et al., 2007; Ochoa-Herrera et al., 2011; Paus et al., 2014; Holtan-Hartwig et al., 2002). Copper is also involved in electron transfer and oxygen transport, as well as in redox reactions of multiple substrates (Dupont 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 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 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 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 stormwater wet detention ponds across the world may have to face this condition. A linear ditch study is one of the closest simulations of actual field conditions. However, a study on nutrient removal via Biosorption-Activated Media at laboratory and field-scale indicated that unknowns regarding external impacts from traffic compaction and animal activities need to be considered to better resemble field conditions (Chang et al., 2019b). 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 such factors may potentially change the hydraulic conditions for stormwater treatment processes, and may compound certain effects, 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 (Glibert et al., 2004; Berg et al., 1997; Berman and Bronk, 2003). 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-ICR-MS) has been widely applied to address complex DOM in marine organics (Koch et al., 2008), surface water (Minor et al., 2012; Stenson et al., 2003), stormwater (Zhang et al., 2016; Chang et al., 2018), biochar (Avneri-Katz et al., 2017; Hagemann et al., 2018), and wetlands (O'Donnell et al., 2016), in addition to numerous aqueous water and petroleum systems (Rodgers and Marshall, 2008; Smith et al., 2009; Headley 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 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 proportional to the number of amplified deoxyribonucleic acid (DNA) molecules (Hall et al., 2002; Harms et al., 2003). Thus, RT-PCR would provide quantitative information about the microbial species as another critical factor in 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 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 influences? (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. Materials and methods

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: (1) Column 1 has external force driven by animal conduits, resulting in a faster infiltration rate to mimic the field condition that has a significant number of conduits of different sizes. (2) Column 2 has external forces driven by both routine traffic compaction and animal conduits with a moderate infiltration rate to mimic the field condition that has a few small size conduits, and (3) Column 3 has external forces driven by traffic compaction only, with a low infiltration rate to mimic the field condition that has few to no conduits.

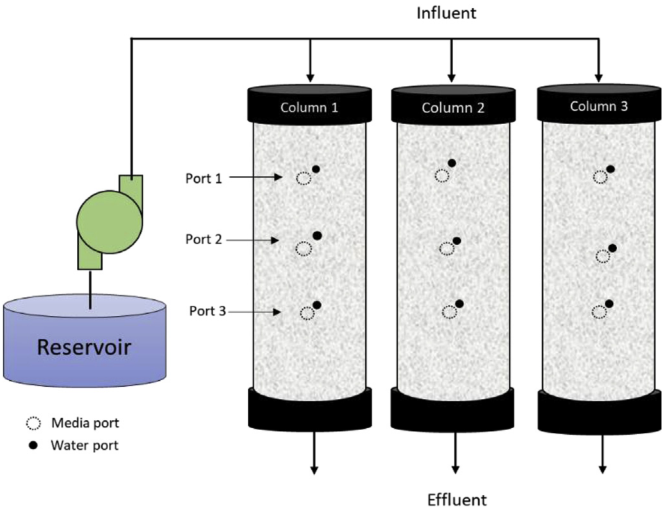


Fig. 1. Column experimental setup.

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 Fig. 1. To conclude the setup, all three columns were filled up to 1.2 m depth with BAM. All columns were 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. 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 with slight compaction. Column 3 was designed by compacting the soil a few times.

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. Water and media samples were collected after one day and after seven days of copper addition. Where the short-term copper assessment (denoted SC, hereafter) corresponded to one day of copper addition, the long-term copper assessment (denoted LC, hereafter) corresponded to seven days of copper addition. 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 preserved for further analysis, as explained in the following sections.

2.3. Water parameters analysis

Water samples (75 ml) were collected in triplicate 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 h of collection at the University of Central Florida (UCF) laboratories for total nitrogen (TN), nitrate, nitrite, ammonia, and alkalinity with the methods specified in Tables S–1. 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 Sec3111 B[66].

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 of 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 mL of diluted Rhodamine dye was injected in the center of the top section of each column. In stage two, water samples from the effluent were collected in 10–15 min intervals and immediately analyzed for dye concentration with the aid of a fluorometer (AquaFlour model: 80000-010).

2.5. Microbial population quantification

Triplicate media samples (10 g) 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, quantitative polymerase chain reaction (qPCR) was applied to 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 μl of qPCR water. The primers and running method are summarized in Tables S–2.

Furthermore, the change in cell volume was calculated based on equation (2), in which n_1 and r_1 correspond to the number of microbial gene copies quantified by qPCR and the radius of the cell before copper addition, respectively. In addition, n_2 and r_2 correspond 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), according to three assumptions. In the first assumption implies that the bacteria cell shape is spherical ($V_{cell} = 4/3 ■ \pi ■ r$ (Luo et al., 2018)). The second assumption suggests that bacteria aim to occupy all the available living volume ($V_1 = n_{1*}\ V_{cell}$). The third assumption expected a constant living volume for microbial growth before and after copper addition ($V_1{=}V_2$).

$$n_1\left(1.33\pi r_1^3\right) = n_2\left(1.33\pi r_2^3\right) \tag{1}$$

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

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

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 $\left(\frac{r_2}{r_1}\right)^3$

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

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 et al., 2008) After SPE, all final samples were kept under −20 °C until analysis. Sample analysis for DON was performed at the National High Magnetic Field Laboratory at the Florida State University (FSU) in Tallahassee, FL. DOM extracts were analyzed at FSU with a custom-built FT-ICR-MS (Kaiser 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 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 (Smith et al., 2009) 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© (Smith et al., 2018). The full operation details of FT-ICR-MS can be viewed in an external link (Smith et al., 2018).

3. Results

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 S-1. 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 min, respectively. On the other hand, the largest HRT value was retrieved from scenario 3 with 240 and 300 min in NC and LC cases, respectively. In scenario 2, the HRT in the case of NC was 140 min, and the value of HRT after copper addition (LC) exhibited an average value of 145 min.

3.2. Water parameters

The pH values fluctuated within the range from 7.4 to 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 S-2. Tables S–3 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—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. Fig. 2 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 Fig. 3. The removals within influent and port 1 ranged from 30% to 82% in scenario 1, 65%—83% in scenario 2, and 73%—88% in scenario 3. Such results indicate that the highest copper removal occurred within the first 30 cm of the column (influent to port 1). A progressive decrease in total copper removal efficiency was observed. After day 7, the removal efficiencies in scenario 1, scenario 2, and scenario 3 decreased from 84% to 70%, 83%—80%, and 95%—82%, respectively. Thereby, we can conclude that copper removal efficiency is negatively impacted by the long-term addition of copper.

3.3. DON results

The variations of inlet and outlet DON composition in each scenario are presented via Van Krevelen diagrams in Fig. 4. In the case of NC (Fig. 4 a-c), the inlet and outlet DON compositions are essentially the same, and the outlet showed dense dots, which indicate 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 (Fig. 4 d-f), there was no additional production of proteins or 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 (Fig. 4 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 partially confirmed in Fig. 2 due to the increased DON concentration.

The peak number assigned for CHON classes from the inlet and outlet of each scenario over different cases are plotted in Fig. 5. 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 (Fig. 5 a), similar CHON species distribution and peak numbers were found between the inlet and outlet samples across all scenarios. In the case of SC (Fig. 5 b), both

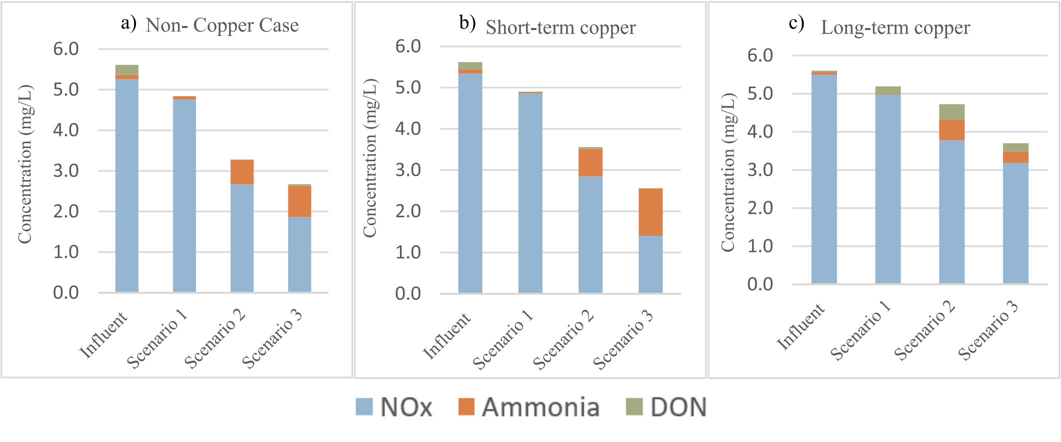


Fig. 2. Total nitrogen (TN) concentration in mg/L and composition for influent and effluent for scenarios 1, 2, and 3 for NC (a), SC (b), and LC case (c).

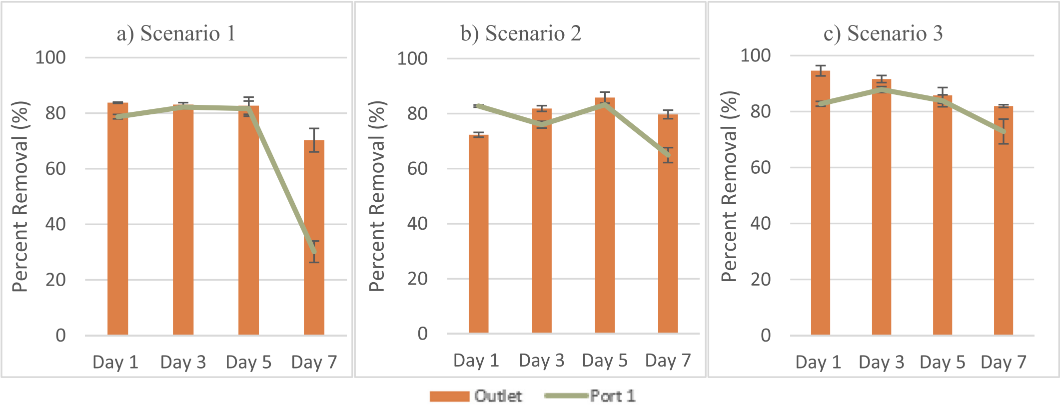
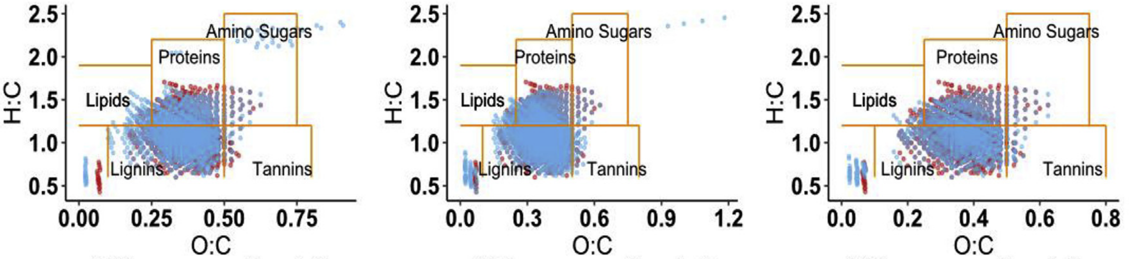
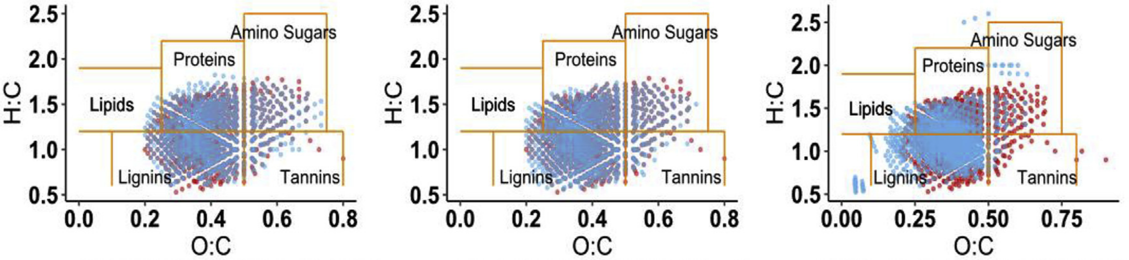


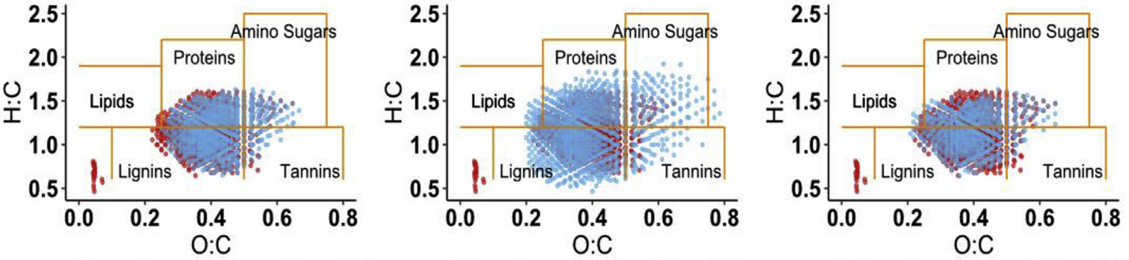
Fig. 3. Copper removal in time series for scenario 1 (a), scenario 2 (b), and scenario 3 (c).



(a) Non-copper case (Scenario 1)
(b) Non-copper case (Scenario 2)
(c) Non-copper case (Scenario 3)



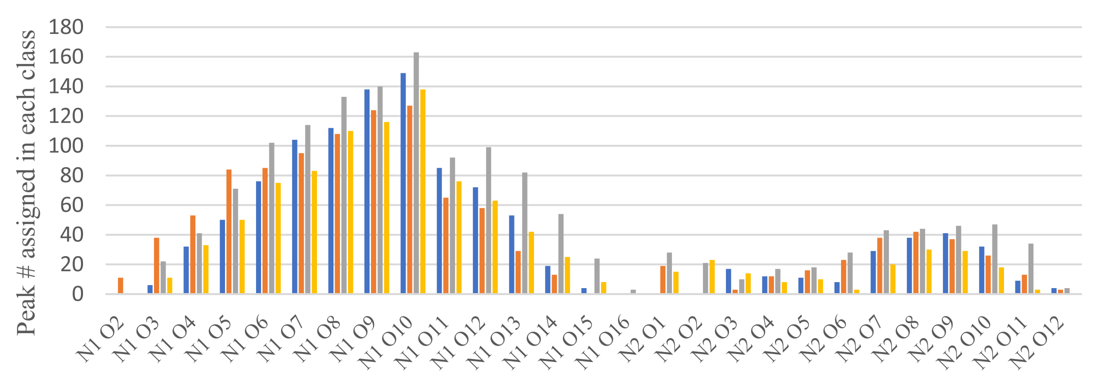
(d) Short-term copper impact (Scenario 1)
(e) Short-term copper impact (Scenario 2)
(f) Short-term copper impact (Scenario 3)



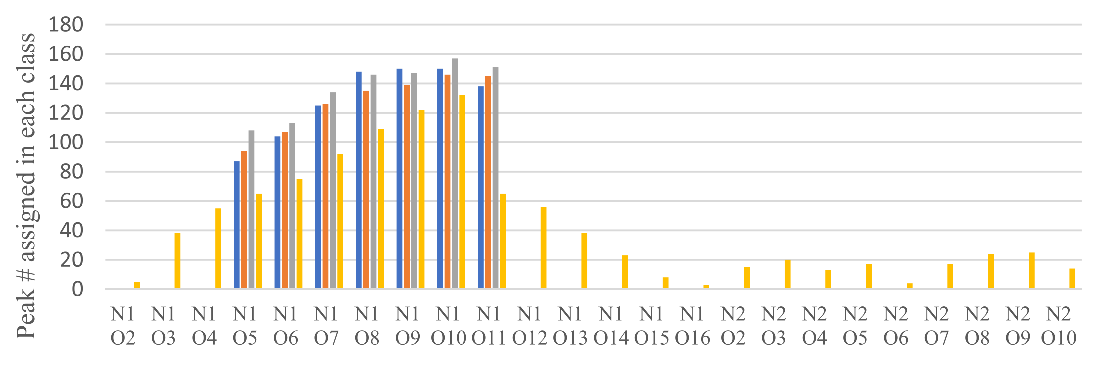
(g) Long-term copper impact (Scenario 1)
(h) Long-term copper impact (Scenario 2)
(e) Long-term copper impact (Scenario 3)

Fig. 4. Van Krevelen diagram of DON composition for the 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).

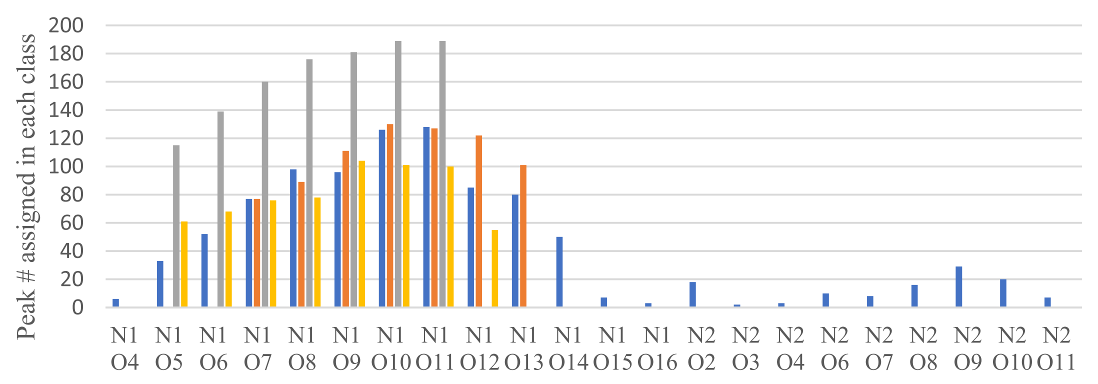
scenarios 1 and 2 showed identical influent and effluent CHON species distribution and peak numbers, which are also very similar to the inlet, while the scenario 3 effluent species exhibited fewer peak numbers with wider CHON distribution. In the case of LC (Fig. 5 c), all scenarios showed similar CHON 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 displayed the highest peak numbers.



(a) Non-copper case



(b) Short-term Copper impact



(c) Long-term Copper impact

Fig. 5. 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).

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; therefore, they were not included here. The population density of comammox was quantified at the top and the different ports in each scenario. Peculiarly, small quantities of comammox were evidenced at the top of each column. Furthermore, short-term presence of copper negatively impacted the population density of comammox, indicating a decrease of 29%, 49%, and 65% in the average population density in each port (excluding the top one) in scenario 1, scenario 2, and scenario 3, respectively (Fig. 6). The longer-term presence of copper negatively impacted scenarios 1 and 3 by decreasing the average population density at each port by 63% and 29%, while in scenario 2 the average population density increased by 52%. 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 (Fig. 7a). 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 (Fig. 7b). Thus, in

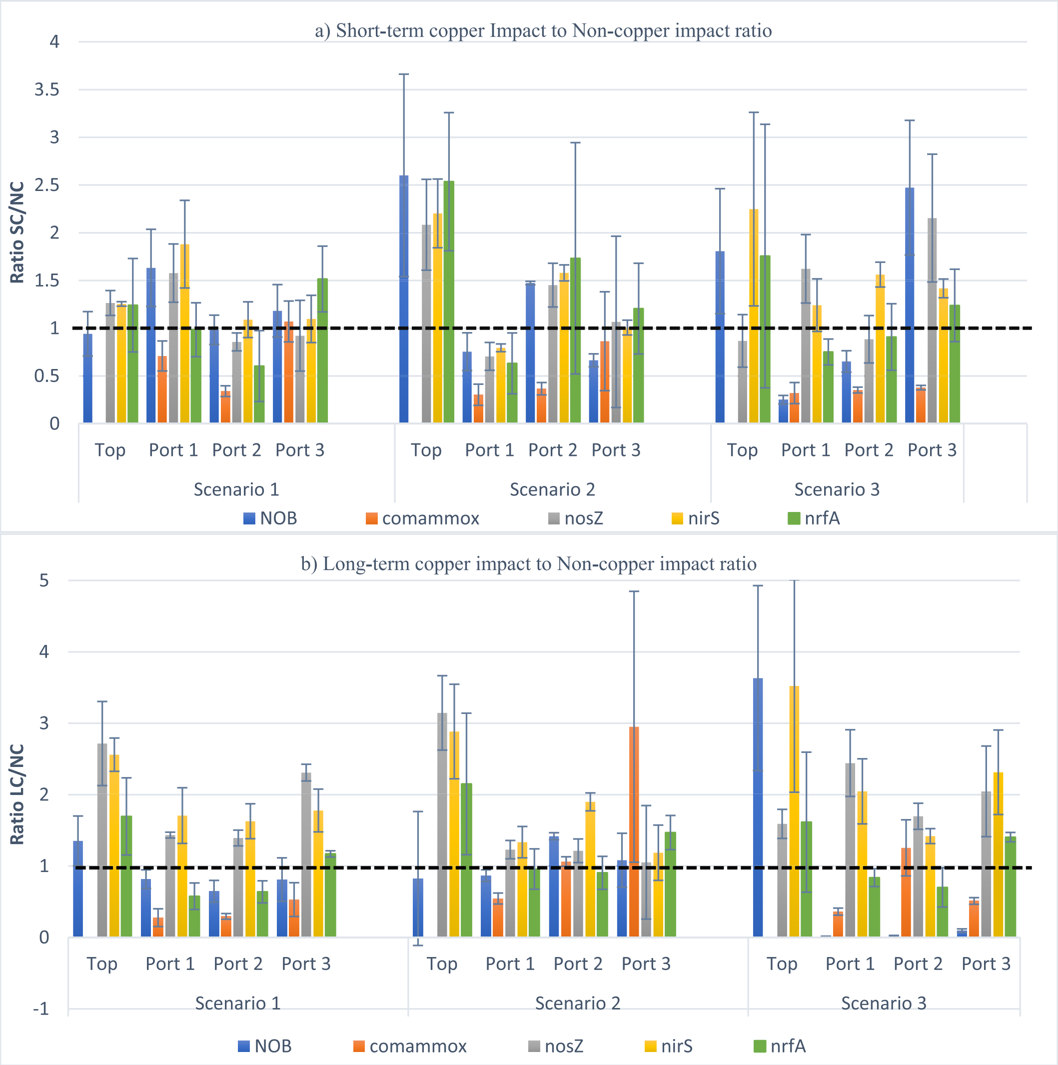


Fig. 6. Microbial population ratio between short-term copper impact and non-copper case (a), and long-term copper impact and non-copper case (b).

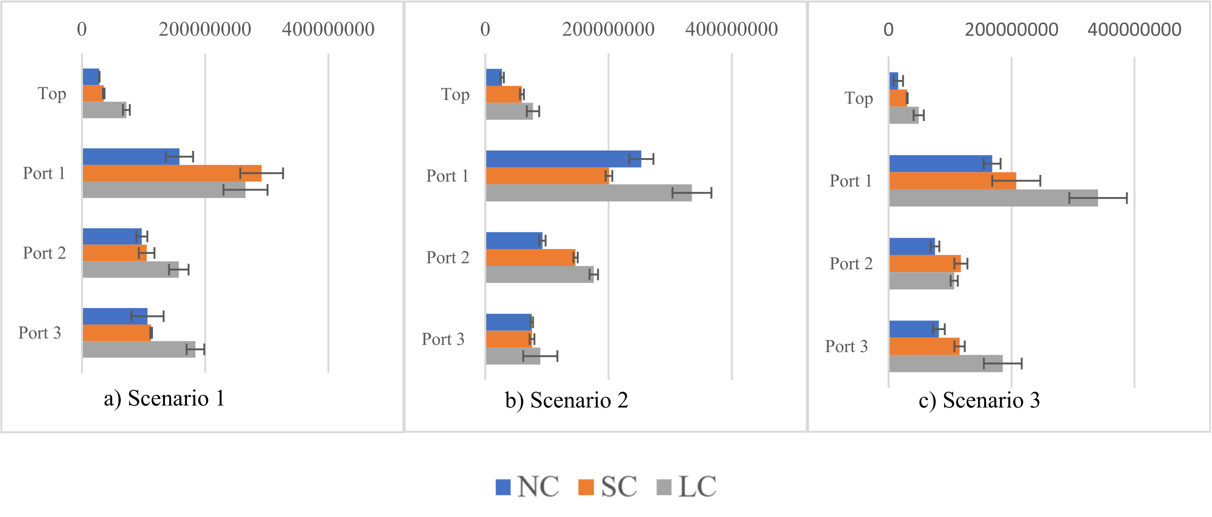


Fig. 7. Denitrifiers (gene nirS) absolute quantity in copy/gram for non-copper case (NC), short-term copper impact (SC) case and long-term copper impact case (LC) under scenario 1 (a), scenario 2 (b) and scenario 3 (c).

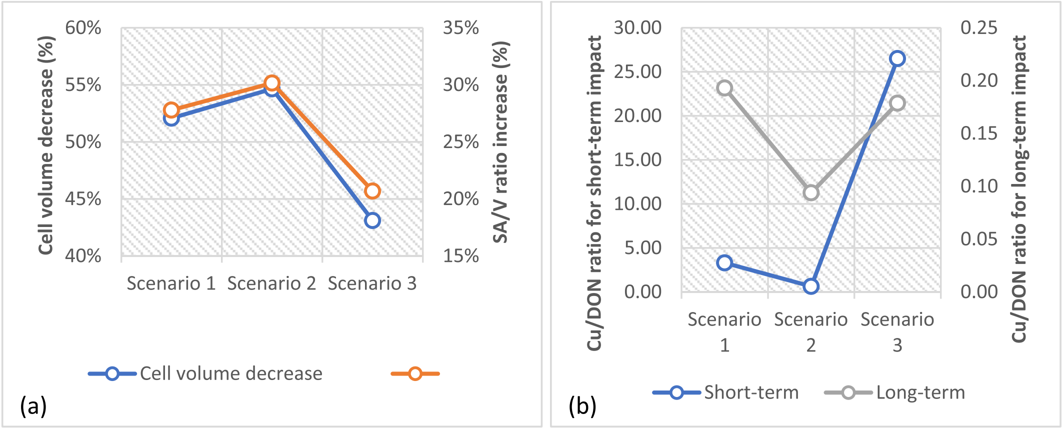


Fig. 8. (a) The theoretical change of cell volume and SA/V ratio in percentage between NC and LC cases (assume all bacteria are in sphere) and (b) The inlet copper concentration to the effluent DON concentration ratio for short- and long-term copper impacts.

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. Fig. 8 indicates the distribution of the denitrifier 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.

4. Discussion

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. Denitrifiers and DNRA microbial population densities increased in the cases of SC and LC when compared to the case of NC, as described in section 3.4 (Fig. 6). 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 S-1). For this reason, scenario 2 triggered the most intensive enzymatic cascade effects and resulted in a large growth of the 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 (Harms et al., 2003) such as the N_2O reductase for the last step of denitrification to convert the N_2O into N_2 (Wen et al., 2018; Magalhães et al., 2011), excessive exposure to copper may cause serious damage to the metabolic processes in bacteria (Dupont et al., 2011). This can be realized by looking at the DON concentration and composition changes from Figs. 4 to 5. 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 notable 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 Fig. 5 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 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 (Fig. 2 and Table-S3).

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 (Lawson and Lücker, 2018; Roots et al., 2019), 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% of all detected species) continued to increase significantly

(Figs. 6 and 7). 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) (Fig. 8a), through which the bacteria could slow down the copper diffusion from cell to cell. Fig. 8a was theoretically determined based on the assumptions that the microbial population intends to occupy all the space available, and that each cell's 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 (Fig. 2). 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 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 (Fig. 8b), 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 (Fig. 4 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 (Fig. 3) and the distribution of effluent CHON classes tended to be similar to 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.

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 (see Figure S-1 and Fig. 2). Higher HRT usually resulted in more efficient nutrient removal, since the nutrients could be thoroughly consumed by bacteria due to the longer contact time, an observation supported by the lower ORP value from scenario 3 (Figure S-2). 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 S-2), 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 (Freinbichler et al., 2011; Yoshida et al., 1993). 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 2 and 3. The increase in DNRA population density may also have contributed to the accumulation of ammonia in scenarios 2 and 3. In scenario 1, the faster HRT due to conduits provided less contact time, which may be the reason for less or no ammonia increase. 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 et al., 2011). This means the incomplete denitrification might be the major cause of the failure of nutrient removal in the end.

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 copper, the microbial community reproduced more smaller cell-sized bacteria through enzymatic cascade and extracellular sequestration and released more DON to minimize the copper toxicity impact via both inter- and extracellular approaches. Denitrifiers are the main contributors to 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 resulted in a minimally 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 by external factors. This understanding is important for a proper application of BAM in watershed systems to mitigate pollutants from non-point sources to reach different receiving water bodies (rivers, lakes, aquifers).

Author contribution

Dan Wen was in charge of experimental design of the column study, nutrient analysis and paper writing; Diana Ordonez was in charge of column setting, qPCR analysis and paper writing; Andrea

Valencia was in charge of column setting; Amy M. McKenna was in charge of DON analysis, and Ni-Bin Chang was in charge of methodology screening and integration, as well as paper editing.

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Appendix A. Supplementary data

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