Nitrogen Removal in Greywater Living Walls: Insights into the Governing Mechanisms

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Abstract: Nitrogen is a pollutant of great concern when present in excess in surface waters. Living wall biofiltration systems that employ ornamentals and climbing plants are an emerging green technology that has recently demonstrated significant potential to reduce nitrogen concentrations from greywater before outdoor domestic re-use. However, there still exists a paucity of knowledge around the mechanisms governing this removal, particularly in regards to the fate of dissolved organic nitrogen (DON) within these systems. Understanding the fate of nitrogen in living wall treatment systems is imperative both to optimise designs and to predict the long-term viability of these systems, more so given the growing interest in adopting green infrastructure within urban cities. A laboratory study was undertaken to investigate the transformation and fate of nitrogen in biofilters planted with different climbing plants and ornamental species. An isotropic tracer ($^{15}$N-urea) was applied to quantify the amount removed through coupled nitrification-denitrification. The results found that nitrification-denitrification formed a minor removal pathway in planted systems, comprising only 0–15% of added $^{15}$N. DON and ammonium were effectively reduced by all biofilter designs, indicating effective mineralisation and nitrification rates. However, in designs with poor nitrogen removal, the effluent was enriched with nitrate, suggesting limited denitrification rates. Given the likely dominance of plant assimilation in removal, this indicates that plant selection is a critical design parameter, as is maintaining healthy plant growth for optimal nitrogen removal in greywater living wall biofilters in their early years of operation.

Keywords: green infrastructure; denitrification; plant-soil system; biofiltration; plant assimilation; $^{15}$N isotropic tracer

1. Introduction

Nutrient management remains one of the most challenging environmental problems. The presence of excess nutrients impacts negatively on the ecological health of our aquatic ecosystems through promotion of eutrophication, oxygen depletion, death of aquatic life, and loss of biodiversity [1]. This, in turn, has deleterious influences on human health and economy [2].

Greywater is increasingly being considered as a viable alternative water source for non-potable water re-use because it is consistently generated in large volumes close to demand. Yet, it contains nutrients at concentrations that present a health threat to aquatic ecosystems [3,4]. This is applicable in all instances where the greywater is re-used for outdoor applications. For instance, using untreated
greywater for irrigation introduces a high risk of its transport to nearby streams after excessive rainfall or low underlying groundwater aquifers and its unintended percolation (overflows) to stormwater drains [5]. It is thus imperative that appropriate measures be adopted to protect ground and surface waters from potential nutrient enrichment.

Living walls are a green technology that is gaining momentum in the field of sustainable development. Living walls comprise climbing plants and flowers grown in trenches or planter boxes at the base of a building using the wall or a separate structural system adjacent or attached to the wall as support. A schematic representation of a typical greywater living wall and a photograph of a living wall located in Melbourne Australia are presented in Figure S1 (Supplementary Material). Whilst they have been traditionally employed as aesthetic features of buildings, living walls possess other functional benefits, including city cooling, improving the building thermal performance and increasing the amenity value of their surrounding environment. Recent research revealed their potential to serve as an effective greywater treatment option [6]. In this latter study, a sand-based filter media was used to effectuate treatment and support ornamental vegetation (including climbers); treatment occurs as incoming water percolates down through the engineered sand media and during temporary storage in the bottom saturated zone (created through a raised outlet pipe and supplemented with a carbon source). The purpose of the saturated zone in living wall design is to extend the water residence time within the system for improved nitrogen processing as well as to provide an anoxic environment for denitrifying bacteria to reduce oxidized nitrogen. The laboratory-scale systems planted with ornamentals and climbing plants were able to achieve in excess of 80% and 90% reductions in concentrations of TSS and BOD respectively. Removal of nitrogen and phosphorus were, however, more variable. For instance, removal efficiencies of nitrogen (N) ranged between 7% and 90% after one year of biofilter operation, with presence and type of vegetation found to have a significant effect on performance [6].

In order to optimise designs and to predict long term system viability, a better understanding of the processes governing nitrogen fate in these novel greywater systems is necessary (the authors recently found that phosphorus retention in greywater living walls is governed by direct plant uptake [7]). Drawing from studies of other vegetated water treatment systems, e.g., wetland systems, it is hypothesised that N retention mechanisms are possibly restricted to denitrification, plant assimilation and to a lesser extent ammonium (NH$_4^+$) adsorption and organic N burial [8]. Assimilation was found to be the dominant nitrate (NO$_3^-$) removal pathway in comparison to denitrification in laboratory scale stormwater biofilters [9]. In vertical flow subsurface wastewater wetlands receiving higher N loads, denitrification is similarly low while plant harvesting and accumulation of organic matter in soil can make slightly greater contributions towards removal [10]. In contrast, coupled nitrification-denitrification has mostly been found to be the dominant process removing N in sub surface horizontal flow wastewater wetland systems (e.g., [11]).

However, a living wall that is a form of biofiltration for treatment of either stormwater or greywater differs from wetland systems. Stormwater biofilters are dry most of the time, i.e., they receive inflow only during storm days, while wetland systems are continuously wet systems. Greywater biofilters more closely resemble stormwater biofilters in design; yet, their difference lie in their inflow dynamics and pollutant concentrations. Greywater biofilters will have a higher moisture regime than stormwater biofilters in that inflows are more frequent. Greywater biofilters will also typically receive inflow only when greywater is produced. For instance, for households going to work during the day, the system will only receive inflow in the morning and evening; in this sense greywater biofilters have the opportunity to dry-up and are thus not permanently wet systems compared to wetlands. While we know a lot about stormwater biofilters (with respect to their performance and nitrogen removal mechanisms, e.g., [9]), greywater biofilters have only recently introduced to practice with a very small number of studies available. Since, greywater biofilters receive higher nutrient and organic inflows in contrast to stormwater biofilters, it is not certain whether the same N assimilation processes identified for stormwater systems will continue to dominate N retention. Indeed, it has previously been observed
that the proportion of N denitrified increases with nitrogen loading [9]. Moreover, the main form of N in greywater is typically dissolved organic nitrogen (DON) [3]. To the best of our knowledge, the removal pathway of DON has not previously been investigated in biofiltration systems. DON can be converted to more bioavailable forms, e.g., to NO$_3^-$, which is highly mobile and can easily leach and have more damaging consequences in the environment (e.g., eutrophication, aquatic and biodiversity loss as discussed earlier) if a reliable process to remove this contaminant is absent within the treatment system.

This study investigates, for the first time, the transformation and fate of dissolved N found in greywater in laboratory-scale biofilters planted with ornamental species. The objective of this study was to elucidate on the mechanisms governing dissolved organic nitrogen removal in greywater biofilters by quantifying the extent of nitrification-denitrification occurring within these systems using an isotope tracer. The implications of these for greywater biofilter design are subsequently made.

2. Materials and Methods

2.1. Experimental Set-Up

This study was part of a larger column study which tested the performance of a number of different biofilter designs [6]. Briefly, biofilter columns were constructed from 240 mm PVC pipe and installed in an open air greenhouse with a clear impermeable roof. The columns were filled with 500 mm of washed sand. The outflow pipe was raised to create a permanently saturated zone (SZ) of 440 mm, consisting of a layer of washed sand mixed with 5% by volume of cedar mulch followed by coarse sand and gravel layers (Figure 1a). Three sampling ports were installed within this section to allow collection of SZ pore water. A novel SZ design type was also tested with the aim to improve NO$_3^-$ removal efficiency by facilitating the injection of an external carbon source and hence speed up the rate of denitrification. The novel SZ was constructed from a series of Atlantis panels with a total depth of 160 mm and a porosity of >95% (Figure 1b). It maximises contact between externally supplemented carbon substrate and water as well as facilitates periodic replenishment of substrates. The columns were planted with a variety of species, including native, ornamental, and climbing species [6]. Five replicates per species were tested alongside five non-vegetated controls. Due to resource limitations, a sub-set of columns could be investigated in this isotropic study; this included a native species, a selection of both high and poor performing climbers and ornamental species (as per results obtained from the larger study [6]) and the non-vegetated controls (Table 1). Given that the influence of SZ type on total nitrogen performance was found to be insignificant (see [6]), plant species could be effectively compared with respect to each other across SZ designs.

![Figure 1. Laboratory biofilter configuration: (a) standard saturated zone design; (b) novel saturated zone (NSZ) design.](image)
Table 1. Summary of experimental details.

| Design Variables | Factors |
|------------------|---------|
| **Plant species**| Standard Saturated Zone  
Carex appressa (nv), Canna lilies (om), Phormium (om), Boston Ivy (cl), non-vegetated  
Novel Saturated Zone  
Grape vine (cl), Strelitzia reginae (om) |
| **Greywater application**| Synthetic light greywater applied 5 times per week  
5.9 mg/L TN; 3.5 mg/L TP; 110 mg/L BOD; 36–50 mg/L TOC.  
Tracer added twice in October |
| **Composition and dosing frequency**| 110 mm/d (0.5 pore volume)—normal dosing days  
221 mm/d (1 pore volume)—tracer dosing |
| **Hydraulic loading rate**| 48 h—normal dosing days  
24 h—tracer dosing |
| **Retention time**| Two—May and October |

Note: nv = native; om = ornamental; cl = climber.

The columns were established for a total period of eight months after which they were dosed with synthetic light greywater (representing wash basins, showers and baths discharges) at a loading rate of 110 mm/d (5 L/d) five days per week (Table 1). Washing machine discharges were excluded because the idea was to use the least polluted household greywater stream so as to prevent premature system failure due to plant and soil damage as well as clogging of the living wall biofilter. Details regarding the composition and preparation of the synthetic light greywater as well as the dosing procedure and the rationale behind are given in [6]. Typical nutrient and organic pollutant concentrations are given in Table 1. The majority of the nitrogen occurred as DON (>80%).

Saturated Zone type was not found to be a factor influencing nitrogen removal, enabling comparison of plant species across Saturated Zone types.

2.2. Water Sampling and Analysis

In May, that is, seven months after greywater dosing commenced, SZ pore water was sampled to examine the change in water quality parameters over time before labelled N addition. Water was extracted with the aid of a syringe and needle from 350 mm (port S1; Figure 1a) and 110 mm from the bottom of the standard design (port S2; Figure 1a) (corresponding to the middle of the first half and second half of the SZ layer respectively) and from 70 mm from the bottom of the novel SZ design (port S4; corresponding to the middle of the SZ layer). During this event, columns were dosed with 5 L of synthetic greywater per column (corresponding to approximately half SZ pore volume) as per normal operation. As the columns finished draining, a pore water sample was collected for analysis of total dissolved nitrogen (TDN), NH\textsubscript{4}\textsuperscript{+}, oxidised nitrogen (NO\textsubscript{x}), and N\textsubscript{2}. A sub-sample was filtered using a 0.45 µm filter and frozen for analysis of TDN, NH\textsubscript{4}\textsuperscript{+}, and NO\textsubscript{x}. Dissolved oxygen (DO) in the sample was measured using a HQ40d multi-parameter probe (Hach, Loveland, CO, USA). The sampling technique was verified in the laboratory to ensure accuracy of DO values in the measured samples. This was carried out by sampling anoxic water of average DO concentration of 0.6 ± 0.1 mgO\textsubscript{2}/L using a syringe and plastic tubing. After sampling, DO concentrations were measured to be 1.4 ± 0.3 mgO\textsubscript{2}/L. This increase was factored when analysing DO data in the measured samples. A total of four sets of samples were collected over a period of 48 h following this sampling regime. Since the volume of the influent was 0.5 × pore volume of the SZ layer and the hydraulic residence time is approximately 48 h, it is expected that all this water would be flushed from the system after two consecutive dosings. The same cycle will repeat itself after the next dosing and hence SZ pore water was monitored for a total period of 48 h.

At approximately 12 months of operation (October), the influent was enriched with \textsuperscript{15}N-urea (>98% purity). The ratio of \textsuperscript{14}N-urea: \textsuperscript{15}N-urea was 0.4:0.6. Influent samples were collected before and after tracer addition. Before tracer addition, pore water samples were collected and analysed for background concentration of N\textsubscript{2}. The dosing volume changed to 10 L per day for the standard column...
designs and 8 L for the novel column designs. This volume corresponded to 1 SZ pore volume (24 h theoretical retention time) compared to previously to reduce any mixing effect of enriched influent with previous non-enriched influent during water retention in the SZ. Pore water from columns with the standard design were, this time, sampled from a port installed 65 mm from the bottom (port S3 in Figure 1a, located in the gravel layer since sampling from the other ports without introducing significant amount of air in the sample was found to be challenging). Sodium acetate (350 mg) as carbon source was added in the novel SZ biofilter designs immediately after the columns finished draining. Samples were collected 6 to 7 h following dosing (after columns finished draining), measured for DO and processed for nutrient analysis as noted before. For analysis of N₂ concentrations, a sub-sample was transferred into a 12.5 mL glass gas-tight vial to which zinc chloride (250 µL, 50% w/v) was added to preserve the sample. A total of six sets of pore water samples were collected over a period of 48 h, whereby the columns were dosed twice (every 24 h) with the tracer. It was hypothesised that during the first tracer dosing the ¹⁵N-urea would be retained in the upper layer through physical and adsorption processes while in the second tracer dosing, any transformed ¹⁵N urea (to NOₓ) would be carried into the lower SZ layer for denitrification and N₂ production. Effluent samples from each column were collected on a regular dosing day two weeks after as outlined in [6] and analysed for TN, TDN, NH₄⁺, and NOₓ.

Nutrient concentrations in the water samples were analysed in a NATA (National Association of Testing Authorities, http://www.nata.asn.au) accredited laboratory (Water Studies Centre analytical laboratory) using standard flow injection analysis methods. Dissolved ²⁸N₂, ²⁹N₂, and ³⁰N₂ were measured in the water samples after a 4 mL helium headspace was placed in the vials. N₂ was analysed on an ANCA GSL2 elemental analyser (Sercon, Crewe, UK) followed by a Hydra 20–22 isotope ratio mass-spectrometer (Sercon, Crewe, UK).

2.3. Data Analysis

Effluent PON, DON, NH₄⁺, and NOₓ concentrations were compared across designs. One-way ANOVA was used to detect differences in effluent concentrations across treatments. Data was tested for normality using the Shapiro-Wilk test. When the data deviated from normality, the non-parametric Kruskal-Wallis test was used to verify results. Significance was taken at α = 0.05. Pore water DON, NH₄⁺, and NOₓ concentrations were plotted for each treatment to examine influent DON transformations and evolution over time. The peak in ²⁸N₂ and ³⁰N₂ production occurred 6 h following the second tracer addition (30 h following the first tracer addition). At this point, it can be assumed that all denitrification had occurred. The amount of ¹⁵N denitrified was, thus, calculated from the production of ²⁹N₂ and ³⁰N₂ and the ratio of ¹⁴N-urea/¹⁵N-urea. The rate of gas loss was calculated to be insignificant compared to the production rate (<10%).

3. Results and Discussion

3.1. N Speciation of Biofilter Effluents

The concentrations and proportion of N species in the effluent of the different biofilter treatments are given in Figure 2. The influent N was mostly in dissolved organic form (66%) while dissolved inorganic nitrogen (DIN) made up only 7% of TN. Treatments corresponding to high TDN retention, namely, Carex appressa, Canna lilies, and Boston Ivy, had low amounts of DIN in their effluent (0%, 10% and 30% of effluent TN respectively). In contrast, treatments corresponding to low TDN retention, namely, Phormium and non-vegetated control had elevated DIN concentrations, in particular NOₓ, in their effluent (93% and 71% of effluent TN respectively). PON and DON effluent concentrations did not vary significantly (p > 0.05) across designs, ranging between 0.20–0.37 mgN/L (except for grape vine, PON = 0.08 mgN/L) and 0.13–0.43 mgN/L (except for Phormium, DON was <0.01 mgN/L) respectively. PON and DON concentrations in the effluent may essentially represent background concentrations, internally generated by residuals and biofilter return fluxes. For example, background organic nitrogen
concentrations in the range of 0.5–2.0 mgN/L are typically reported in wetland systems [12]. Hence, the influent PON and DON associated with greywater, which makes up >90% of the influent TN, are effectively retained or transformed to other N forms in the biofilter. PON is primarily retained through sedimentation processes, although a portion of the PON can be subsequently nitrified to NO$_3^-$ during inter-event periods [13]. DON undergoes mineralisation to NH$_4^+$ likely in the upper filter layers (oxygenated zone) where mineralisation rates are fastest [8]. Although there was a significant difference in effluent NH$_4^+$ concentrations across designs ($p < 0.001$), they were relatively low (between 0 and 0.35 mgN/L). This indicates that processes that remove NH$_4^+$ from solution, such as nitrification to NO$_x$, plant and microbial assimilation, and media adsorption are occurring favourably within the biofilter environment. High NH$_4^+$ removal has been similarly recorded in stormwater biofilters and vertical sub-surface flow wetland systems which operate under similar mechanisms, being a result of the oxygenated environment imposed in these systems [14–16]. However, not all of the mineralised NH$_4^+$ is assimilated or nitrified in the standard biofilter designs during inter-dosing events. Untransformed (or unassimilated) NH$_4^+$ sorbed on the media surface is partly released on subsequent dosing events [12] which could explain the small amounts of NH$_4^+$ observed in the effluent (Figure 2). NH$_4^+$ release from subsequent microbial biomass turnover could also be contributing to NH$_4^+$ levels in the media. Indeed, mineralisation proceeds more rapidly than nitrification [8] and creates the potential for NH$_4^+$ to build up with nowhere to go.

Figure 2. Cont.
On the other hand, effluent NO\textsubscript{x} concentrations were relatively higher and more variable across plant species, ranging between 0 and 3.58 mgN/L. There could be several reasons behind this variation as discussed in the following sections.

While the SZ design type has been found to not be a significant factor in influencing nitrogen removal in these systems \cite{6}, it influences nitrogen speciation in the effluent. The novel SZ experiences a greater degree of alternating oxic and anoxic conditions (with/without external C additions) which allows nitrification to proceed, leading to zero NH\textsubscript{4}\textsuperscript{+} in effluent and higher NO\textsubscript{x} levels (both in effective—grape vine and less effective—\textit{S. Reginae} species). The pipe used for external C additions may have a role to play in increasing DO content in the NSZ. In contrast, owing to more anoxic conditions in the standard SZ, little nitrification of NH\textsubscript{4}\textsuperscript{+} desorbed from upper layer leads to comparatively higher NH\textsubscript{4}\textsuperscript{+} concentration in the effluent.

### 3.2. DON, NH\textsubscript{4}\textsuperscript{+}, and NO\textsubscript{x} Time Series

In May, the columns were dosed with half SZ pore volume (replicating normal dosing as opposed to 1 pore volume in the October tracer tests). DON, NH\textsubscript{4}\textsuperscript{+}, and NO\textsubscript{x} concentrations in the pore water at a given time (6 to 48 h after dosing) were not significantly different between the upper and lower SZ sections (i.e., between ports S1 and S2 Figure 1; \( p > 0.05 \)) except for non-vegetated columns where NO\textsubscript{x} production was observed in the pore water of the upper SZ section (Figure S2, supporting documentation). Excluding the non-vegetated controls, profiles followed similar trends to October sampling over time.

During \textsuperscript{15}N tracer testing in October, concentrations of DON in the SZ pore water (Figure 3) were low with respect to influent DON (3.9 mgN/L) from the first sampling itself (i.e., after approximately six h since dosing) and were comparable to effluent DON concentrations, confirming that DON is likely retained in the upper filter layer where it undergoes transformation to other forms. Concentrations of NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{x} in the pore water varied significantly across plant species and SZ design (\( p < 0.001 \) and \( p < 0.05 \), respectively). NH\textsubscript{4}\textsuperscript{+} concentrations were near zero (<0.01 mgN/L) in the columns planted with \textit{Carex appressa} or equipped with a NSZ design (grape vine and \textit{Strelitzia reginae}) while they were between 0.09 mgN/L (Canna) and 0.33 mgN/L (Boston Ivy) 6 h after dosing (first tracer dosing) and did not show a significant change over time until the next dosing event. Yet, these values are an order of magnitude smaller than the influent TDN concentrations, implying effective NH\textsubscript{4}\textsuperscript{+} removal by the biofilter designs.
Figure 3. Variation of concentrations of DON, NH$_4^+$, and NO$_x$ in saturated zone pore water over time in October 2015: (a) Carex appressa; (b) Canna lilies; (c) Boston Ivy; (d) Phormium; (e) Non-vegetated control; (f) Grape vine and (g) Strelitzia Reginae. Red arrow denotes dosing at 0 h and 24 h. Columns planted with grape vine and S. Reginae (novel SZ design) were supplemented with sodium acetate as external carbon source.
NO\textsubscript{x} concentrations in the order of <0.01 mgN/L were measured in the pore water of columns planted with Carex appressa, Canna lilies, Boston Ivy, and grape vine (Figure 3); these columns also achieved high TDN removal efficiencies. The others demonstrated a general decline in NO\textsubscript{x} concentration with time. Low or absence of NO\textsubscript{x} in the SZ pore water of effective species (i.e., those with high TDN removal) suggests that assimilation may be dominating NH\textsubscript{4}\textsuperscript{+} processing at the detriment of nitrification (conversion of NH\textsubscript{4}\textsuperscript{+} to NO\textsubscript{x}). Indeed, nitrification rates is known to increase only when the NH\textsubscript{4}\textsuperscript{+} supply exceeds plant and heterotroph demand since nitrifiers are relatively poor competitors for NH\textsubscript{4}\textsuperscript{+} in the soil solution [17]. Similarly, a greenhouse study involving grassland plant species found that plants were more effective in acquiring added inorganic N than were soil microbes [18]. It is highly likely that after plant and heterotroph demand is met, left-over NH\textsubscript{4}\textsuperscript{+} adsorbed on the media surface gets nitrified during the next dosing event (during which time the upper filter layer is re-oxygenated). In the absence of subsequent adequate assimilation or denitrification, produced NO\textsubscript{x}, being relatively more mobile, leaches out in the subsequent dosing event and the effluent becomes enriched with NO\textsubscript{x} as evidenced by elevated NO\textsubscript{x} levels in the effluent of designs with poor TDN removal (Figure 2). NO\textsubscript{x} concentrations in the pore water measured six h following dosing in the presence of less effective species (i.e., those with low TDN removal) were, however, 33- (Phormium) to 3-fold (non-vegetated) lower than the influent TDN concentrations (Figure 3). What could have caused the increase in effluent NO\textsubscript{x} levels despite relatively low concentrations in the SZ pore water? As previously observed in stormwater biofilters in similar settings, the effluent water is likely a mixture of “old” resident water and “new” water [19]. During normal operation, the theoretical water residence time in the system is 48 h during which time the majority of denitrification losses is expected to occur. However, due to preferential flow paths, it can be speculated that the water residence time in the system is reduced; hence the more mobile NO\textsubscript{x} (flushed from the upper filter layer) does not possess sufficient contact time with denitrifying bacteria for its removal from solution. This could explain the elevated levels of nitrified NO\textsubscript{x} (in the absence of effective assimilation in the upper filter layer in between dosing events) in the effluent of columns planted with Phormium, Strelitzia reginae, and non-vegetated columns (and lack of denitrification—see further).

NO\textsubscript{x} concentrations in the pore water of columns planted with Strelitzia reginae (novel SZ design) remained unchanged at 0.36 mgN/L without acetate addition in May (Figure S2, supporting documentation). Addition of acetate in the SZ of these columns in October tracer tests effectively caused a rapid decrease in NO\textsubscript{x} concentration to <0.01 mgN/L within 12 h (1st dosing) and 6 h (2nd dosing) of dosing (Figure 3) (although this was not attributed to be due to denitrification as per the tracer results, see the following section). Average concentrations of DO measured in the SZ varied between 1.1 and 2.3 mg\textsubscript{O}\textsubscript{2}/L for the standard designs and between 1.2 and 2.9 mg\textsubscript{O}\textsubscript{2}/L for the novel designs. The sampling technique introduced a small amount of oxygen (up to 1.3 mg\textsubscript{O}\textsubscript{2}/L); given this, it is likely anoxic conditions are prevalent in the SZ. Concentrations were stable over time. Vegetated columns had lower concentrations of DO than non-vegetated columns.

3.3. Extent of Nitrification-Denitrification

Concentrations of \textsuperscript{29}N\textsubscript{2} and \textsuperscript{30}N\textsubscript{2} were negligible in the first 24 h following the first dosing with the \textsuperscript{15}N tracer but increased sharply following the second tracer application and thereafter stabilised in the next 20 h (Figure 4). The fact that no gas was measured in the SZ in the first dosing with the tracer but a sharp increase was found within 6 h of the next tracer dosing further confirms our initial hypothesis that incoming DON gets trapped in the upper layers, is rapidly mineralised to NH\textsubscript{4}\textsuperscript{+}, which is nitrified to NO\textsubscript{x}. Produced NO\textsubscript{x} is transported to the lower SZ layers during the next dosing event, where denitrification occurs (as the SZ presents more conducive conditions for the process). The tracer results show that the combined process of mineralisation-nitrification-denitrification can occur quite rapidly within a span of 30 h. There might, nevertheless, be a delay in response in any of these steps which could signify that denitrification of N associated with a particular dosing event could be happening over several dosing events over a longer time period. For instance, as [11] noted, sediment
storage could represent a certain proportion of N storage, uncertainty about the long-term fate of this N could mean that denitrification losses are being underestimated. However, in this early biofilter stage, the system was relatively new with comparatively very little sediment accumulation; hence, sediment storage is likely to be minor.

The amount of $^{15}$N loss through nitrification-denitrification accounted for a minor removal pathway in the planted columns, ranging between 0% and 15% of added $^{15}$N (Figure 5). The non-vegetated columns had a greater amount of $^{15}$N that was denitrified (up to 21% of added $^{15}$N). The amount of $^{15}$N denitrified decreased in the treatments with increasing TDN removal efficiency. Low nitrification-denitrification observed in the presence of plant species that showed low NH$_4^+$ and NO$_x$ levels in their effluent partly confirms that assimilation may be the dominating mechanism of DIN retention in these systems. Adsorption is believed to not be a significant NH$_4^+$ retention mechanism in the long term since sand has low cation exchange capacities [20]. The extent of nitrification-denitrification increased in the presence of less effective species and non-vegetated control but was minor and most probably not high enough for complete NO$_x$ reduction. This was also noted by [9] in their laboratory stormwater biofilter study tracing NO$_3^-$ removal pathways.

![Figure 4](image_url)  
**Figure 4.** Evolution of excess $^{29}$N$_2$ and $^{30}$N$_2$ following dosing with $^{15}$N-urea at t = 0 h and t = 24 h in laboratory columns planted with (a) Phormium and (b) non-vegetated columns.

![Figure 5](image_url)  
**Figure 5.** Amount of $^{15}$N denitrified 30 h following first tracer ($^{15}$N-urea) dosing in the different designs calculated as a percentage of added $^{15}$N.
Nitrification rates are, nevertheless, potentially higher than denitrification rates in these systems. TDN removal is limited by insufficient denitrification rates, as more evidently illustrated by the non-vegetated control performance (Figure 2). A previous study found that influent greywater possesses enough BOD to generate satisfactory denitrification rates under anoxic conditions [21]. In this case, it is very likely that the BOD is biologically degraded in the upper filter layer (the first point of contact and an oxygenated zone) with biological degradation rates known to proceed faster under aerobic than anoxic conditions. Hence, although carbon availability may be high in upper filter layers, it is low in lower layers, which could have restricted denitrification in the submerged zone where more conducive conditions for the process (e.g., anoxia) is present. Carbon derived from the woodchips may not be sufficiently biodegradable to engender high denitrification rates within the short time period that NO\textsubscript{X} is in the SZ before being flushed out of the system. In the long-term, as the plants become more established, carbon substrates from plant root exudates [22–24] may satisfy requirements of denitrifying bacteria which could potentially augment denitrification rates [9], particularly in biofilter planted with species having a poorer nutrient demand.

Columns with external C additions (e.g., Strelitzia reginae) demonstrated similar low nitrification-denitrification. Strelitzia reginae is likely a poor performer, as seen by the elevated NO\textsubscript{X} effluent concentrations in columns planted with this species (in the absence of C addition). However, the low proportion denitrified in the presence of a carbon substrate (acetate) suggests that microbial immobilisation may be dominating NO\textsubscript{X} reduction; although effluent concentrations were not measured during C additions, the rapid decline in NO\textsubscript{X} concentration in the SZ pore water (Figure 3) compared to the unchanged NO\textsubscript{X} in the absence of C additions (Figure S2, supplementary material) suggests low effluent NO\textsubscript{X}. DO in the novel SZ was around 4 mg O\textsubscript{2}/L while the columns were still draining and decreased to around 1 mgO\textsubscript{2}/L 3 h after columns finished draining (results not shown; acetate was added soon after columns finished draining). When acetate was added, heterotrophic microbes may have augmented NO\textsubscript{3}− immobilisation while anoxic conditions were still being established.

3.4. Implications for Living Wall Design

The results of this study suggest several design implications. Plants played a vital role in capturing DIN in these systems and reducing nitrogen release, however, some species were more effective than others. Hence, plant selection as a design criterion should perhaps be given high importance particularly for system successful performance during its start-up phase. Plant traits related to resource capture such as root proliferation, root length density and specific root length [18] are likely one of the important factors to be considered when selecting species. Plants relative growth rate is also important [25]. Ornamental species (e.g., Canna lilies or Boston Ivy) demonstrated great potential for use in these systems. It should also be noted that nitrification and denitrification rates may increase over time as plants nutrient uptake diminish once plants are past their active growth phase [9]. In lightly loaded wetland systems, BOD of the influent as well as the organic exudates from plant roots can satisfy carbon demand of denitrifying bacteria for denitrification [12,26]. While the BOD in the greywater is degraded in the upper filter layer and is not directly available to denitrifying bacteria in the SZ as discussed previously, future studies should be conducted on mature greywater biofilters to verify whether assimilation continues to be a prominent N removal pathway and whether carbon exudates is sufficient for sustainable denitrification rates or an additional carbon substrate need to be provided. Since most of the N is transformed to NO\textsubscript{X}, this is a matter of concern since it is relatively more mobile than DON or NH\textsubscript{4}+ and can pose a greater risk in the environment. For example, in the presence of low groundwater aquifers, it can lead to aquifer pollution. If strict removal target needs to be met for N, an external substrate with the capacity to promote rapid denitrification is required since the residence time of NO\textsubscript{3}− may be lower than expected as a result of preferential flow paths.
Future work should trace N₂ loss over a longer time period for a more precise account of N removal caused by denitrification losses in these systems. The amount of N stored in the media and associated organic matter and in plant biomass should be determined for an estimation of the amount of retained N that could potentially be permanently removed from the system through infrequent plant harvests.

4. Conclusions

The extent of DON removal via the biological process of nitrification-denitrification in greywater living wall biofilters were for the first time examined. Most of nitrogen removal, primarily present as DON, took place in the upper filter layers and quite rapidly as the incoming water percolated downwards. Nitrification-denitrification represented a minor dissolved N removal pathway, highlighting the importance of adsorption and assimilation as dominant N retention processes in greywater biofilters at least in their early years of operation. The results indicate that nitrification-denitrification increases after plant nutrient demand is satisfied. Mineralisation of DON primarily present in influent greywater was effective. Nitrification rates were higher than denitrification rates, which were quite limited across biofilter designs. Consequently, in designs with poor N removal, the effluent is enriched with NOₓ which is relatively more mobile; this has significant implications for surrounding aquatic ecosystems. From the results of this study, it can be inferred that plant selection plays a critical role in nitrogen removal in these systems. To ascertain the importance of plants in these systems, future research should look into conducting a mass balance to investigate on the proportion of N stored in plant biomass and retained onto the filter media, Measures to promote rapid denitrification rates in these systems—for instance, by providing an effective carbon substrate—could represent an alternative to improve the N removal capacity over the long term as plant N storage may diminish with time in the event of sustained greywater loadings.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/10/4/527/s1. Figure S1. Example of living wall system located in Melbourne, Australia (left) and schematic of the system for greywater recycling (right); Figure S2. Variation of concentrations of DON, NH₄⁺, and NOₓ as a function of time in saturated zone pore water sampled from upper (continuous line) and lower (broken line) ports in May. Dosing occurred at time t = 0 h.

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