Aerobic nitrification and denitrification among heterotrophic bacterial isolates

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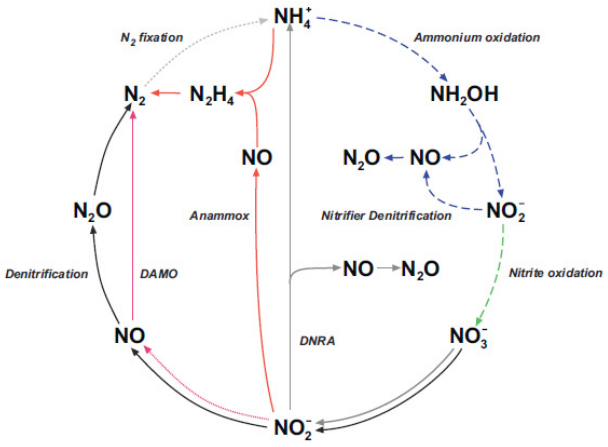
Abstract

In addition to being an integral part of the planet's nitrogen cycle, bacterially facilitated nitrification is a process of great commercial importance in the wastewater treatment, aquaculture and agronomy industries. Twelve distinct species of heterotrophic bacteria, belonging to the genera *Bacillus, Pediococcus* and *Lactobacillus*, were isolated from BiOWiSH's commercially available water treatment products (Aqua, Aqua FOG, AquaFarm). These isolates were then examined for aerobic nitrification activity using colorimetric analysis of ammonia (via a modified Berthelot reaction), nitrite and nitrate (via a modified Griess reaction). Rates of aerobic ammonia degradation ranged from 0.927 ppm h^{-1} to 5.89 ppm h^{-1} in wastewater reactor flasks. The use of heterotrophic, aerobic bacteria in water treatment systems may present economic advantages as well as novel biological management of undesirable nitrogenous intermediates.

Introduction

The biological removal of inorganic nitrogenous compounds, such as ammonia (NH_4^+) and nitrate (NO_3^-) from aquatic systems has long been a topic of interest for wastewater engineers and other water treatment professionals. These compounds contribute to eutrophication and are toxic to many aquatic organisms; therefore their presence in treated wastewater and in clean water systems, such as ponds, lakes, and reservoirs, is undesirable (Shannon *et al*, 2008). In the past, combinations of autotrophic nitrifying and denitrifying bacteria (which convert NH_4^+ to N_2, with NO_3^- as an intermediate) were believed to be the only method for effecting such remediation. However, the discovery of novel metabolic pathways among several bacterial taxa during the latter part of the 20th century forced a reevaluation of this paradigm (Schmidt *et al*, 1987).



Scheme 1 – Diagram of the microbial nitrogen cycle which visualizes the various metabolic pathways through which bacteria convert NH_4^+ to N_2. Dashed lines indicate pathways generally carried out in the presence of oxygen, whereas solid lines indicate pathways generally believed to be anoxic (Schreiber, 2009).

Nitrifying and denitrifying bacteria are an integral part of the planet's Nitrogen Cycle. Three main types of bacteria catalyze the conversions shown above. Ammonia oxidizing bacteria (AOBs) are aerobic chemolithoautotrophs belonging to the phylum Proteobacteria, which contains species such as *Nitrosomonas, Nitrosococcus*, and *Nitrospira* (Koops and Pommererening-Röser, 2001). These convert ammonia (NH_4^+) into hydroxylamine (NH_2OH) through the action of ammonia monooxygenase (Equation 1). Hydroxylamine is then converted to nitrite (NO_2^-) by hydroxylamine oxidoreductase (Equation 2). Doubling time for these organisms ranges from 8-24 hours depending on nutrient availability (Hommes *et al*, 2003).

$$NH_3 + O_2 + 2H^+ + 2e^- \longrightarrow NH_2OH + H_2O \qquad (1)$$

$$NH_2OH + H_2O \longrightarrow NO_2^- + 5H^+ + 4e^- \qquad (2)$$

A second group of Proteobacteria, called nitrite oxidizing bacteria (NOBs), then converts nitrite into nitrate (Equation 3) with the enzyme nitrite oxidoreductase (Prosser, 1989). These are also aerobic chemolithoautotrophs, among the most common being members of the genus *Nitrobacter*. These organisms have a maximum doubling time of 20 hours (Tramper and Grootjen, 1986).

$$1.\ NO_2^- + H_2O \longrightarrow NO_3^- + 2H^+ + 2e^- \qquad (3)$$

Nitrate is then converted into N_2 in a process called denitrification (Equation 4), which was long believed to be limited to bacteria such as *Thiosphaera, Paracoccus* and *Pseudomonas* and to eukaryotes such as algae and fungi (Shapleigh, 2006). However, recent studies have found that members of the genus *Bacillus* (heterotrophic organisms of the phylum Firmicutes) can perform denitrification as well (Verbaendert, 2011). During denitrification, nitrate is substituted for oxygen as a terminal electron acceptor; therefore, because oxygen is an energetically preferable electron acceptor, denitrification generally occurs in anoxic environments. As shown in Scheme 1, nitrate is converted to nitrite before being ultimately converted to N_2.

$$2\ NO_3^- + 10\ e^- + 12\ H^+ \longrightarrow N_2 + 6\ H_2O \qquad (4)$$

The discovery of anaerobic ammonia oxidizers, collectively referred to as "anammox" bacteria, of the phylum Planctomycetes and belonging to genera such as *Brocadia* provided a new method for remediating inorganic nitrogenous compounds in wastewater (Strous *et al*, 1999). Organisms such as *B. anammoxidans* carry out denitrification of nitrite, using ammonia as an electron donor, with H_2O and N_2 as end products (Equation 5). Though their metabolism of ammonia was seen as quite novel, these bacteria are notoriously slow growing (doubling time approaches 11 days) and their anaerobic ammonia metabolism is completely, albeit reversibly, inhibited by oxygen at concentrations as low as 2μM (Jetten *et al*, 2001).

$$NH_4^+ + NO_2^- \longrightarrow N_2 + 2H_2O \qquad (5)$$

Practical applications of these bacterial systems are numerous. In Partial Nitrification reactors (Hellinga *et al*, 1998), AOBs are utilized to convert ammonia into nitrite. Rather than allowing the nitrite to be converted to nitrate by NOBs (which must be inhibited in these systems through temperature and pH controls) the nitrite enriched wastewater is instead added to a denitrification reactor and converted to N_2 by denitrifying bacteria. This allows the denitrifying bacteria to conserve energy, as they do not need to derive their NO_2^- from NO_3^-. The Partial Denitrification process can also be coupled with an anammox reactor in a process known as SHARON (single reactor system for high activity ammonium removal over nitrite), which allows the anammox Planctomycetes to utilize both NH_4^+ and NO_2^- to effect denitrification (Hellinga *et al*, 1998). Canon (completely autotrophic nitrogen removal over nitrite) reactors employ aerobic nitrifying bacteria from the phylum Proteobacteria for nitrification and anaerobic Planctomycetes for denitrification (Third *et al*, 2001). Aerobic AOBs oxidize NH_4^+ to NO_2^- while consuming oxygen, which creates an anoxic environment in which anammox bacteria can thrive. In addition to being hindered by the extended startup times of Planctomycetes, this system is prone to a buildup of NO_2^- in the presence of excess O_2. Finally, NO_x processes (Bock *et al* 1996) involve augmenting cultures of aerobic Proteobacteria such as *Nitrosomonas* with nitrogen oxides, which stimulates the bacteria to perform nitrification and denitrification concurrently (Bock *et al*, 1996).

Heterotrophic nitrification involves the conversion of NH_4^+ to NO_2^- by heterotrophic bacteria which, unlike the autotrophic *Nitrosomonas*, rely on organic compounds as a carbon and energy source (Schreiber, 2009). Though known to take place among some bacteria such as *Thiosphaera pantotropha* and some species in the genus *Pseudomonas*, rates of nitrification and denitrification were observed to be slower among heterotrophs (Schmidt *et al,* 2003). Therefore, autotrophs were viewed as superior organisms for remediating inorganic nitrogenous compounds in wastewater. However, Kim *et al* (2005) observed aerobic nitrification and denitrification among several strains of *Bacillus* (phylum Firmicutes) at higher rates than had been observed previously among heterotrophs. Nitrogen balance revealed that some ammonia nitrogen had been completely lost from the system, presumably as N_2. This suggested a less complicated metabolic pathway among *Bacillus* than exists among Proteobacteria and Planctomycetes, as well as a potential alternative to the current nitrification and denitrification systems dominated by autotrophs.

Bacteria of the genus *Bacillus* offer a number of potential advantages over members of the phyla Proteobacteria and Planctomycetes. As endospore formers, suspensions are hardier than preparations of vegetative cells and can remain viable under a wider range of environmental conditions. Additionally, while Proteobacteria such as *Nitrosomonas* and *Nitrobacter* have doubling times of 8-24 hours and anammox species have doubling times in excess of seven days, members of the genus *Bacillus* have doubling times as low as 40 minutes under optimal conditions (Hageman *et al*, 1984). Thus, these bacteria may offer several economic advantages over their more common wastewater treatment counterparts.

BiOWiSH's water treatment products (Aqua, Aqua FOG, AquaFarm) comprise a proprietary mixture of bacteria in the phylum Firmicutes and belonging to the genera *Bacillus*, *Pediococcus* and *Lactobacillus*, formulated to enhance carbon degradation in aqueous environments. Quantitative data collected during subsequent field applications revealed these products to be effective at removing ammonia and nitrates from aquatic systems under aerobic conditions. In the present study we examined twelve different bacterial isolates from BiOWiSH's water treatment products for aerobic nitrification activity, including nitrite and nitrate production, in order to determine the metabolic fate of ammonia for each species. Additionally, we examined four selected species for aerobic denitrification activity. Ammonia, nitrite, and nitrate were determined spectroscopically using a commercially available test kit which utilized a modified Berthelot method (Berthelot, 1859) to assay ammonia and a modified Griess method (Greiss, 1879) to assay nitrite and nitrate. Aerobic nitrification was observed among two heterotrophic genera, *Bacillus* and *Pediococcus*, in a minimal DI water medium and in untreated wastewater. Aerobic denitrification was observed in 3 species of *Bacillus* under identical conditions. A wastewater treatment system utilizing these species, capable of aerobic, heterotrophic nitrification and denitrification, could possess several economic advantages and be of potential interest to water treatment professionals.

Methodology

Chemicals and Materials – Ammonium Phosphate ($NH_4H_2PO_4$; > 98%) and sodium nitrate ($NaNO_3$; > 99%) were purchased from Sigma-Aldrich. Dextrose monohydrate was purchased from My Spice Sage. The dissolved oxygen test kit was purchased directly through LaMotte. API ammonium, nitrite, and nitrate kits were purchased locally.

Bacterial isolates were screened for aerobic nitrification potential in a minimalist deionized (DI) H_2O medium (reactor flasks run in duplicate) and in untreated wastewater collected from a local wastewater treatment plant (reactor flasks run in duplicate). Selected isolates were screened for aerobic denitrification activity under the same conditions. Additionally, unaltered product (BiOWiSH® Aqua) was dosed into duplicate DI H_2O and wastewater reactors to determine the impact of endospore germination upon the product's nitrogen ability to metabolize nitrogen.

Bacterial isolation and identification – Serial dilutions in sterile phosphate buffered saline (PBS) solutions were performed upon the component bacterial formulations that comprise BiOWiSH's water treatment products. Dilution aliquots of 100 µL were dispensed onto plates of trypticase soy agar (TSA), and the plates were incubated for 48 hours at 35°C. Morphologically distinct colonies exhibiting diagnostic characteristics of the genus *Bacillus* were selected and streaked for isolation on TSA. Pure cultures were shipped to Nelson Laboratories (Salt Lake City, UT, USA) and identified to the species level using 16s rDNA analysis. Stock cultures of *Lactobacillus plantarum*, *Pediococcus pentosaceous*, *Pediococcus acidilactici* and one strain of *Bacillus* subtilis (designated "KLB") obtained from BiOWiSH Technologies, which had previously been identified via 16s rDNA analysis, were also screened for nitrification ability.

Preparation of bacterial inoculums – Suspensions of vegetative *Bacillus*, *Lactobacillus* and *Pediococcus* were used to inoculate the reactor flasks. A 100 mL broth culture of each target species (*Bacillus* species grown in trypticase soy broth (TSB), and *Pediococcus* and *Lactobacillus* grown in de Man, Rogosa and Sharpe (MRS) broth) was grown for 24 hours at 35°C and shaken at 150 rpm. After 24 hours broth was withdrawn in 2 mL aliquots, dispensed aseptically into sterile microcentrifuge tubes, and centrifuged at 6,000 x g for 10 minutes to pellet vegetative cells. Nutrient broth supernatant was decanted and pellets were rinsed gently with a sterile PBS solution before being re-suspended in 0.5 mL aliquots of sterile PBS. Re-suspended vegetative cells were added to a vial of 5 mL of a sterile PBS solution until an absorbance of 1.000 at OD600 was achieved. An aliquot of 1.0 mL of this inoculum was then added to each reactor flask.

Preparation of DI water reactor flasks – Kim *et al.* (2005) observed aerobic nitrification and denitrification among *Bacillus* species at carbon doses of 800 – 1,600 mg L^{-1}; therefore, the nitrification DI H_2O flasks were supplemented with 1,000 mg L^{-1} dextrose as the carbon source and ammonia (10 mg L^{-1}) as the nitrogen source using ammonium phosphate. The nitrification DI H_2O flasks for *Pediococcus* and *Lactobacillus* was supplemented with dextrose at 3 g L^{-1} as the carbon source and ammonia (10 mg L^{-1}) as the nitrogen source using ammonium phosphate.1 The denitrification DI H_2O medium was prepared in the same manner, but was supplemented with 25 mg L^{-1} NO_3^- instead of NH_4^+ using sodium nitrate. The medium was dispensed in 150 mL aliquots into 500 mL Erlenmeyer flasks, which were capped with foil and autoclaved at 121°C, 15 psi for 15 minutes. Flasks were stored at 4°C until needed.

Preparation of wastewater reactor flasks – Filtered, untreated wastewater was collected from the Sycamore Creek Wastewater Treatment Plant (Cincinnati, OH, USA) and transported back to the laboratory in a disinfected, capped plastic carboy. For nitrification assays, wastewater was centrifuged at 6,000 x g for ten minutes to remove visible biosolids which may have interfered with spectrophotometric analysis, then filtered using 0.22 µm cellulose acetate membrane filter syringes. Wastewater was then dispensed in 150 mL aliquots into 500 mL Erlenmeyer flasks, which were capped with foil and autoclaved at 121°C, 15 psi for 15 minutes in order to remove any potentially pathogenic enteric bacteria. Flasks were stored at 4°C until needed. The denitrification wastewater medium was prepared in the same manner, but was supplemented with 25 mg L^{-1} NO_3^- (while the medium tested positive for NH_3, no detectable NO_3^- was present).

Sampling of reactor flasks – Inoculums of each bacterial isolate were added to duplicate DI H_2O and wastewater reactor flasks, from which a 15 mL sample (T=0) was immediately, aseptically removed using a sterile serological pipette and stored inside a sterile, screw-capped 15 mL centrifuge tube. Reactor flasks were then placed inside an incubator/shaker set to 40°C (for *Bacillus*) or 35°C (for *Lactobacillus* and *Pediococcus*). Flasks were periodically removed for sampling as described above at hours 2, 3, 4, 5, 6 and 24. Sample tubes were centrifuged at 6,000 x *g* for 10 minutes to remove suspended cells, which may have interfered with spectrophotometric analysis. Following centrifugation, 5 mL aliquots were removed from the tube using an autopipettor and dispensed into 20 mL scintillation vials for colorimetric analysis.

Preparation of BiOWiSH® Aqua Direct Dosing Flasks – Reactor flasks of DI H_2O medium and wastewater medium were prepared as described above and dosed with fully formulated, unaltered BiOWiSH® Aqua at both a high dose (10^9 CFUs) and a low dose (10^6 CFUs). The flasks were incubated at 40°C, 150 rpm for 56 hours and sampled at hours 0, 8, 24, 32, 48 and 56 as described above.

Nitrogen balance – Aliquots of 15mL were removed from duplicate DI H_2O and wastewater reactor flasks of *B. subtilis*, *B. mojavensis* and *B. pumilus* and immediately frozen at T=0, T=6 and T=24. These samples were shipped frozen to Midwest Laboratories (Omaha, NE, USA) for the following analysis using standard methods: Total N, Organic N, Nitrate/Nitrite N, Ammonia N, and Total Kjeldahl N.

Colorimetric Analysis of NH_3, NO_2^-, and NO_3^- – Ammonia, nitrite, and nitrate concentrations were determined colorimetrically using a commercially available test kit (MARS Fishcare). Reactions were performed as indicated by the test kit. 5-7 minutes were allowed for samples to fully react before measuring absorbance. Ammonia was measured using a modified Berthelot reaction method with absorbance calibrated at 696 nm ($y = 3887.8x + 0.0112$; $r^2 = 0.999$). Nitrite and nitrate were measured using a modified Griess reaction method with nitrite absorbance calibrated at 540 nm ($y = 34565x + 0.1404$; $r^2 = 0.998$) and nitrate absorbance calibrated at 546 nm ($y = 1896.7x + 0.0377$; $r^2 = 0.999$). Figures depicting the calibration lines can be found in the Supplemental Information (SI).

Titration of Dissolved Oxygen – A commercial dissolved oxygen test kit (LaMotte) was used as directed to titrate dissolved oxygen within the reactor flasks. These data are approximate trends for qualitative comparisons. Samples were not kept air-tight during reaction flask incubation.

Instrumental – Reactor flask samples (15mL) were centrifuged using a Centra CL3 centrifuge (Thermo). A Galaxy 14D microfuge (VWR) was used to centrifuge bacterial inoculum samples (2.5mL). All absorbance measurements were performed on a DU-520 spectrophotometer (Beckman-Coulter). Sample pH was tested using a HI 9813-6 pH probe (Hanna Instruments) calibrated with a pH 7.0 standard (Hanna Instruments). Reactor flasks were incubated in a Controlled Environment Incubator Shaker (New Brunswick Scientific). Bacterial inoculums were incubated in a Modell 520 Incubator (Memmert). A RCC7 75GPD 5 Stage Reverse Osmosis Water Filter system (iSpring) provided DI water.

Results

Identification of Bacterial Isolates – Characteristics of bacterial isolates, including the results of 16s rDNA analysis, are displayed in Table 1. Isolates from BiOWiSH water treatment products were found to be comprised of *Bacillus subtilis*, *Bacillus mojavensis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus pumilus*. In cases where several isolates were found to be genetically identical, only one isolate from that group was selected for the nitrification assays.

Nitrification assays – Ammonia degradation curves for six of the eight *Bacillus* species tested are displayed in Figure 1, and NO_2^- generation curves for the same species are displayed in Figure 2. The NH_4^+ degradation and NO_2^- generation curves for *Lactobacillus* and *Pediococcus* are displayed in Figures 3 and 4, respectively. With the exception of *Lactobacillus plantarum*, all isolates tested showed aerobic nitrification at high levels of dissolved oxygen (DO > 2.4 ppm) within twenty four hours, with measurable degradation often occurring within three hours. However, many isolates showed differing rates of ammonia degradation between the DI H_2O and wastewater media.

These data are summarized in Table 2. For all species except *B. mojavensis*, colorimetric analysis revealed no *detectable increase in the concentration of NO_3^- upon degradation of NO_2^-.*

Denitrification assays – Four selected species of *Bacillus* (*B. pumilus, B. subtilis*, *B. licheniformis and B. mojavensis*) were screened for aerobic denitrification. Results of the denitrification assays are displayed in Figure 5. *Bacillus pumilus*, *B. licheniformis* and, to a lesser extent, *B. subtilis* (KLB) all showed NO_3^- degradation activity within 24 hours; however, isolates performed differently in DI H_2O medium than in wastewater medium. These trends are summarized in Table 3.

Nitrogen Balance – Results of nitrogen balance assays (Midwest Laboratories, Omaha, NE, USA) for DI H_2O reactor flasks are displayed in Table 4, and for wastewater flasks are displayed in Table 5.

BiOWiSH® Aqua Direct Dosing Flasks – Results for the BiOWiSH® Aqua product showed a lag in the initial ammonia degradation (accounted for by the germination and lag time of endospore forming species), but after the lag phase the degradation of ammonia and the evolution of nitrogenous intermediates compared favorably with data collected in reactor flasks treated with vegetative inoculums (Figure 6).

Isolate	Gram Stain	Colony Morphology	16s rDNA Idnetification	Percent Similarity
B5	+	Cream, raised, irregular, mucoid, wrinkled	*Bacillus licheniformis*	99.37
B6	+	Cream, raised, irregular, mucoid, wrinkled	*Bacillus amyloliquefaciens*	99.65
B8	+	Creamy tan, flat, irregular	*Bacillus mojavensis*	99.34
B9	+	Cream, raised, irregular, smooth	*Bacillus subtilis*	99.93
B10	+	Cream, raised, irregular, mucoid, wrinkled	*Bacillus amyloliquefaciens*	99.11
B11	+	Cream, flat, irregular	*Bacillus licheniformis*	96.00
B12	+	Cream, flat, irregular, wrinkled	*Bacillus pumilus*	100.00
B13	+	Cream, raised, irregular, mucoid, wrinkled	*Bacillus licheniformis*	99.04

Table 1 – Identification of bacterial isolates from BiOWiSH Water Treatment Products. Genetic sequencing (16s rDNA) was performed by Nelson Laboratories (Salt Lake City, UT, USA). BiOWiSH water treatment products are comprised of proprietary bacterial formulations containing Firmicutes of the genera *Bacillus*, *Pediococcus* and *Lactobacillus*.

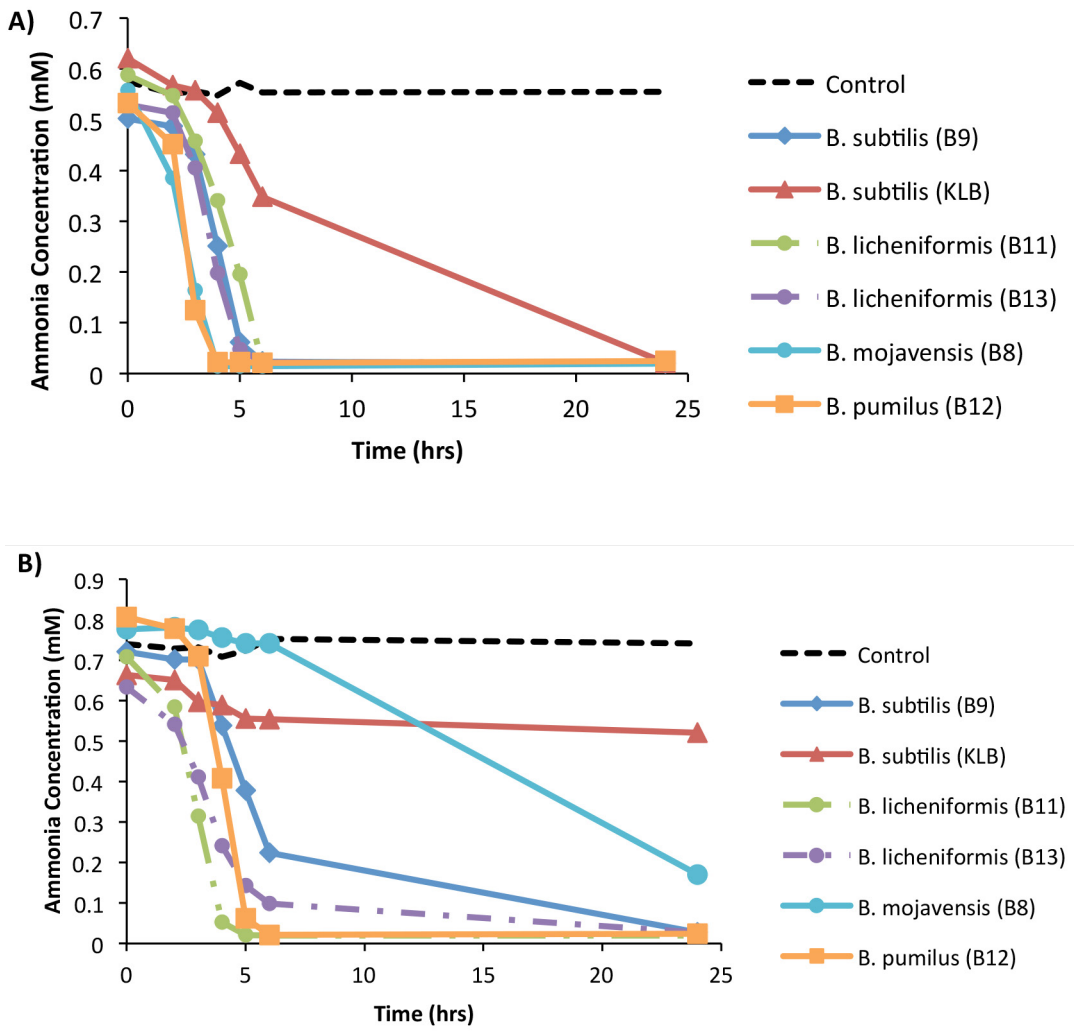


Figure 1 – Disappearance of ammonia over time in the presence of heterotrophic *Bacillus* isolates in A) DI water and B) wastewater reactor flasks.

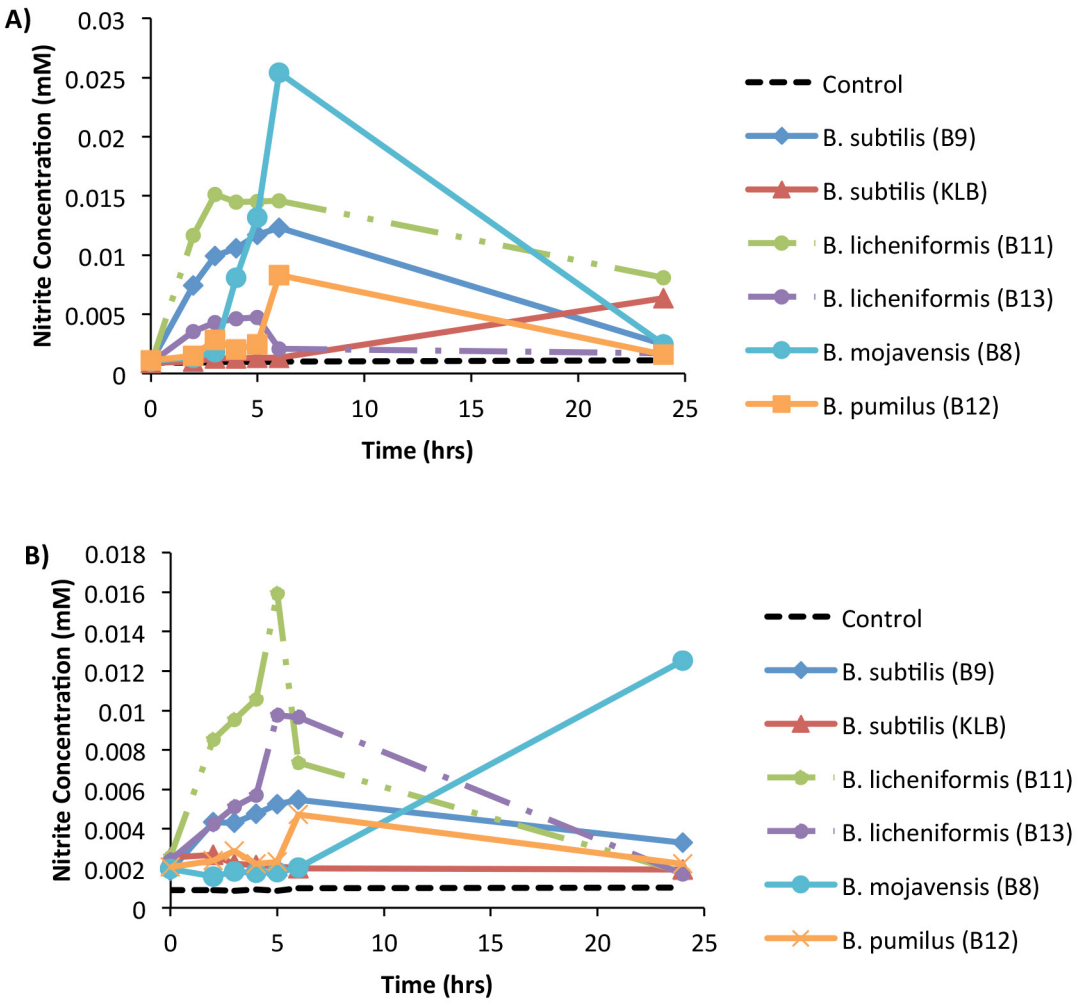


Figure 2 - Appearance of nitrite over time in the presence of heterotrophic *Bacillus* isolates in A) DI water and B) wastewater reactor flasks.

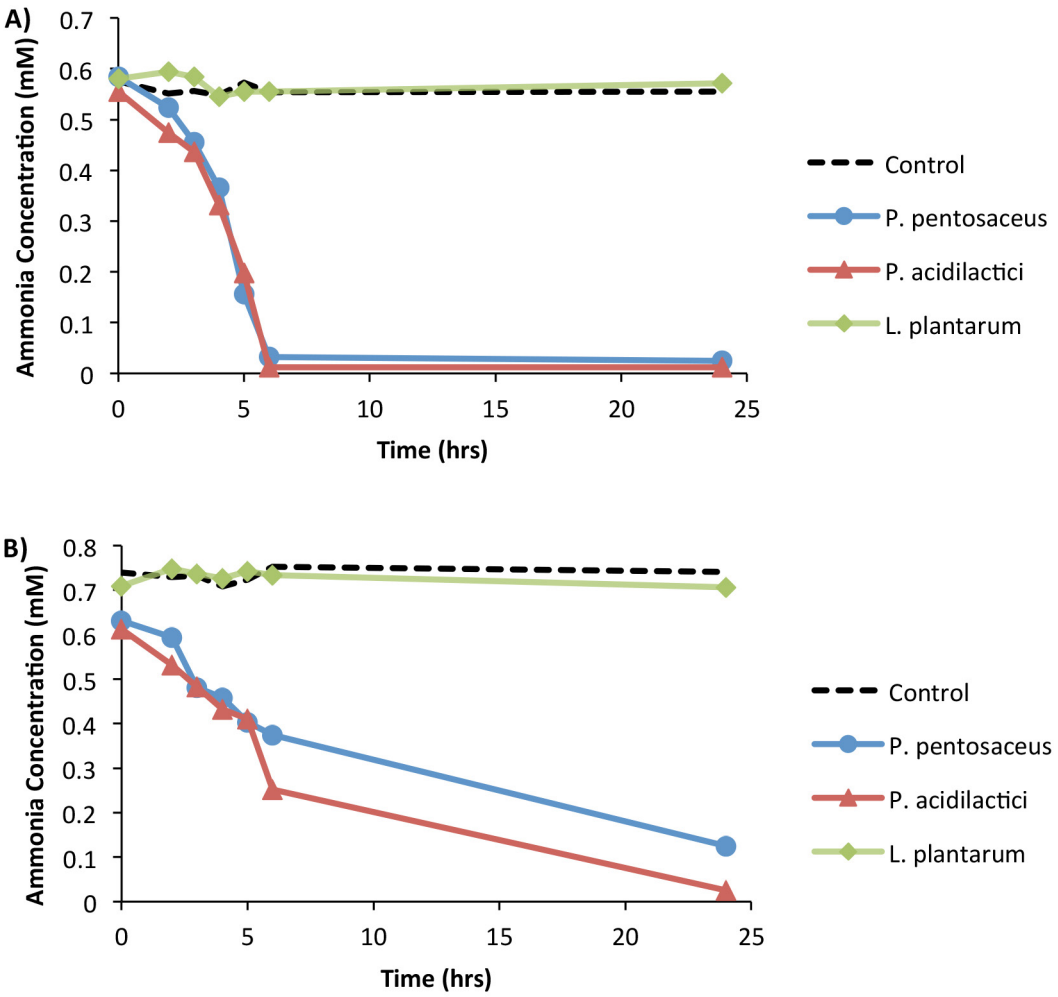


Figure 3 - Disappearance of ammonia over time in the presence of heterotrophic *Lactobacillus* and *Pediococcus* isolates in A) DI water and B) wastewater reactor flasks.

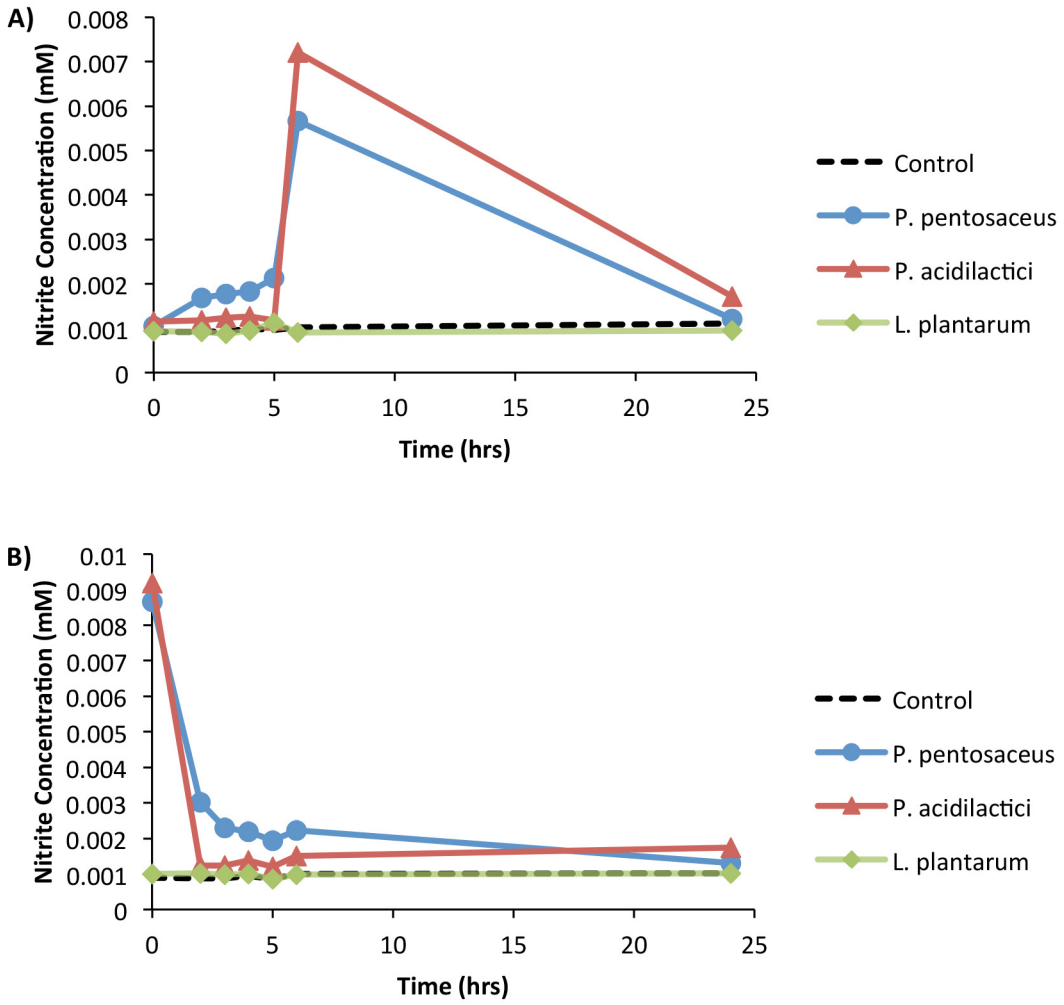


Figure 4 - Appearance of nitrite over time in the presence of heterotrophic *Lactobacillus* and *Pediococcus* isolates in A) DI water and B) wastewater reactor flasks.

Table 2 – Ammonia metabolism in DI H_2O and wastewater reactor flasks. All bacterial isolates with the exception of *L. plantarum* showed aerobic nitrification ability at high levels of dissolved oxygen (DO). The highest rate of degradation in both DI H_2O medium and in wastewater was achieved by *Bacillus pumilus*. Rates of degradation (h^{-1}) are estimations of the first order rate based on a regression analysis of the data in ln(C/CO)/t form.

Isolate	DI Water Reactors			Wastewater Reactors		
	Rate (mM h^{-1})	DO (ppm) T = 0	DO (ppm) T = 24	Rate (mM h^{-1})	DO (ppm) T = 0	DO (ppm) T = 24
B. licheniformis (B5)	0.655	6.6	4.2	0.56	7.9	5.8
B. licheniformis (B11)	0.563	7.2	6.3	0.70	6.7	5.8
B. licheniformis (B13)	0.530	8	6.2	0.69	6.2	5.6
B. amyloliquefaciens (B6)	0.146	8.2	4.2	0.11	5.8	4.4
B. amyloliquefaciens (B10)	0.639	7.9	2.4	0.64	5.5	5
B. subtilis (B9)	0.507	6	5.6	0.14	5	4.8
B. subtilis (KLB)	0.141	6.9	4.6	0.01	7	6.5
B. mojavensis (B8)	0.737	6.1	5.4	0.06	6	3.8
B. pumilus (B12)	0.797	6.1	5.3	0.60	5.9	5.6
P. pentosaceous	0.133	8.4	5.6	0.07	7.1	6.8
P. acidilactici	0.639	7.6	6.5	0.13	6.9	5.4
L. plantarum	0.001	7.8	7.6	2.35E-04	6.3	6.4

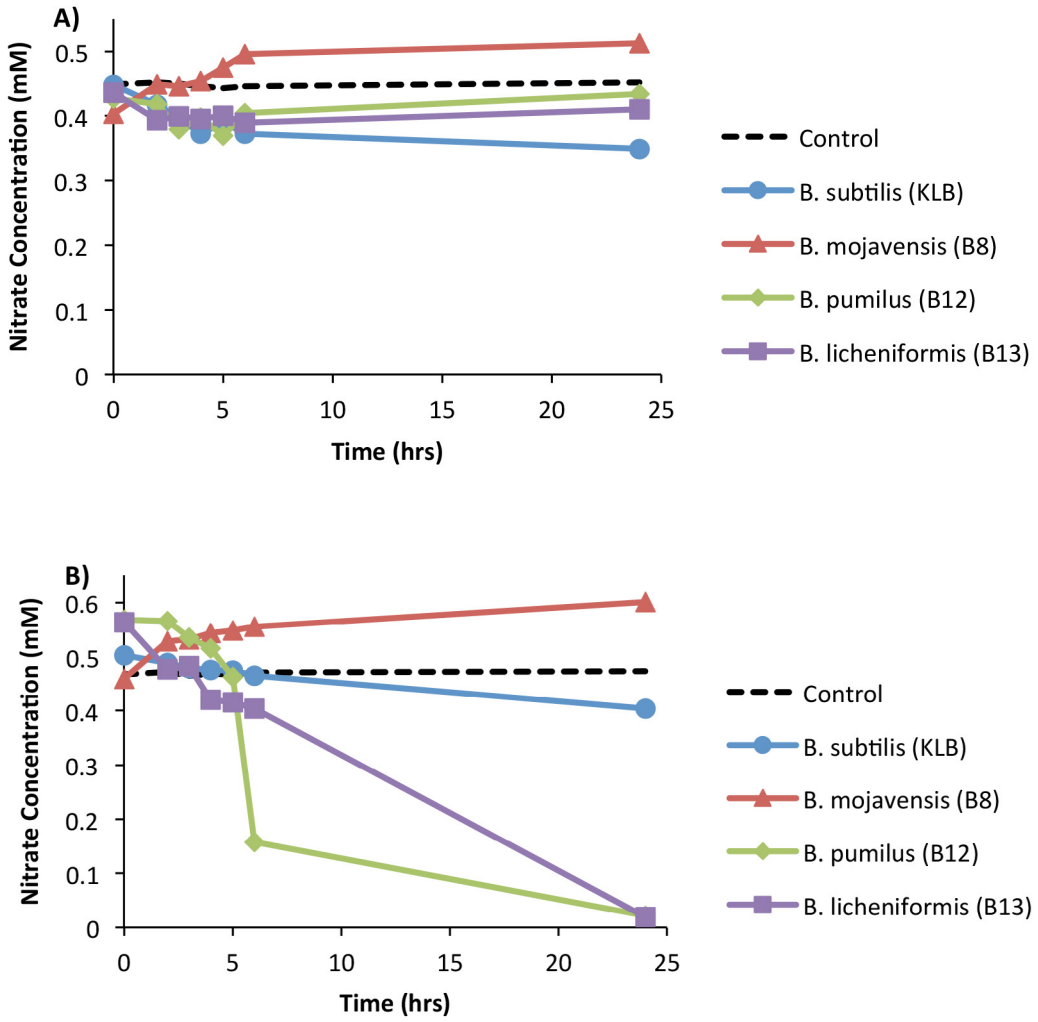


Figure 5 - Disappearance of nitrate over time in the presence of heterotrophic *Bacillus* isolates in A) DI water and B) wastewater reactor flasks.

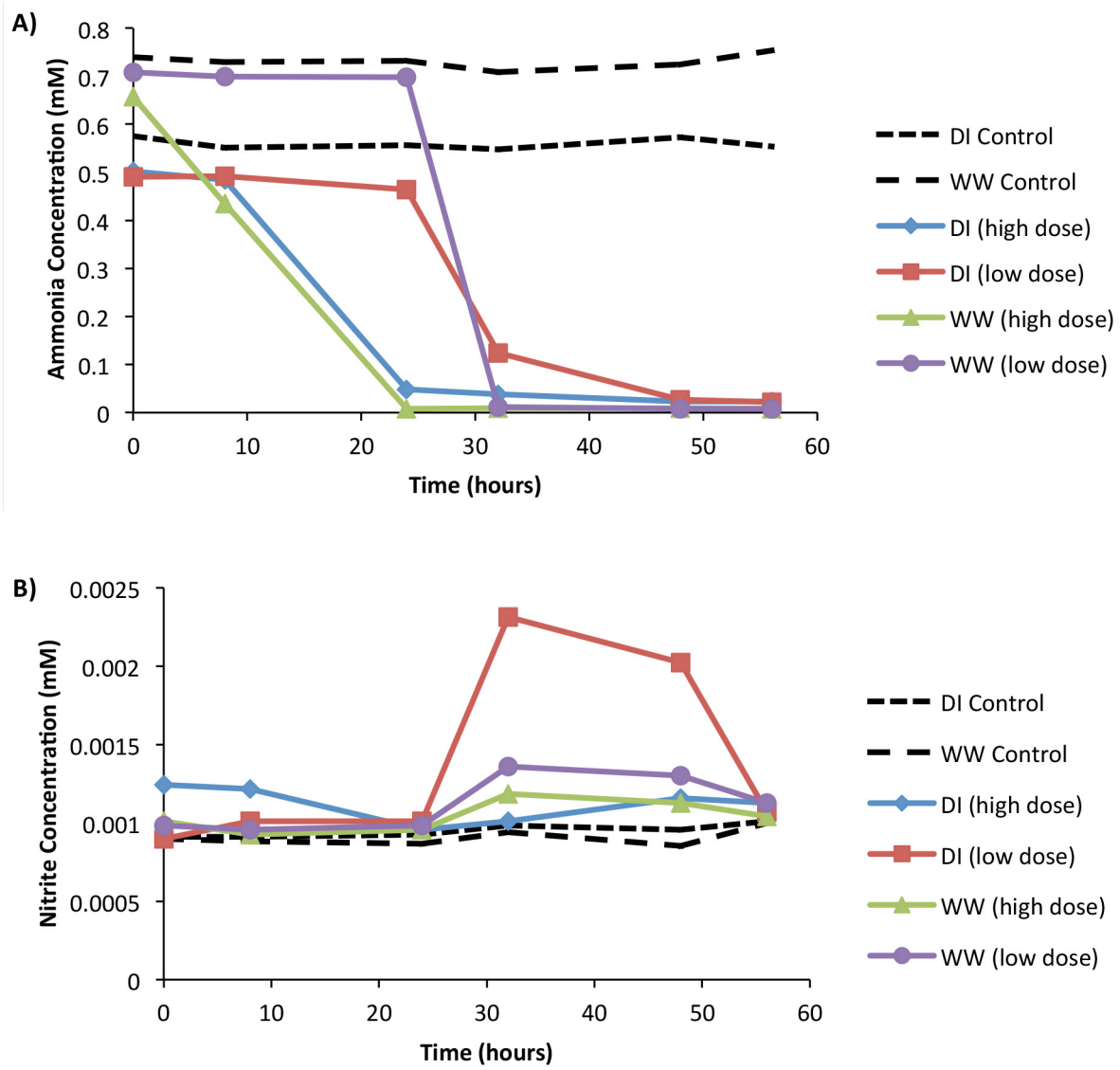


Figure 6 – Ammonia degradation (A) and nitrite production (B) in BiOWiSH® Aqua direct dosing reactor flasks. Although a delay in both curves was observed, attributable to germination and lag time among endospore formers, the overall nitrogen metabolism demonstrated by the fully formulated product compared favorably with the patterns observed in flasks treated with vegetative inoculums.

Table 3 – Nitrate metabolism in DI H_2O and wastewater reactor flasks. *Bacillus pumilus* (B12), *Bacillus licheniformis* (B13) and, to a lesser extent, *Bacillus* subtilis (KLB) showed nitrogen degradation capability at high levels of dissolved oxygen (DO). The highest rate of degradation in DI H_2O medium was achieved by B. subtilis, while the highest rate in wastewater was achieved by *Bacillus pumilus*. Rates of degradation (h^{-1}) are estimations of the first order rate based on a regression analysis of the data in ln(C/CO)/t form. Because nitrate was produced in flasks inoculated with *B. mojavensis*, not degraded, the value for these flasks is shown as negative.

Isolate	DI Water Reactors			Wastewater Reactors		
	Rate (mM h^{-1})	DO (ppm) T = 0	DO (ppm) T = 24	Rate (mM h^{-1})	DO (ppm) T = 0	DO (ppm) T = 24
B. subtilis (KLB)	0.010	7.7	6.4	0.009	7.7	6.4
B. mojavensis (B8)	-0.010	8.5	6.8	-0.011	8.5	6.8
B. pumilus (B12)	0.001	7.7	5.8	0.110	7.7	5.8
B. licheniformis (B13)	0.002	7.3	5.4	0.112	7.3	5.4

	Bacillus mojavensis (B8)			
	Total Organic N	Total Ammonia N	Total NO_2^-/NO_3^-	Total N
T=0	6.845	4.165	0.800	11.010
T=6	9.770	0.265	0.600	10.025
ΔN	**2.925**	**-3.9**	**-0.200**	**-0.985**
	***Bacillus subtilis* (B9)**			
	Total Organic N	Total Ammonia N	Total NO_2^-/NO_3^-	Total N
T=0	4.86	4.16	0.8	9.02
T=6	8.57	0.12	0.7	8.69
ΔN	**3.71**	**-4.04**	**-0.1**	**-0.33**
	***Bacillus pumilus* (B12)**			
	Total Organic N	Total Ammonia N	Total NO_2^-/NO_3^-	Total N
T=0	5.245	4.21	0.8	9.455
T=6	9.285	0.17	0.15	9.455
ΔN	**4.04**	**-4.04**	**-0.65**	**0**

Table 4 – Nitrogen balance for DI H_2O reactor flasks. In all treatments except *B. pumilus*, total N decreased between T = 0 and T = 6. Units for all values are mg L^{-1}. Total Ammonia N and Total NO_2^-/NO_3^- were direct measures taken by Midwest Laboratories, while Total N (TKN$^+$ NO_2^-/NO_3^-) and Total Organic N (Total N$^-$ NH_4^+) were calculations performed according to instructions from the testing laboratory.

Bacillus mojavensis (B8)				
	Total Organic N	**Total Ammonia N**	**Total NO_2^-/NO_3^-**	**Total N**
T=0	6.690	5.510	0.500	12.200
T=6	6.815	5.185	0.550	12.000
ΔN	**0.125**	**-0.325**	**0.050**	**-0.200**
***Bacillus subtilis* (B9)**				
	Total Organic N	**Total Ammonia N**	**Total NO_2^-/NO_3^-**	**Total N**
T=0	7.255	5.695	0.45	12.95
T=6	11.385	0.215	0.5	11.6
ΔN	**4.13**	**-5.48**	**0.05**	**-1.35**
***Bacillus pumilus* (B12)**				
	Total Organic N	**Total Ammonia N**	**Total NO_2^-/NO_3^-**	**Total N**
T=0	6.945	5.745	0.4	12.65
T=6	11.545	0.205	0.4	11.75
ΔN	**4.6**	**-5.54**	**0**	**-0.9**

Table 5 – Nitrogen balance for wastewater reactor flasks. In all treatments, total N decreased between T = 0 and T = 6. Units for all values are mg L^{-1}. Total Ammonia N and Total NO_2^-/NO_3^- were direct measures taken by Midwest Laboratories, while Total N (TKN + NO_2^-/NO_3^-) and Total Organic N (Total N – NH_4^+) were calculations performed according to instructions from the testing laboratory.

Discussion

The current paradigm for wastewater treatment involves utilization of bacteria in the phyla Proteobacteria (AOBs and NOBs such as *Nitrosomonas*, *Nitrospira and Nitrobacter*) and Planctomycetes (anammox organisms such as *Brocadia*) to convert aqueous NH_4^+ into gaseous N_2 through the biochemical processes of nitrification and denitrification. However, the use of these bacterial taxa to achieve this goal is not without limitations. Though aerobic, Proteobacteria have doubling times ranging from eight to twenty four hours, and NOBs such as *Nitrobacter* can be sensitive to changes in temperature and pH. Planctomycetes, though their anaerobic metabolism of ammonia has been viewed as revolutionary among wastewater treatment professionals, require careful mitigation of oxygen levels (886 nM O_2 results in 50% inhibition) and have temperature-dependent doubling times approaching 11 days (Dalsgaard *et al* 2014). Therefore, the examination of other bacterial species, specifically from aerobic and non-fastidious taxa, is an area of potential interest for the wastewater industry.

Bacteria of the phylum Firmicutes, which includes the genera *Bacillus* and *Pediococcus*, are aerobic heterotrophic organisms. The formation of endospores is diagnostic of the genus *Bacillus*, and these organisms have doubling times as low as 40 minutes under optimal conditions. Though not endospore formers, members of the genus *Pediococcus* have doubling times as low as one hour under optimal conditions (Kurniasari *et al*, 2012). Heterotrophic bacteria were long believed to perform aerobic nitrification and denitrification at rates much lower than those achieved by chemoautotrophs such as Proteobacteria and Planctomycetes. However, Kim *et al*. (2005) discovered that some heterotrophic bacteria are capable of performing aerobic nitrification and denitrification at rates comparable to those achieved by conventional chemoautotrophs.

The water treatment products produced by BiOWiSH Technologies inc. comprise a proprietary mixture of bacteria in the phylum Firmicutes belonging to the genera *Bacillus*, *Pediococcus* and *Lactobacillus*. In the present study, we isolated and identified eight species of the genus *Bacillus* from these products. These species, as well as four known organisms from stock cultures (*Bacillus* subtilis isolate "KLB", *Pediococcus pentosaceous*, *Pediococcus acidilactici*, and *Lactobacillus plantarum*), were screened for aerobic nitrification in both DI H_2O medium and in sterilized wastewater. Ammonia degradation and nitrite generation were measured via colorimetric analysis, and three selected isolates were subjected to a nitrogen balance to help elucidate the metabolic fate of NH_4^+. Four isolates were also tested for aerobic denitrification, and the degradation of NO_3^- was measured via colorimetric analysis.

With the exception of *Lactobacillus plantarum*, all isolates tested showed some degree of aerobic nitrification. Rates of nitrification were observed to be higher in wastewater reactor flasks for strains of *B. licheniformis*, *B. amyloliquefaciens* and *B. pumilus*, whereas higher nitrification rates were observed in DI H_2O flasks for *B. subtilis*, *L. plantarum*, *P. pentosaceous* and *P. acidilactici*. Since dextrose was the only available carbon source in DI H_2O reactor flasks, compared to the putative abundance of organic carbon sources in wastewater reactor flasks (despite autoclaving and filtering, N balance showed elevated levels of nitrogen in wastewater reactor flasks at T=0, supporting the hypothesis that more micronutrients were present in wastewater reactors than in DI H_2O reactors), these differences could be attributed to a carbon source affinity between species. The highest rates of nitrification in the wastewater medium were achieved by *B. licheniformis* (B11, 0.704 mM h^{-1}), *B. amyloliquefaciens* (B10, 0.641 mM h^{-1}) and *B. pumilus* (B12, 0.600 mM h^{-1}). The present study did not measure the impact of biomass upon ammonia degradation, and this topic is an area for future research.

Though only qualitative in nature, observations of dissolved oxygen (DO) indicate that these rates were achieved in the presence of oxygen levels far above the 30% saturation observed by Kim *et al*. (2005). Data collected from direct dosing reactor flasks (inoculated with commercially available BiOWiSH® Aqua) showed similar trends.

All reactor flasks experienced a spike in nitrite (NO_2^-) subsequent to the onset of ammonia degradation; in some cases (such as with *B. subtilis* isolate B9) the generation of nitrite was concurrent with the degradation of ammonia, while in other cases (such as with *B. pumilus* isolate B12) nitrite did not begin to appear to be generated in appreciable quantities until ammonia levels had been falling for several hours. The nature of this delay, which could indicate either a preferential conversion of ammonia nitrogen into biomass (an explanation potentially supported by the rise in organic nitrogen from T = 0 to T = 6) or a delay in the conversion of hydroxylamine (which was not measured) to nitrite by hydroxylamine oxidoreductase, is a topic for further study. Additionally, the analysis revealed no generation of NO_3^- subsequent to the degradation of NO_2^-. This observation, coupled with the decrease in total nitrogen detected between T = 0 and T = 6, suggests that a gaseous nitrogenous compound, not NO_3^-, is the metabolic fate of ammonia among the isolates tested.

Schrieber (2009) describes the aerobic microbial conversion of NO_2^- to N_2O, with NO as an intermediate, subsequent to the conversion of NH_4^+ to NO_2^- via hydroxylamine. Because the metabolic process described by Kim *et al*. (2005) involves the conversion of NH_4^+ directly to N_2, this system is unlikely to describe the nitrogen metabolism of the isolates tested during the present study. Rather, the decrease in generated NO_2^- coupled with a decrease in total nitrogen in the reactor flasks suggests that N_2O may be the metabolic fate of NH_4^+ among the bacteria examined here. Verification of this hypothesis requires more precise instrumentation to test for the presence of N_2O and is an area of future study. Furthermore, to the authors' knowledge this is the first report of aerobic heterotrophic nitrification among bacteria of the genus *Pediococcus*. These organisms also achieved a rate of nitrification in DI H_2O reactor flasks comparable to that achieved by strains of *B. licheniformis* and B*. subtilis*. The molecular mechanism of nitrification among members of this genus is an additional area for future study.

Of the isolates screened for denitrification, one (*B. subtilis* isolate KLB) showed the ability to remove NO_3^- in DI H_2O medium, and two (*B. pumilus* isolate B12 and *B. licheniformis* isolate B13) showed prodigious NO_3^- reduction in sterilized wastewater medium. As with the nitrification reactor flasks, the differences in nitrate reduction rate between different culture media could be attributable to carbon source affinity or the availability of other organic and inorganic micronutrients (which would be much more numerous in wastewater than in DI water). A more detailed examination of these species, including performing a nitrogen balance to determine the ultimate metabolic fate of nitrate, is another area for future study. Because the ultimate fate of nitrate is not known (and, thus, any denitrification activity among these species is putative), care should be taken when interpreting these results.

Conclusion

Bacteria of the phylum Firmicutes are aerobic heterotrophic organisms with rapid doubling times. Members of the genus *Bacillus* are non-fastidious and capable of withstanding unfavorable environmental conditions as endospores. Though not endospore formers, members of the genus *Pediococcus* are known inhibitors of pathogenic enteric bacteria (Kang *et al* 1999). Combined with the aerobic denitrification observed during the present study and the genus' rapid doubling times, this genus may be of interest to wastewater professionals (especially in combination with bacteria of the genus *Bacillus*). Formulations such as BiOWiSH® Aqua, BiOWiSH® Aqua FOG, and BiOWiSH® AquaFarm may represent the beginning of a paradigm shift from autotrophs to heterotrophs as the preferred microbial catalysts for the remediation of inorganic nitrogenous compounds in water treatment applications.

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