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ASSESSMENT OF MICROBIAL DENITRIFYING CAPACITY IN VEGETATED BIOREACTORS TREATING GREENHOUSE EFFLUENT

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Abstract: The discharge from food production greenhouses (greenhouse effluent) contains high nutrient concentrations, which if directly released, can have adverse effects on the environment. Wood-chip bioreactors are increasingly popular passive water treatment systems favoured for their economical denitrification in treating agricultural field tile drainage. Microbial communities are central to denitrification, however little is known about the maturation of microbial communities in wood-chip bioreactors treating greenhouse effluents. Multiple subsurface flow wood-chip bioreactors, each vegetated with a different plant species, together with an unplanted unit, received synthetic greenhouse effluent with elevated nitrate concentrations. The hybrid bioreactors were operated for over 2 years, during which water samples were collected from the inlet, outlet and within the reactors. The denitrification rate in the bioreactor planted with Typha angustifolia (narrowleaf cattail) was shown to be higher with increasing microbial activity and metabolic richness, measured by the carbon utilization patterns in Biolog® EcoPlates. The increased denitrifying gene (*nirS*) copies (determined by quantitative polymerase chain reaction, qPCR), and near-complete nitrate removal were observed in the *T. angustifolia* and unplanted reactors after 16 and 23 months of operation respectively. The findings suggested that an acclimation period of at least one year can be expected in unseeded bioreactors planted with *T. angustifolia*, while bioreactors without vegetation may require a longer time to maximize their denitrification capacity. These results are important for the designers and operators of wood-chip bioreactors, which are expected to be more commonly applied in future.

Keywords: Denitrification; wood-chip bioreactor; greenhouse; microbial community

1 INTRODUCTION

Denitrifying bioreactors are passive treatment systems supplied with an external carbon source such as wood chips that are used for nitrate removal from wastewaters. The early studies showed that the available carbon source in the riparian zones near surface waters had an important effect on the denitrification of the ground water that occurred in the soil (e.g. Schipper et al., 1991). These observations led to the innovation of in-situ systems with externally supplied solid carbon for enhanced nitrate removal (e.g. Robertson and Cherry, 1995). In addition to providing an electron donor for heterotrophic denitrification, these carbon substrates create a favourable anoxic environment by serving as an energy source for the aerobic microorganisms (Schipper et al., 2010). As reviewed by Addy et al. (2016), there have been many recent

studies focused on using denitrifying bioreactors to reduce the adverse impact of the nitrates released from agricultural tile drainage. However, the nitrate concentrations found in the discharges from food production greenhouses (greenhouse effluent) are notably greater than those in the typical agricultural runoff. These high nutrient levels in the greenhouse effluent are typically due to the unused portion of the dissolved nutrients that are added to the irrigation water. High nitrate concentrations can lead to hypoxia in surface waters and cause potential health implications if consumed in drinking water. Therefore, detrimental environmental and health impacts can occur from the continuous release of the untreated greenhouse effluent.

Most of the studies on denitrifying bioreactors are comprised of unplanted systems, where employing cover vegetation has received little attention. This is potentially due to the common understanding that plants in passive treatment systems, such as constructed wetlands (CWs) can facilitate oxygen transfer (Kadlec and Wallace, 2009), which in turn might compromise the anoxic conditions necessary for denitrification. However, up to 99% nitrate removal from the greenhouse effluent was recently achieved in a series of pilot-scale vegetated bioreactors (Fatehi Pouladi et al., 2016a).

Despite the high performance of denitrifying bioreactors in reducing nitrate loads, the evidence to confirm the presence or function of microbial communities, presumed as the key driver of the reported denitrification, is limited. The few published studies investigating the microbial communities in these bioreactors (e.g. Warneke et al., 2011; Healy et al., 2015) were primarily conducted on unplanted reactors designed for treatment of discharges other than the greenhouse effluent. Among the microbial assessment methodologies employed for investigating treatment wetlands, the tools developed for functional assessment provide an understanding of what the existing microbial community is able to accomplish without directly identifying the species. This is a useful approach, as the presence of microbial species with a certain gene does not necessarily indicate that the environmental conditions are favourable for that gene to be expressed, and result in the expected function (Weber, 2016). The quantitative polymerase chain reaction (qPCR) and Community-Level Physiological Profiling (CLPP) are two examples of these methods than can assess the functional capacity of microbial communities.

The qPCR technique is a standard and powerful molecular biological method in which a targeted gene is amplified and measured in real time. This method provides information about the activity of the targeted enzymes and their potential function, such as those involved in the denitrification process. In a CLPP method using Biolog® microplates, the environmental sample is inoculated with various types of carbon sources and the colour change developed through the production of NADH over the incubation period provides information regarding metabolic function of the mixed microbial communities (Weber and Legge, 2010). In many recent studies, the parameters derived from the CLPP method have helped researchers to understand the microbial function of treatment wetlands of different scales and designs (e.g. Weber and Legge, 2011; Bissegger et al., 2014; Button et al., 2015).

The aim of the initial stage of this study was to investigate the potential development of microbial communities in vegetated wood-chip bioreactors with no prior seeding by measuring the mixed metabolic function of microbial communities via the CLPP method. Moreover, the study aimed to evaluate the bioreactors for their utilisation patterns of specific organic substrates in the CLPP Biolog® EcoPlates. The other important goal was the characterization of the denitrifying capacity of the bacterial community by investigating the denitrifying gene copies present in the bioreactors. These research objectives were defined to describe whether the nitrate removal exhibited from the water quality measurements could be explained by the microbial activity assessments. As each of the vegetated bioreactors was planted with a different plant species including halophytes and non-halophytes, the role of the plants in the microbial development of denitrifying bioreactors was also of interest. Overall, the study investigated the microbial denitrification capacity of vegetated wood-chip bioreactors as a potentially effective mechanism in treating the effluents from greenhouse operations.

2 MATERIALS AND METHODS

2.1 Timeline and Reactor Design

The pilot-scale project was built and operated at the laboratory of the Department of Civil Engineering at Queen's University (Kingston, ON, Canada). Each of the 5 bioreactors was constructed from open-top 220-L barrels (diameter: 56 cm, height: 90 cm) and filled with Maple hard wood chips, acquired from Quebec, Canada, in a single 80-cm layer. A vertical PVC tube (inner diameter: 2.54 cm) was positioned in the centre of each reactor through the wood-chip media as a platform to support 3 interstitial water sampling tubes (inner diameter: 1.6 mm), which were installed at 20, 40 and 60 cm below the top surface of the wood chips.

The synthetic greenhouse effluent was prepared in the laboratory per the common greenhouse effluent characteristics reported in the literature and by greenhouse operations in Ontario. The bioreactors were operated in vertical subsurface-flow (VSSF) mode. The influent was continuously distributed over the surface of each reactor by means of a peristaltic pump (hydraulic loading rate: 12 cm d^{-1}) and a square distribution grid, while the outflow was discharged from the bottom. The 4 vegetated bioreactors together with the unplanted (control) unit were in continuous operation indoors (ambient air temperature: 24° C) under a 1,000 W Metal Halide grow light set to a 16/8 on/off cycle to simulate the sunlight. More details about the experiment, including its design, schematic of the reactors, plant selection criteria and influent characteristics have previously been published (Fatehi Pouladi et al., 2016a, 2016b).

The project timeline, various test dates and operational phases are shown in Figure 1. The total operation was divided in the High Loading (average influent NO_3-N: 307 mg L^{-1}) and Low Loading phase (average influent NO_3-N: 202 mg L^{-1}). The active plant species were grouped as A, B and C. The A selection included softstem bulrush (*Schoenoplectus tabernaemontani* C.C. Gmel. Palla), switchgrass (*Panicum virgatum* L.), narrowleaf cattail (*Typha angustifolia* L.) and Canada wildrye (*Elymus canadensis* L.). The species present in the B group were *S. tabernaemontani* and *T. angustifolia* as the other two plants could not survive the experimental conditions. The C group including *S. tabernaemontani*, *T. angustifolia*, prairie cordgrass (*Spartina pectinata* Bosc ex Link) and saltgrass (*Distichlis spicata* L. Greene) resumed the operation starting in June 2015.

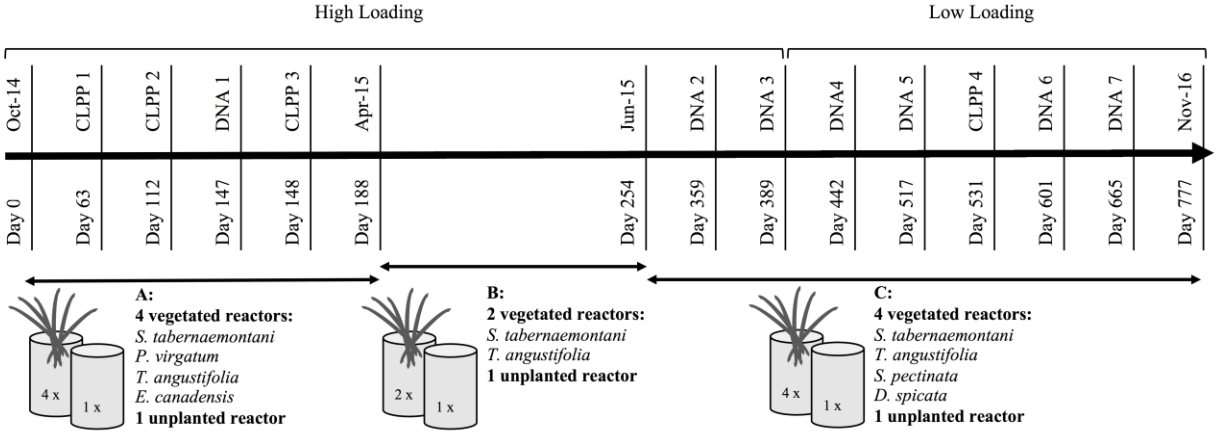

Figure 1. The operation timeline, loading phases, sampling dates and plant species. "CLPP" and "DNA" refer to the sampling events for the total 4 CLPP and 7 qPCR analyses respectively (time intervals not drawn to scale).

2.2 Microbial Assessment

The interstitial water samples were drawn from the spatial vertical profile using a set of dedicated 50 mL plastic syringes and were then transferred to air-tight sterile polypropylene tubes. Samples were consequently transported to the laboratory of the Environmental Sciences Group (ESG) at the Royal Military College of Canada (Kingston, ON, Canada) for further preparation.

2.2.1 CLPP

Biolog® EcoPlates (BIOLOG Inc., Hayward CA, USA) with 31 different carbon sources and one blank well in triplicates (96 wells per plate) were inoculated with each water sample at room temperature on the same day the samples were collected. Sample preparation was performed in an aseptic environment using sterile laboratory consumables and 70% ethanol spray for sterilizing the tools and bench tops. 100 μL of the unfiltered samples was pipetted to each well, after which the plates were stored in the dark during the incubation period. The optical densities of the plates were read using a microplate absorbance reader (TECAN Infinite®, Tecan US Inc., Morrisville, NC, USA) at an absorbance of 590 nm at several intervals after inoculation.

The first two CLPP tests (CLPP 1 and 2), were performed per the commonly practiced aerobic protocol where sample collection, preparation, inoculation and incubation procedure were exposed to natural oxygen in air. CLPP 3 and 4 were conducted anaerobically to better preserve the anaerobic microbial communities and inhibit aerobic respiration. Nitrogen gas was used to purge oxygen from the sample tubes by de-airing them prior to sample collection. The tubes were then sealed using multi layers of adhesive tape. The plate inoculation was done in an oxygen-free environment inside a glove box (or a glove bag) purged with a high flow of nitrogen gas. All the laboratory equipment including the samples and plates were stored inside the de-aired chamber while a small continuous flow of nitrogen ensured a low-oxygen environment. The plates, petri dishes and other tools were similarly purged prior to use, and the inoculated plates were sealed using adhesive tape around each plate ensuring the top lids and bottom surfaces remained intact. The plates were stored in the chamber covered with opaque black plastic bags during the incubation period, and were only removed temporarily to record their absorbance values. The lids remained taped to the plates during the entire process.

The CLPP data analysis was performed according to the previously established methods (Weber et al., 2007; Weber and Legge, 2010). The absorbance readings at 48 to 50 h were selected as the suitable time point for further analysis. The absorbance reading of each plate, associated with one sample from a specific reactor and depth resulted in 96 carbon source utilisation patterns (CSUPs). The overall average well colour development (AWCD) of one sample was calculated as:

[1] $$AWCD = \frac{1}{31}\sum_{i=1}^{31}(A_i - A_0)$$

where A_i is the average of the triplicate absorbance readings of the carbon source i, and A_0 is the average absorbance reading of the blank well with no carbon source. In the case of negative numbers for (A_i-A_0), indicating very low recorded absorbance, the values were recorded as zeros. For the statistical analysis where replicates were needed, 3 AWCD values were associated with each reactor's depth, in which case A_i and A_0 represented the absorbance values of one of the three replicates for the carbon source i and the blank well respectively, rather than the average value of the three replicates. The richness of the microbial community was described as the metabolic potential of the community, calculated as a ratio of the number of the carbon sources that were utilized by the inoculated microorganisms, over the total number of carbon sources, presented in percentage (%). A carbon source was counted as 'utilized' when the well's blanked value (A_i-A_0) was greater than 0.25. The carbon substrates in each Biolog® plate have been grouped in 5 major guilds, namely carbohydrates, carboxylic/acetic acids, polymers, amines/amides and amino acids (Weber and Legge, 2009, adapted from Zak et al., 1994). Moreover, 8 of the total 31 carbon substrates have recently been identified for their common occurrence in various plant root exudates (Button et al., 2016). The plant root exudate richness of a sample (%) was also reported as the microbial community's richness by calculating the percentage ratio of the utilized number of the carbon sources associated with plant root exudate over the total number of substrates (8).

2.2.2 qPCR

The expression level of the nitrite reductase genes *nirK* and *nirS* was determined by real-time RT-PCR using the SYBR Green detection system. The primers and programs used for the amplification of *nirK* and *nirS* have been previously designed and optimized (Ligi et al., 2014). The influent and interstitial water samples were first filtered using glass-fiber filter papers (0.2 μm pore size, diameter: 47mm, Millipore,

Etobicoke, ON, Canada). Filters with the captured residues were then placed in sterile tubes and stored at -20 ºC until the DNA extraction was conducted. The total community DNA extraction was performed by rolling the thawed filters inside the extraction vials provided in the FastDNA SPIN Kit for Soil (MP Biomedical, Solon, OH, USA), following the manufacturer's protocol. The nucleic acid concentrations were measured by pipetting 1 µL of the extracted samples using the NanoDrop 2000 spectrophotometer (Fisher Scientific, Toronto, ON, Canada). The samples were analyzed undiluted in duplicates on Eco™ Real-Time PCR System (Illumina, San Diego, CA, USA) using Maxima SYBR Green Master Mix (Fisher Scientific, Toronto, ON, Canada) and 0.4 µM of forward and reverse primers. The reaction efficiencies calculated by EcoStudy Software (Illumina, San Diego, CA, USA) were 100 ± 10 % with an $R^2 > 0.990$. On each plate, a standard curve (0.025 to 2.5 ng) and no template control were run with the samples. The qPCR standard curves were prepared using 6 serial dilutions (25.00, 6.25, 1.56, 0.40, 0.10 and 0.025 ng) of the DNA extracted from thermophilic sludge from Ravensview wastewater treatment plant (Kingston, ON, Canada).

The gene copy concentrations were derived relative to the standard curves. Each value was normalized for both the nucleic acid concentration of the extracted DNA sample, as well as the volume of the filtered water sample. The normalized values for the interstitial or influent water samples were calculated as:

[2] $$nirS\ or\ nirK = nirS_1\ or\ nirK_1/(V \times N_C)$$

where *nirS* (or *nirK*) represents the sample's normalized amplified target gene copy quantity (no unit), $nirS_1$ (or $nirK_1$) is the recorded amplified target gene copy quantity (ng) of the sample, V is the initial water sample volume (µL) and N_C is the nucleic acid concentration (ng μL^{-1}) of the respective sample after DNA extraction. The normalized gene copies of all the samples were standardized against that of the influent sample, which was selected as the calibrator to report the gene copy fold-change as shown in Eq. 3:

[3] $$nirS\ or\ nirK\ fold\ change = nirS\ or\ nirK\ /(nirS_{inf}\ or\ nirK_{inf})$$

where '*nirS* or *nirK* fold change' (no unit) describes the times-increase of the amplified target gene quantity to that of the calibrator (influent) and $nirS_{inf}$ or $nirK_{inf}$ is the normalized amplified target gene copy quantity (no unit) of the influent sample per Eq. 2. It is important to note that the reported *nirS* and *nirK* quantities are not absolute and are relative numbers. Nitrate concentrations in the influent and outflow grab samples were analyzed at an external laboratory according to Standard Methods (APHA, 2005). All the statistical analyses including one-way ANOVA, the Tukey's and Dunnett's tests were performed using XLSTAT software (©Addinsoft, New York, NY, USA) and a difference was reported significant when *p-value*<0.05.

3 RESULTS AND DISCUSSION

The AWCD values of the CLPP analyses and the richness of the mixed microbial communities were generally highest in the bioreactor planted with *S. tabernaemontani* in the first CLPP test conducted in the early stages of the experiment, and in the *T. angustifolia* reactor in the rest of the measurements (Table 1 and Figure 2). Each of these reactors were identified as significantly different from the other units for their metabolic activity within each CLPP analysis. A statistical analysis of the AWCD values with depth (top, middle and bottom) as an independent factor suggested some paired significant differences in CLPP 1 and CLPP 3. However, these differences were small, showing no apparent depth trend for the activity values. Moreover, the depth variations as shown in Figure 2 made it difficult to identify a consistent pattern in the AWCD values along the vertical profile. The AWCD numbers in the *T. angustifolia* reactor increased from 0.32 in CLPP 1 to 1.07 in CLPP 4 (over 3-times increase), while the denitrification rate of this reactor reached its maximum of 99% (32 g N day^{-1} m^{-3}) after about 16 months of continuous operation (Day 491: Figure 3.a and 4.a).

The water quality measurements indicated the denitrification rate in the *T. angustifolia* reactor remained above 30 g N day^{-1} m^{-3} (99% NO_3-N removal) during a period of at least 9 months after the last CLPP test was performed on Day 531 (Figure 4.a), suggesting that suitable conditions for successful denitrification continued to be maintained. Similarly, the greatest microbial activity measurement of 0.72 in the *S. tabernaemontani* bioreactor in CLPP 1 was concurrent with the highest nitrate reduction of 99% (49 g N day^{-1} m^{-3}) observed in this reactor in the beginning of the experiment. However, the nitrate removal rate in

this reactor decreased rapidly as the operation progressed, and the metabolic activity dropped to 0.35 in CLPP 4 as well. The decreased activity and denitrification of this reactor were likely due to the loss of organic carbon from wood chips after the initial leaching, which deprived the heterotrophic community of the energy required for their metabolism (outflow BOD_5 < 2 mg L^{-1} on Day 559).

Table 1. Mean AWCD values ± standard deviation and mean total richness (%) in each bioreactor (n: 3 depths) and influent. Reactors grouped with similar lower-case letters did not show significant statistical difference in their AWCD values within each CLPP test. Asterisk (*) indicates a significant difference from the unplanted reactor.

	CLPP 1		CLPP 2		CLPP 3		CLPP 4	
	AWCD	Rich. (%)	AWCD	Rich. (%)	AWCD	Rich. (%)	AWCD	Rich. (%)
Influent	0.36	45	0.36	39	0.20 ± 0.25	29	0.24 ± 0.23	42
Control	0.54 ± 0.18 b	48	0.24 ± 0.09 c	35	0.32 ± 0.01 c	32	0.51 ± 0.16 c	45
E. canadensis	0.54 ± 0.04 b	58	0.10 ± 0.02 d*	13	0.20 ± 0.08 d*	26	-	-
P. virgatum	0.33 ± 0.02 c*	39	0.30 ± 0.08 b, c	42	0.30 ± 0.01 c	45	-	-
S. tabernaem.	0.72 ± 0.02 a*	64	0.35 ± 0.08 b*	42	0.42 ± 0.13 b*	45	0.35 ± 0.06 d*	35
T. angustifolia	0.32 ± 0.01 c*	39	0.54 ± 0.05 a*	55	0.55 ± 0.11 a*	55	1.07 ± 0.13 a*	74
S. pectinata	-	-	-	-	-	-	0.64 ± 0.02 b*	48
D. spicata	-	-	-	-	-	-	0.32 ± 0.03 d*	32

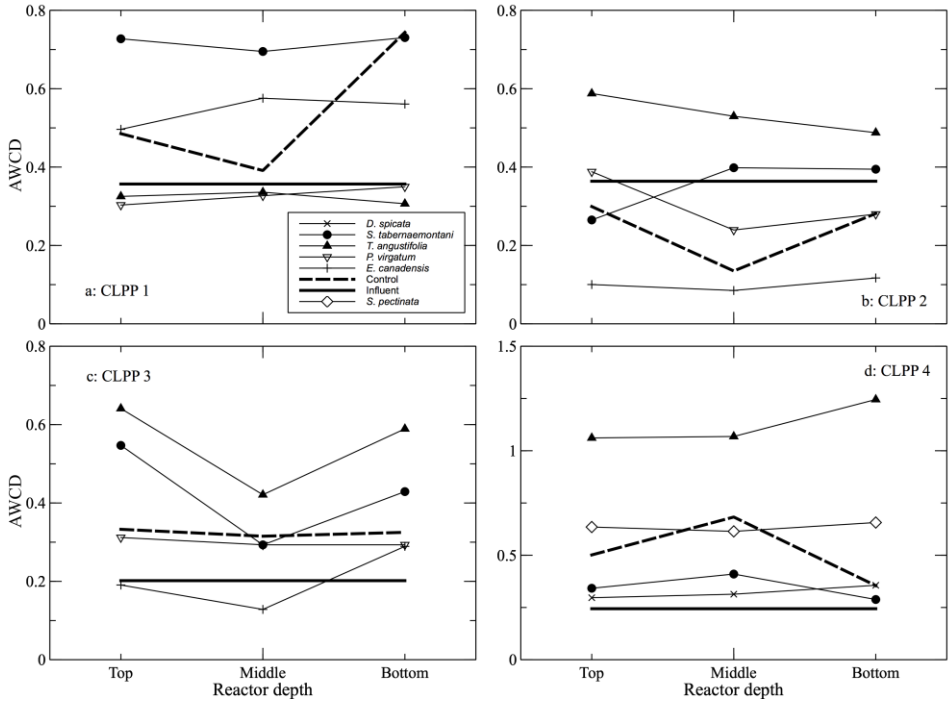

Figure 2. AWCD values for each bioreactor and influent measured by 4 CLPP tests on water samples from the reactors' top, middle and bottom depths.

Of the four CLPP tests, the results of CLPP 4 can be considered the most representative of the matured state of the system, as it followed an anaerobic protocol, and was conducted approximately 17 months after the project start-up. Despite the statistical difference that was reported between the control reactor and all the other units (Table 1), the *T. angustifolia* reactor was the only unit with considerably higher metabolic activity, while the other reactors showed lower, or in case of *S. pectinata*, only slightly higher numbers than control. In addition to the AWCD values, the carbon utilization richness associated with plant root exudates for the *T. angustifolia* reactor displayed a consistent increase from 37% in CLPP 1 to 81% in CLPP 4 (Figure

3.b), suggesting that the planted reactor could utilize a higher number of root exudate carbon substrates as the bioreactor matured. This can potentially indicate that the presence of *T. angustifolia* and its roots played a role in the maturation of the microbial community that developed within the reactor. These results should however be interpreted with caution, as the metabolic activity and root exudate richness values of the influent also ranged from 0.20 to 0.36 and 25% to 62% respectively over the same period, which can be an indication of a natural microbial community establishment inside the influent reservoirs. In addition, the observed activity in the influent samples might suggest that a background chemical reaction occurred between the substrates in the EcoPlates and the nutrient solution, which did not happen in the blank wells.

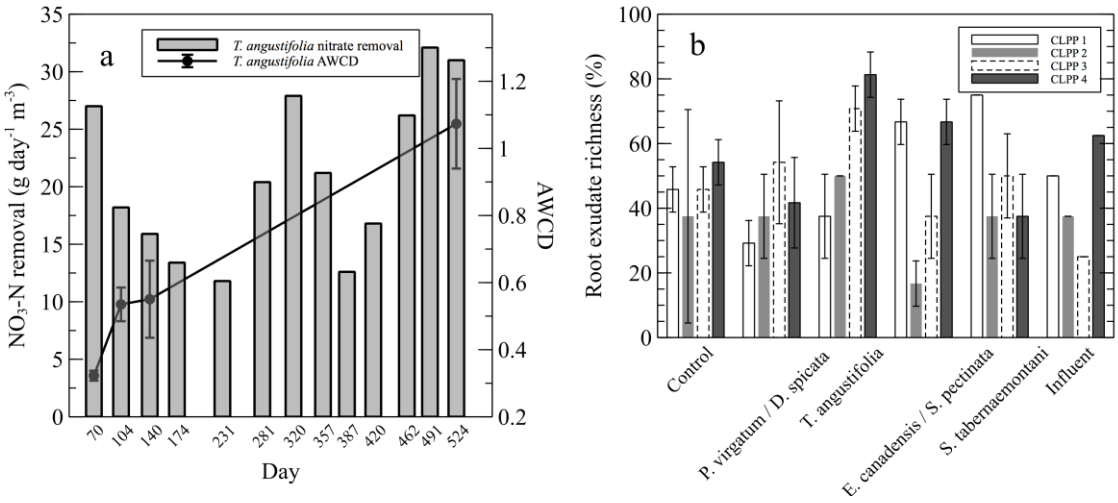

Figure 3. a: Temporal trend of NO_3-N removal rate of the bioreactor planted with *T. angustifolia* (left vertical axis) and the same reactor's AWCD (right axis). b: Average microbial community richness based on the utilized carbon sources associated with plant root exudates. Error bars represent the standard deviation of 3 depths in both figures.

The *nirS* gene copies were positively correlated with the denitrification rate (Figures 4.b and c). Similar to the mixed microbial activity results, the highest *nirS* copies were measured in the bioreactor planted with *T. angustifolia*. The average fold-change of *nirS* in this reactor revealed a sudden boost in the second half of the operation with an average of 1,732, 715 and 679-times increase on Days 517, 601 and 665 respectively (Figure 5.a). Similar to Healy et al. (2015), who investigated the denitrification genes along their experimental wood-chip columns, we did not observe a consistent trend in the denitrification gene copies with the depth.

The nitrate removal rate and the ln (*nirS* fold-change) were positively correlated in the *T. angustifolia* reactor (Figure 5.b, R^2=0.6). A correlation between the total copies of nitrite reductase genes (∑*nirS*, *nirK* and *nosZ*) and nitrate removal rate was previously shown in a study of denitrifying bioreactors with carbon-rich media (Warneke et al., 2011), where the reductase gene copies were found to be lower in hard and soft wood chips in comparison with the other carbon substrates such as maize cobs, wheat straws and sawdust. The *nirK* fold-changes in our study were found to be negligible and hence not reported. However, *nirK* populations in other denitrifying systems were typically found in abundance, with a *nirS*/*nirK* ratio ranging from 1 to 13 in different environmental conditions (Warneke et al., 2011; Ligi et al., 2014). The low *nirK* copies in our system can be attributed to the fact that our bioreactors were constructed and operated in a laboratory setting and received synthetic influent in reasonably controlled conditions. Thus, it was expected that the developed microbial communities were not as diverse as reported in other systems.

In the *T. angustifolia* reactor, the higher activities of the mixed microbial communities interpreted by the AWCD values, together with the higher nitrite reductase gene (*nirS*) populations are strong markers to indicate the elevated denitrification performance in this reactor was caused by microbial pathways involving denitrifying bacteria. However, future work to analyze the absolute quantities of *nirS* and *nirK* would be useful to determine the actual denitrification rates. It has been shown that CWs planted with *Typha latifolia*,

another species of the same family, encouraged the growth of *nirK* containing bacteria, while the addition of the plant's litter could stimulate the growth of the bacteria expressing *nirS* genes (Chen et al., 2014). These findings reinforce the possibility that the dead and decaying biomass of *T. angustifolia* in our study might have contributed to the higher growth of the bacterial community. Since the results of the *T. angustifolia* unit stood out in all the different analyses, the presence of *T. angustifolia* can be regarded as an important factor in providing the favourable conditions for growth of heterotrophic bacteria.

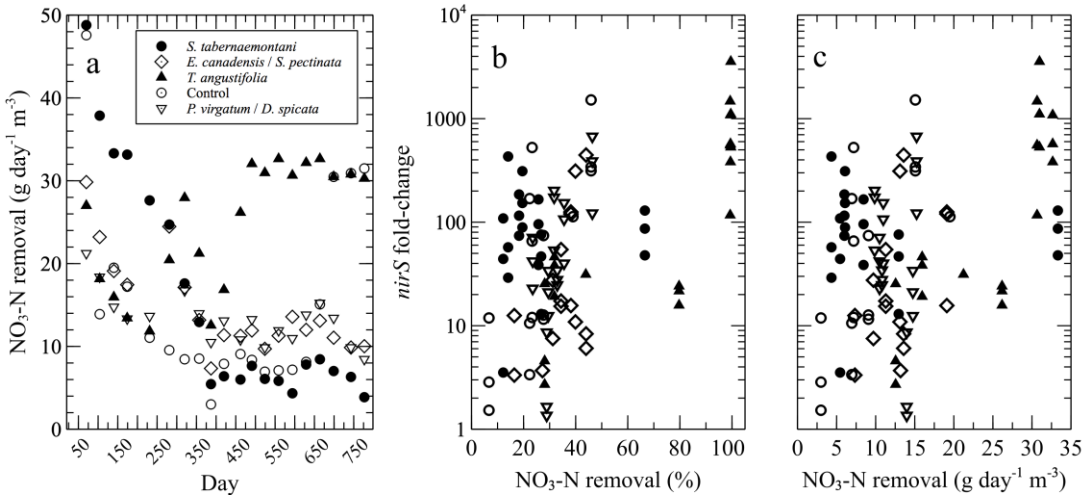

Figure 4. Nitrate mass removal during the entire period of operation (a), *nirS* fold-change in bioreactors vs. nitrate treatment rate (b), and nitrate mass removal rate (c).

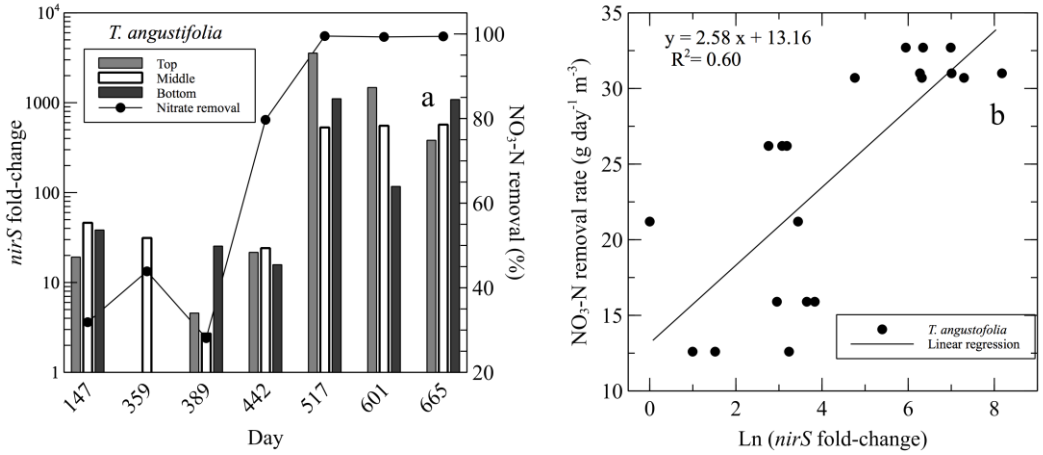

Figure 5. a: Temporal *nirS* fold-change in *T. angustifolia* bioreactor at 3 depths (left axis) and bioreactor's nitrate removal rate (right axis); b: *T. angustifolia* bioreactor's nitrate removal rate as a function of ln (*nirS* fold-change).

An important observation that ensued was a relatively high *nirS* copies in the unplanted reactor (Figures 4.b and c) despite nitrate maximum removal of 46% around the time when the final DNA sampling was performed (Day 665). It should however be noted that the nitrate reduction in this reactor reached 99% (30 g N day^{-1} m^{-3}) on Day 699, about one month after the final DNA testing (Figure 4.a). This delayed high denitrification performance demonstrated the unplanted reactor required a longer acclimation time to attain its full denitrification capacity. Furthermore, the recognizable higher *nirS* fold-change observed in this reactor was likely captured during the exponential growth phase of its bacterial community, which proved an effective measure predicting the next phase, where denitrification rate was maximized and nitrate concentrations became the limiting growth factor.

It was earlier suggested that the substantially higher release of organic carbon (shown by outflow BOD_5 and COD measurements) in the *T. angustifolia* reactor was the main driver for the near-complete denitrification (Fatehi Pouladi et al., 2016a). The degradation of the wood chips can be a major factor in leaching organic matter that provides food and energy source for the heterotrophic denitrifying bacteria. The appearance of white substances on the surface of the wood chips in our bioreactors could hint at the presence and growth of fungi species as well. Fungi has been shown to contribute to co-denitrification and production of nitrogen gas in soils (Long et al., 2013). In addition to a potentially direct role, the fungi species may have an indirect role by aiding the denitrifying bacteria by degrading the cellulose and lignin in wood chips and making the organic carbon bio-available for the bacteria (Appleford et al., 2008; Porter, 2011). Thus, it is possible that fungi growth assisted the denitrification performance of the *T. angustifolia* and unplanted reactor by decomposing the wood chips. Therefore, further investigations are needed and recommended as future steps to gain deeper insights into the function of the fungi and denitrifying bacteria in enhancing denitrification in direct or indirect ways. Furthermore, although the interstitial water sampling method has previously been used in microbial studies of CWs to avoid destructive core sampling techniques (e.g. Button et al., 2015), the microbial characteristics of the biofilm attached to the wood chips are still of great interest. This component will be carried out in the next phase of the project, which will provide a basis for comparison with the current investigation of the interstitial water samples.

The results of this study provide useful information for the greenhouse owners and managers who are interested in employing low-cost treatment systems for nutrient management of their food production operations. The long-term nature of the pilot-scale experiments presented in this paper can inform designers about the dynamic nature of wood-chip bioreactors in long-term operations. It was also shown that bioreactors vegetated with capable species such as *T. angustifolia*, can host and develop a more active microbial community than other species such as *D. spicata* and result in a better denitrification performance with a shorter start-up period.

4 CONCLUSIONS

The results of this study demonstrated that high denitrification performance of wood-chip bioreactors in treating greenhouse effluent could be linked to the mixed microbial community and bacterial population encoding the denitrifying gene of *nirS*, thus confirming the biologically mediated denitrification in these bioreactors. The presence of *T. angustifolia* resulted in a shorter acclimation period, whereas the unplanted reactor followed the same trend with an approximately 7-month delay. Based on these findings, the presence of *T. angustifolia*, its rhizosphere and decaying biomass, as well the growth of fungi on wood chips were discussed as the potential factors that enhanced microbial denitrification. A closer look at the fungi population and the microbial characteristics of the biofilm attached to the media substrate were recommended as the future steps.

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