

Aerobic Nitrification and Denitrification among Heterotrophic Bacterial Isolates from a Commercial Water Treatment Product (BiOWiSH™ Aqua)

J. P. Gorsuch^{1*}, J. J. P. Roberts², E. A. Lenhoff³, M. S. Showell⁴

ABSTRACT

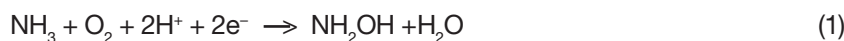
In addition to being an integral part of the planet's nitrogen cycle, bacterially facilitated nitrification and denitrification are processes of great commercial importance in the wastewater treatment, aquaculture and agronomy industries. Twelve distinct species of heterotrophic bacteria, belonging to the genera *Bacillus*, *Pediococcus* and *Lactobacillus*, were isolated from BiOWiSH™ Aqua, a commercially available water treatment product. These isolates were then examined for aerobic nitrification and denitrification activity using colorimetric analysis of ammonia (via a modified Berthelot reaction), nitrite (via a modified Griess reaction) and nitrate (via Cataldo's assay). Bacterial isolates mediated ammonia and nitrate removal in reactor flasks, as well as a decrease in total nitrogen (TKN) under aerobic conditions within 24 hours. The use of heterotrophic, aerobic bacteria in water treatment systems may present economic advantages as well as novel biological management of undesirable nitrogenous intermediates.

Keywords: aerobic nitrification, aerobic denitrification, heterotrophic nitrification, water treatment, *Bacillus*

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, consortiums 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).

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).



^{1*} BiOWiSH Technologies, 2724 Erie Avenue, Suite B, Cincinnati, OH, 45208; e-mail: jgorsuch@biowishtech.com (R&D Research Associate).

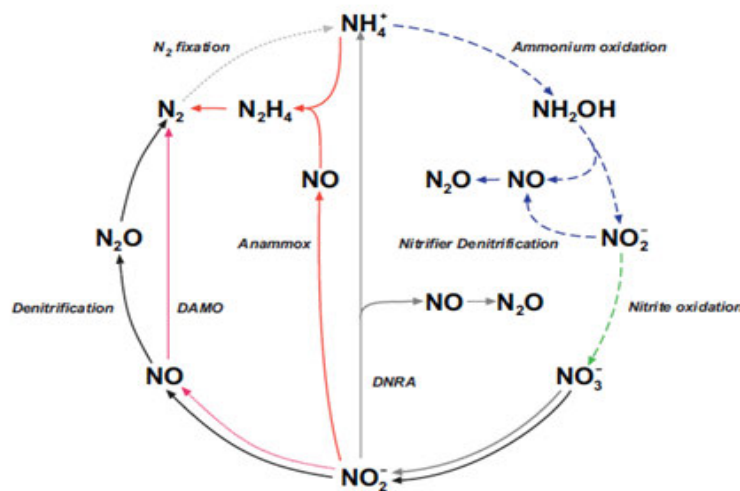
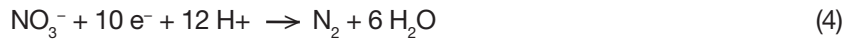
² Avila University, Kansas City, Missouri

³ Vice President of Research and Development, BiOWiSH Technologies, Cincinnati, Ohio

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).



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 .



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).

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 $2\mu\text{M}$ (Jetten et al, 2001).



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_3^- 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™ Aqua is a proprietary mixture of bacteria in the phylum Firmicutes and belonging to the genera *Bacillus*, *Pediococcus* and *Lactobacillus* that is formulated to enhance carbon degradation in aqueous environments. Quantitative data collected during subsequent field applications revealed BiOWiSH™ Aqua 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™ Aqua for aerobic nitrification and denitrification. Our null hypothesis (H_0) was that members of the BiOWiSH™ Aqua bacterial consortium were acting as conventional AOBs and NOBs to effect nitrification under aerobic conditions, and that aerobic nitrate remediation was attributable to biological assimilation. An alternative hypothesis was that the bacterial consortium present in BiOWiSH™ Aqua was carrying out a variation of the aerobic nitrification/denitrification process elucidated by Kim et al (2005). To test this hypothesis, we monitored the disappearance of NH_4^+ and the appearance of $\text{NO}_2^-/\text{NO}_3^-$ (for nitrification), and monitored the disappearance of NO_3^- and the appearance of NO_2^- (for denitrification). Ammonia and nitrite 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 (Griess, 1879) to assay nitrite. Nitrate concentrations were examined spectroscopically using the protocol of Cataldo (1975). Furthermore, we performed a nitrogen balance to elucidate the role of biological assimilation and the possibility of a gaseous metabolic fate for NH_4^+ and NO_3^- . To determine the potential role of nutrient availability in mediating nitrogen metabolism among the bacterial isolates tested, assays were conducted both in mineral medium (which contained only once source each for C and N, respectively) and in wastewater extract medium. In the case of aerobic denitrification assays, mineral medium reactor flasks served to remove any potential interference by ammonia oxidation, ensuring that NO_3^- was the only possible source for any observed NO_2^- .

Aerobic ammonia removal, followed by a transient nitrite spike, was observed among multiple *Bacillus* isolates in mineral medium and in untreated wastewater. The aerobic reduction of NO_3^- to NO_2^- and subsequent removal of NO_2^- (putatively referred to as aerobic denitrification) was observed in multiple *Bacillus* isolates under identical conditions. These processes were accompanied by a measured decrease in total nitrogen (TKN) from reactor flasks, suggesting a gaseous fate for both ammonia and nitrate nitrogen. A wastewater treatment system utilizing these heterotrophic species, capable of performing simultaneous nitrification and denitrification, could possess several economic advantages and be of potential interest to water treatment professionals.

METHODOLOGY

Chemicals and Materials – Ammonium Phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$; > 98%) and sodium nitrate (NaNO_3 ; > 99%) were purchased from Sigma-Aldrich. Dextrose monohydrate was purchased from My Spice Sage. Salicylic acid, concentrated sulfuric acid (95–98%), and sodium hydroxide pellets (> 97%) were purchased from Sigma-Aldrich. The dissolved oxygen

test kit was purchased directly through LaMotte. Aquarium Pharmaceuticals International (API) ammonium, nitrite, and nitrate kits were purchased locally.

Bacterial isolates were screened for aerobic nitrification potential in a modified mineral medium (reactor flasks run in duplicate) and in untreated wastewater collected from a local wastewater treatment plant (reactor flasks run in duplicate). Isolates were also screened for aerobic denitrification activity under the same conditions. Additionally, unaltered BiOWiSH™ Aqua was dosed into duplicate DI H₂O and wastewater reactors to determine the impact of endospore germination upon the product's 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™ Aqua. 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, Utah, USA) and identified to the species level using 16s rDNA analysis. Stock cultures of *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *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 × 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 mineral medium reactor flasks – Mineral medium was prepared according to standard methods (OECD Test Protocol 301), with the exception that all nitrogenous compounds were included as described below. 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 mineral medium 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 mineral medium 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. The denitrification mineral medium was prepared in the same manner, but was supplemented with 25 mg L⁻¹ NO₃⁻ instead of NH₄⁺ 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, Ohio, USA) and transported back to the laboratory in a disinfected, capped plastic carboy. For nitrification assays, wastewater was centrifuged at 6,000 × g for ten minutes to remove visible biosolids which may have interfered with spectrophotometric analysis, and 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 NO₃⁻ (while the medium tested positive for NH₃, no detectable NO₃⁻ was present).

Sampling of reactor flasks – Inoculums of each bacterial isolate were added to duplicate mineral medium 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 for ammonia and nitrite determination. Due to the length of time required to carry out nitrate determination assays, nitrate was sampled only at hours 3, 6, and 24 after collection of T = 0 hours samples. Sample tubes were centrifuged at 6,000 × 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 mineral medium and wastewater medium were prepared as described above and dosed with fully formulated, unaltered BiOWiSH™ Aqua at both a high dose (109 CFUs) and a low dose (106 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 mineral medium and wastewater reactor flasks of *B. subtilis*, *B. mojavensis* and *B. pumilus* and immediately frozen at T = 0 hours, T = 6 hours and T = 24 hours. These samples were shipped frozen to Midwest Laboratories (Omaha, NE, USA) for the following analysis using standard methods: Total N (THN + NO₃⁻/NO₂⁻), Organic N (standard methods), Nitrate/Nitrite N (EPA 353.2), Ammonia N (SM 4500-NH₃ G-1997), and Total Kjeldahl N (colorimetric block digestion).

Colorimetric Analysis of NH₃, NO₂⁻, and NO₃⁻ – Ammonia and nitrite concentrations were determined colorimetrically using a commercially available test kit (MARS Fishcare). Reactions were performed as indicated by the test kit. Five to seven 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). Nitrate concentrations were determined colorimetrically using a nitration of salicylic acid method (University of Wisconsin, Dept. of Agronomy, 1975). Sample aliquots of 0.2 mL were dispensed into 25-mL Erlenmeyer flasks, which were then mixed with 0.8 mL of concentrated sulfuric acid (H₂SO₄). A blank was also made with distilled water, mineral medium, or wastewater, depending on the medium of the samples. The samples were left for 20 minutes, and then 19 mL of 2M NaOH were added. The samples were cooled to room temperature and then analyzed for absorbance with an Agilent Cary-60 spectrophotometer calibrated at 410 nm ($y=0.00143x-0.00431$; $r^2=0.999$).

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.

Statistical Analysis of Nitrification Results – Absorbance values for ammonia, nitrite, and nitrate were recorded at each time point in duplicate. Calibrations for ammonia, nitrite, and nitrate were then used to determine each concentration in millimoles (mM) per liter. The average concentration and standard deviation were calculated using MS Excel, and 95% confidence intervals were determined using the procedure for confidence limits in *McDonald's Handbook of Biological Statistics* (2014).

Statistical Analysis of Denitrification Results – Absorbance values for nitrite and nitrate were recorded at each time point in duplicate. Calibrations for nitrite and nitrate were then used to determine each concentration in millimoles (mM) per liter. The average concentration and standard deviation calculated using MS Excel, and 95% confidence intervals were determined using the procedure for confidence limits in *McDonald's Handbook of Biological Statistics* (2014).

RESULTS

Identification of Bacterial Isolates – Characteristics of bacterial isolates, including the results of 16s rDNA analysis, are displayed in Table 1. Isolates from BiOWiSH™ Aqua 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 *Bacillus* species are displayed in Figure 1, and NO₂⁻ generation curves for the same species are displayed in Figure 2. All isolates tested showed aerobic ammonia removal followed by a transient nitrite spike at high levels of dissolved oxygen (DO > 2.4 ppm) within 24 hours, with measurable degradation often occurring within three hours. Colorimetric analysis revealed no detectable increase in the concentration of NO₃⁻ upon degradation of NO₂⁻.

Denitrification assays – Nitrate degradation curves and nitrite generation curves for four *Bacillus* species in mineral medium are displayed in Figure 3. All isolates tested showed aerobic reduction of nitrate to nitrite at high levels of dissolved oxygen (DO > 3.8 ppm) within 24 hours, with measurable degradation often occurring within three hours. Many isolates showed the ability to completely remove NO₂⁻ from reactor flasks within 24 hours. Because wastewater medium also contained ammonia, and thus a second potential source of NO₂⁻ from ammonia oxidation, only nitrate degradation was monitored for bacterial isolates in wastewater medium flasks.

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

Table 1 – Identification of bacterial isolates from BiOWiSH™ Aqua. Genetic sequencing (16s rDNA) was performed by Nelson Laboratories (Salt Lake City, Utah, USA). BiOWiSH™ Aqua is comprised of several proprietary bacterial formulations containing Firmicutes of the genera *Bacillus*, *Pediococcus* and *Lactobacillus*.

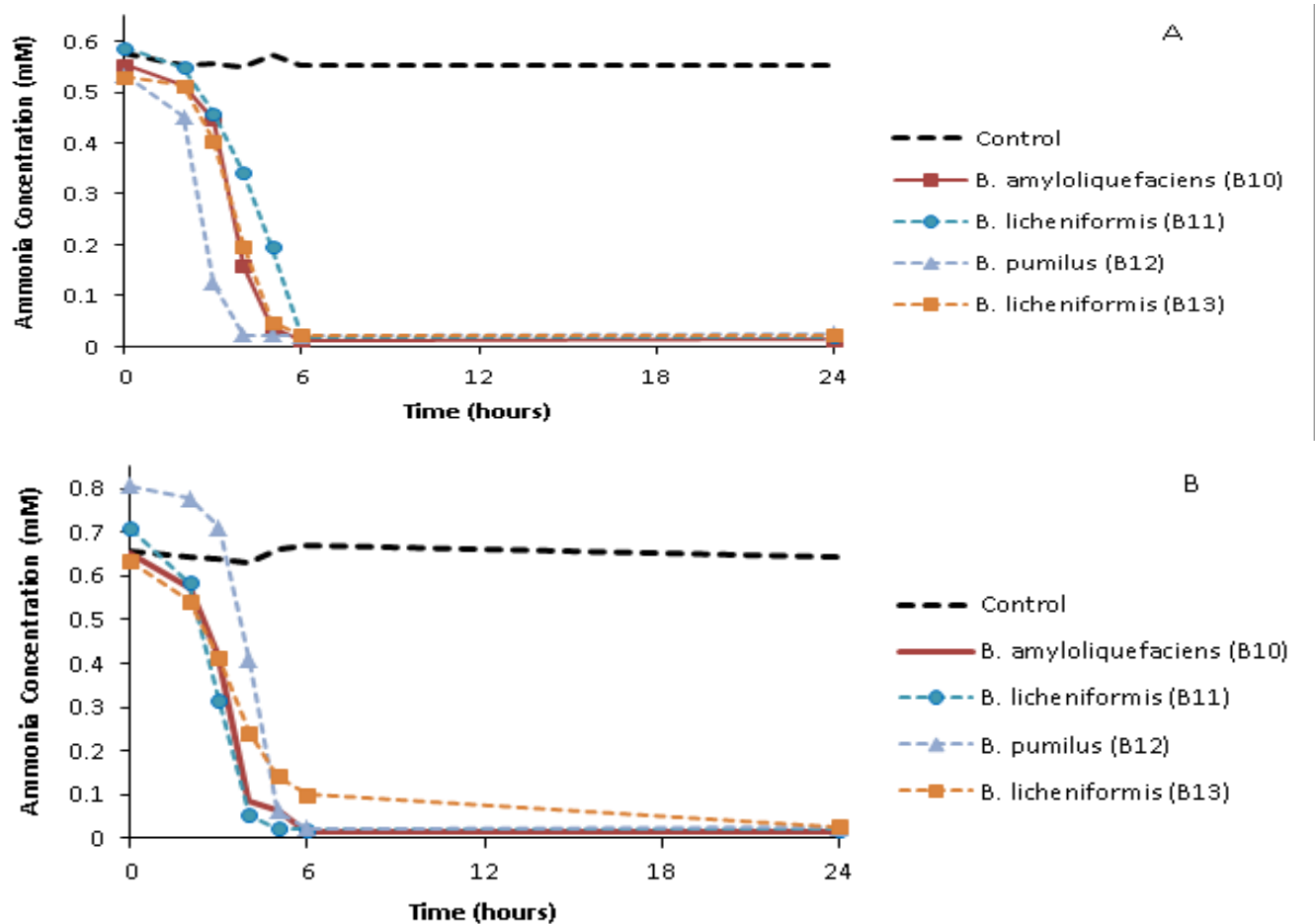


Figure 1 – Disappearance of ammonia over time in the presence of heterotrophic *Bacillus* isolates in (A) mineral medium and (B) wastewater nitrification reactor flasks.

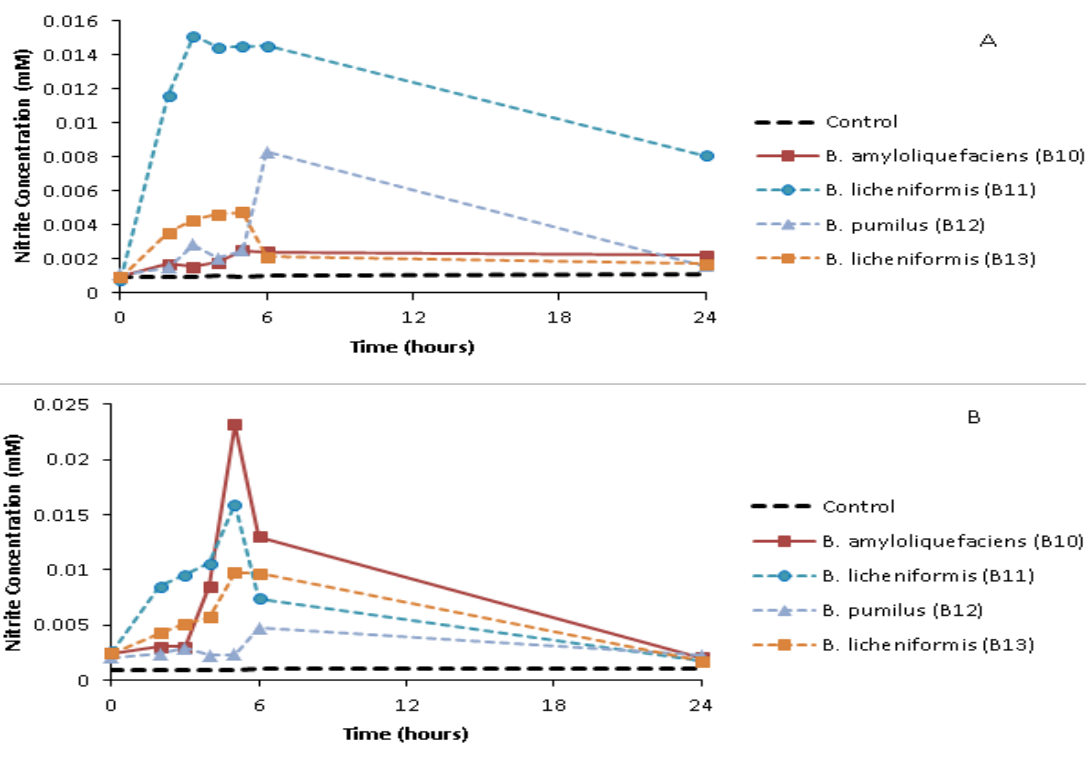


Figure 2 – Appearance of nitrite over time in the presence of heterotrophic *Bacillus* isolates in (A) mineral medium and (B) wastewater nitrification reactor flasks.

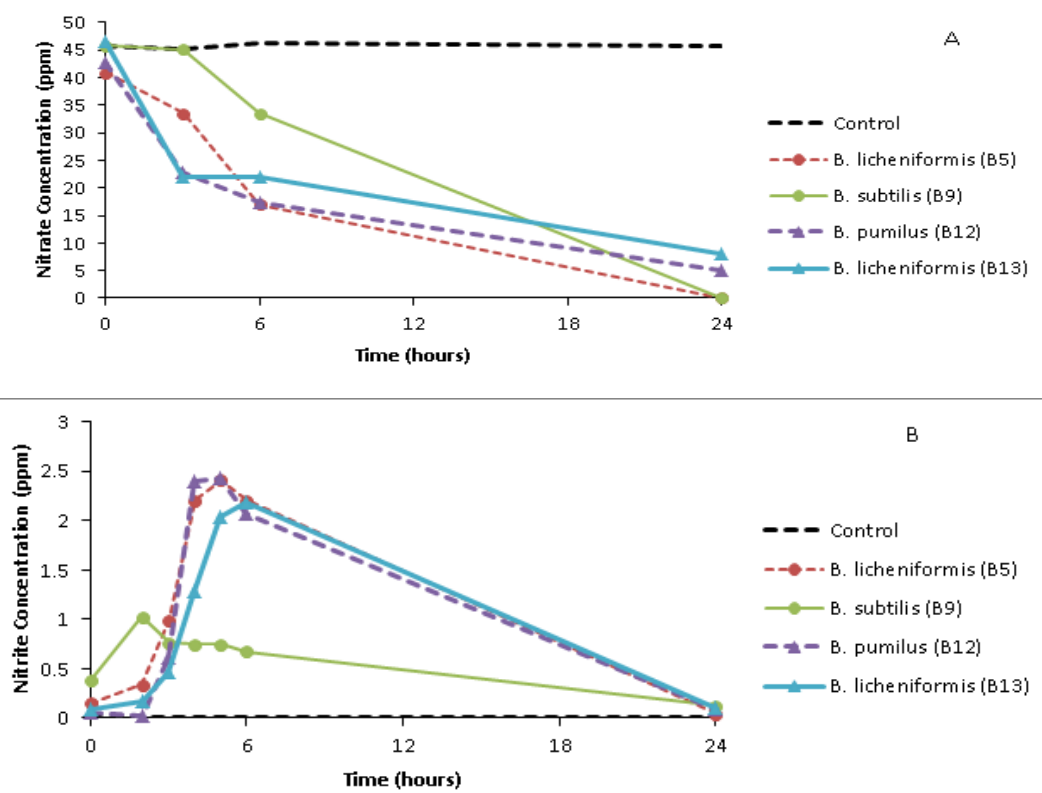


Figure 3 – Disappearance of nitrate (A) and appearance of nitrite (B) over time in the presence of heterotrophic *Bacillus* isolates in mineral medium reactor flasks

Nitrogen Balance – Results of nitrogen balance assays (Midwest Laboratories, Omaha, Nebraska, USA) for mineral medium reactor flasks are displayed in Table 2, and for wastewater flasks are displayed in Table 3.

<i>Bacillus mojavensis</i> (B8)				
	<i>Total Organic N</i>	<i>Total Ammonia N</i>	<i>Total NO₂⁻/NO₃⁻</i>	<i>Total N</i>
T=0	6.845	4.165	0.800	11.010
T=6	9.770	0.265	0.600	10.025
ΔN	2.925	-3.900	-0.200	-0.985
<i>Bacillus subtilis</i> (B9)				
	<i>Total Organic N</i>	<i>Total Ammonia N</i>	<i>Total NO₂⁻/NO₃⁻</i>	<i>Total N</i>
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
<i>Bacillus pumilus</i> (B12)				
	<i>Total Organic N</i>	<i>Total Ammonia N</i>	<i>Total NO₂⁻/NO₃⁻</i>	<i>Total N</i>
T=0	5.245	4.21	0.80	9.455
T=6	9.285	0.17	0.15	0.455
ΔN	4.040	-4.040	-0.65	0

Table 2 – Nitrogen balance for mineral medium nitrification 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 NO₂⁻/NO₃⁻ were direct measures taken by Midwest Laboratories, while Total N (TKN + NO₂⁻/NO₃⁻) and Total Organic N (Total N – NH₄⁺) were calculations performed according to instructions from the testing laboratory.

<i>Bacillus mojavensis</i> (B8)				
	<i>Total Organic N</i>	<i>Total Ammonia N</i>	<i>Total NO₂⁻/NO₃⁻</i>	<i>Total N</i>
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
<i>Bacillus subtilis</i> (B9)				
	<i>Total Organic N</i>	<i>Total Ammonia N</i>	<i>Total NO₂⁻/NO₃⁻</i>	<i>Total N</i>
T=0	7.255	5.695	0.45	12.95
T=6	11.385	0.215	0.50	11.60
ΔN	4.130	-5.480	0.05	-1.35
<i>Bacillus pumilus</i> (B12)				
	<i>Total Organic N</i>	<i>Total Ammonia N</i>	<i>Total NO₂⁻/NO₃⁻</i>	<i>Total N</i>
T=0	6.945	5.745	0.4	12.65
T=6	11.545	0.205	0.4	11.75
ΔN	4.600	-5.540	0	-0.90

Table 3 – Nitrogen balance for wastewater nitrification 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 NO₂⁻/NO₃⁻ were direct measures taken by Midwest Laboratories, while Total N (TKN + NO₂⁻/NO₃⁻) and Total Organic N (Total N – NH₄⁺) were calculations performed according to instructions from the testing laboratory.

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 4).

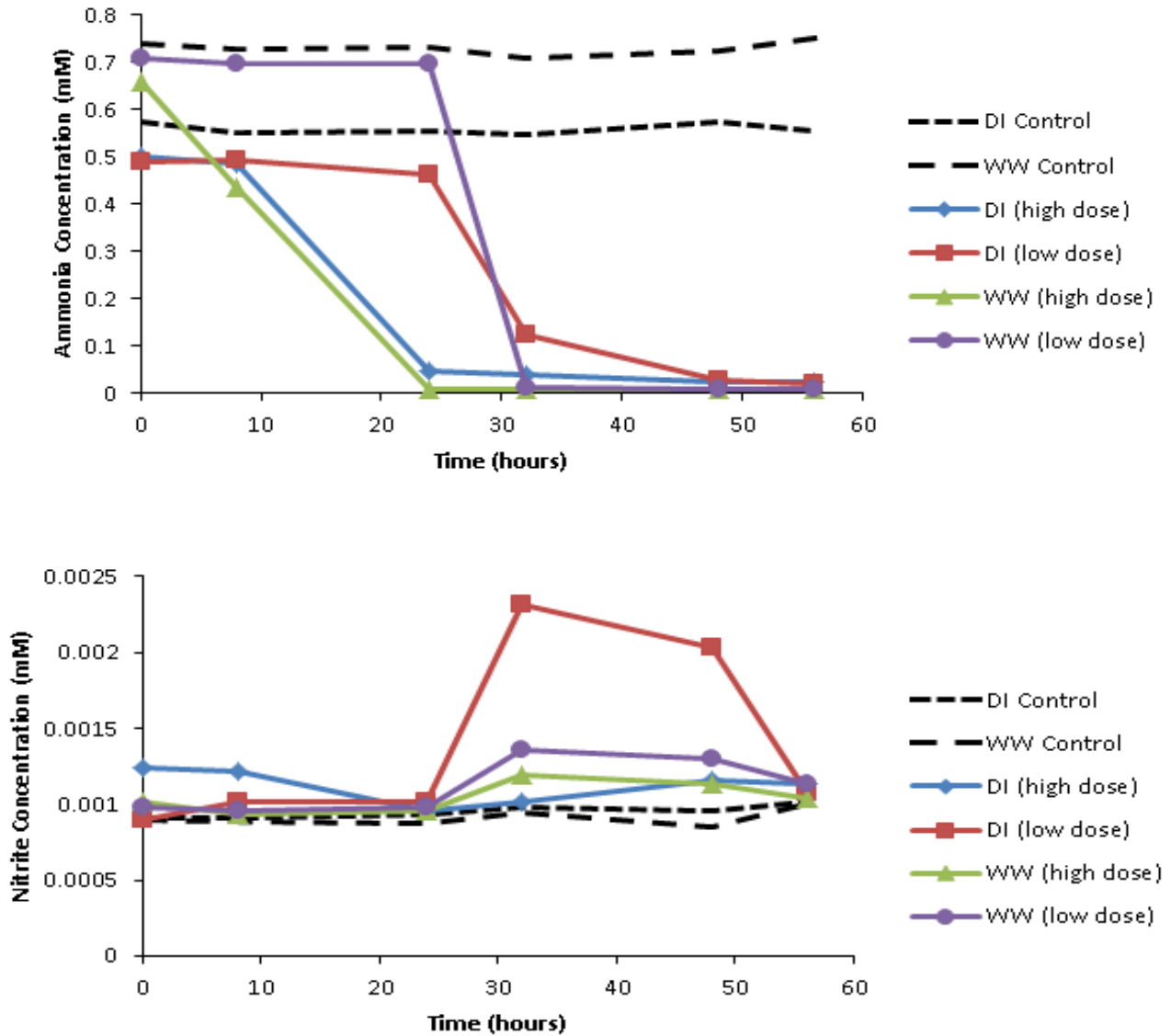


Figure 4 – Ammonia degradation (A) and nitrite production (B) in *BiOWiSH™ Aqua* direct dosing reactor flasks. Although a delay in both curves was observed, attributable to germination and lag time among endospore formers, the overall nitrogen metabolism demonstrated by the fully formulated product compared favorably with the patterns observed in flasks treated with vegetative inoculums.

Statistical Analysis of Nitrification Results – Calculation of 95% confidence limits for the data displayed in Figures 1 and 2 are displayed in Table 4.

Isolate	Time (hours)	Ammonia					Nitrite					
		Trial A (mM)	Trial B (mM)	Avg. (mM)	Std. Dev.	95% Conf. Int.	Trial A (mM)	Trial B (mM)	Avg. (mM)	Std. Dev.	95% Conf. Int.	
Bl0	0	0.6541	0.6536	0.654	0.0004	+/-0.0033	0.0024	0.0025	0.0024	0.00004	+/-0.0004	
	2	0.5782	0.5659	0.572	0.0087	+/-0.0784	0.0031	0.003	0.003	0.00004	+/-0.0004	
	3	0.4594	0.3719	0.416	0.0618	+/-0.5556	0.0031	0.003	0.0031	0.00002	+/-0.0002	
	4	0.098	0.0687	0.083	0.0207	+/-0.1863	0.0063	0.0108	0.0085	0.00321	+/-0.0289	
	5	0.0839	0.0442	0.064	0.028	+/-0.2517	0.022	0.0243	0.0232	0.00165	+/-0.0149	
	6	0.0141	0.0136	0.014	0.0004	+/-0.0033	0.0134	0.0126	0.013	0.00057	+/-0.0052	
	24	0.0157	0.0147	0.015	0.0007	+/-0.0065	0.0019	0.0022	0.0021	0.00022	+/-0.0020	
	Bl1	0	0.7091	0.7084	0.709	0.0005	+/-0.0044	0.0033	0.0018	0.0026	0.0011	+/-0.0095
	2	0.5744	0.5931	0.584	0.0133	+/-0.1188	0.0089	0.0082	0.0085	0.0005	+/-0.0044	
	3	0.3184	0.3097	0.314	0.0062	+/-0.0553	0.01	0.0091	0.0095	0.0006	+/-0.0057	
	4	0.0592	0.0463	0.053	0.0091	+/-0.0820	0.011	0.0101	0.0106	0.0006	+/-0.0057	
	5	0.0211	0.0219	0.022	0.0005	+/-0.0051	0.0165	0.0153	0.0159	0.0009	+/-0.0076	
	6	0.0203	0.0198	0.02	0.0004	+/-0.0032	0.0087	0.006	0.0074	0.0019	+/-0.0172	
	24	0.0185	0.0208	0.02	0.0016	+/-0.0146	0.0019	0.0017	0.0018	0.0002	+/-0.0013	
Bl2	0	0.8038	0.8097	0.807	0.0042	0.038	0.002	0.0021	0.0021	0.00004	+/-0.0006	
		2	0.7809	0.7752	0.778	0.004	0.038	0.0024	0.0024	0.0024	0	0
		3	0.7256	0.6942	0.71	0.0222	0.203	0.0029	0.0029	0.0029	0	0
		4	0.4584	0.358	0.408	0.0709	0.635	0.0016	0.0028	0.0022	0.00084	+/-0.0076
		5	0.098	0.025	0.062	0.0517	0.464	0.0022	0.0024	0.0023	0.00012	+/-0.1245
		6	0.0237	0.0195	0.022	0.0029	0.025	0.0022	0.0073	0.0047	0.00362	+/-0.0324
		24	0.0255	0.0219	0.024	0.0025	0.019	0.002	0.0024	0.0022	0.00027	+/-0.0025
	Bl3	0	0.6446	0.6212	0.632	0.0165	+/-0.1487	0.0024	0.0025	0.0024	0.00006	+/-0.0006
	2	0.5332	0.5507	0.542	0.0124	+/-0.1067	0.0044	0.0041	0.0042	0.00027	+/-0.0024	
	3	0.4069	0.4172	0.412	0.0073	+/-0.0654	0.0052	0.0051	0.0051	0.00008	+/-0.0005	
	4	0.241	0.2433	0.242	0.0016	+/-0.0148	0.0059	0.0055	0.0057	0.00029	+/-0.0025	
	5	0.1376	0.1479	0.143	0.0073	+/-0.0654	0.0118	0.0078	0.0098	0.00284	+/-0.0255	
	6	0.0983	0.1006	0.099	0.0016	+/-0.0174	0.0089	0.0104	0.0097	0.00108	+/-0.0095	
	24	0.0255	0.0283	0.027	0.002	+/-0.0180	0.002	0.0014	0.0017	0.00045	+/-0.0040	

Table 4 – Statistical analysis for nitrification reactor flasks. Confidence intervals (95%) were calculated using *McDonald's Handbook of Biological Statistics* (2009).

Statistical Analysis of Denitrification Results - Calculation of 95% confidence limits for the data displayed in Figures 3 and 4 are displayed in Table 5.

Isolate	Time (hours)	Nitrate					Nitrite				
		Trial A (mM)	Trial B (mM)	Avg. (mM)	Std. Dev.	95% Conf. Int.	Trial A (mM)	Trial B (mM)	Avg. (mM)	Std. Dev.	95% Conf. Int.
B5	0	0.7519	0.727	0.7395	0.0175	+/-0.1582	0.0085	0.0079	0.0082	0.0004	+/-0.0038
	2	-	-	-	-	-	0.0217	0.0228	0.0223	0.0008	+/-0.0070
	3	0.6898	0.8015	0.7457	0.079	+/-0.7096	0.0162	0.0173	0.0167	0.0008	+/-0.0070
	4	-	-	-	-	-	0.0157	0.0168	0.0162	0.0008	+/-0.0070
	5	-	-	-	-	-	0.0159	0.0166	0.0163	0.0005	+/-0.0044
	6	0.5409	0.5409	0.5409	0	+/-0	0.0148	0.0144	0.0146	0.0003	+/-0.0025
	24	0	0	0	0	+/-0	0.0024	0.003	0.0027	0.0004	+/-0.0038
	B9	0	0.603	0.7146	0.6588	0.079	+/-0.7115	0.004	0.0026	0.0033	0.001
2		-	-	-	-	-	0.0077	0.007	0.0073	0.0004	+/-0.0040
3		0.5658	0.5161	0.5409	0.0351	+/-0.3177	0.0214	0.0219	0.0217	0.0004	+/-0.0032
4		-	-	-	-	-	0.0499	0.046	0.048	0.0028	+/-0.0251
5		-	-	-	-	-	0.051	0.0538	0.0524	0.002	+/-0.0178
6		0.2432	0.3052	0.2742	0.0439	+/-0.3939	0.048	0.0478	0.0479	0.0001	+/-0.0013
24		0	0	0	0	+/-0	0.0009	0.001	0.001	0.0001	+/-0.0006
B12		0	0.5533	0.4913	0.5223	0.0439	+/-0.3940	0.0013	0.0012	0.0013	7E-05
	2	-	-	-	-	-	0.0006	0.0007	0.0006	8E-05	+/-0.00070
	3	0.3548	0.3672	0.361	0.0088	+/-0.0760	0.0129	0.0137	0.0133	0.0005	+/-0.00483
	4	-	-	-	-	-	0.0528	0.0515	0.0522	0.001	+/-0.00870
	5	-	-	-	-	-	0.0542	0.5169	0.053	0.0018	+/-0.01630
	6	0.268	0.2804	0.2742	0.0088	+/-0.0760	0.045	0.0452	0.0451	0.0002	+/-0.00178
	24	0.0819	0.0819	0.0819	0	+/-0	0.0023	0.0017	0.002	0.0005	+/-0.00400
	B13	0	0.7146	0.4665	0.5906	0.1755	+/-1.5762	0.0017	0.0022	0.002	0.0003
2		-	-	-	-	-	0.0032	0.004	0.0036	0.0006	+/-0.0051
3		0.3672	0.3424	0.3548	0.0175	+/-0.1576	0.0083	0.0121	0.0102	0.0027	+/-0.0432
4		-	-	-	-	-	0.023	0.0322	0.0278	0.0062	+/-0.0559
5		-	-	-	-	-	0.0398	0.0486	0.0442	0.0063	+/-0.0559
6		0.3772	0.3474	0.3474	0.0421	+/-0.3786	0.0452	0.0497	0.0474	0.0032	+/-0.0286
24		0.1067	0.0943	0.1005	0.0088	+/-0.0788	0.0019	0.0027	0.0023	0.0005	+/-0.0051

Table 5 – Statistical analysis for denitrification reactor flasks. Confidence intervals (95%) were calculated using *McDonald's Handbook of Biological Statistics* (2009).

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 anaerobic denitrification. However, the use of these bacterial taxa to achieve this goal is not without limitations. Though aerobic, Proteobacteria have doubling times ranging from 8 to 24 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.

BiOWiSH™ Aqua is one of a group of water treatment products produced by BiOWiSH™ Technologies Inc. comprising 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 BiOWiSH™ Aqua. These species, as well as four known organisms from stock cultures (*Bacillus subtilis* isolate “KLB”, *Pediococcus pentosaceus*, *Pediococcus acidilactici*, and *Lactobacillus plantarum*), were screened for aerobic nitrification in both mineral 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^+ . All isolates were also tested for aerobic denitrification under identical conditions, and the degradation of NO_3^- and subsequent generation of NO_2^- (as a transient intermediate) was measured via colorimetric analysis.

All *Bacillus* isolates tested showed some degree of aerobic ammonia oxidation ability. Bacterial isolates were observed to produce different degradation curves in mineral medium than in wastewater media, which may be attributable to difference in micronutrient abundance between the two media. Since dextrose was the only available carbon source in mineral medium 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 mineral medium reactors), these differences could be attributed to a carbon source affinity between species. Because no detectable production of nitrate was observed in either medium subsequent to ammonia oxidation, we propose that these data do not compare favorably with known pathways of nitrification; thus, our null hypothesis is not supported. We propose that these data support the hypothesis that the consortium of heterotrophic bacteria present in BiOWiSH™ Aqua carries out a variation of the aerobic nitrification/denitrification metabolic pathway proposed by Kim et al (2005).

Though only qualitative in nature, observations of dissolved oxygen (DO) indicate that these results 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 nitrification 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. Further, 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. Verification of this hypothesis requires more precise instrumentation to test for the presence of N_2 and N_2O in the head space of reactor flasks, and is an area of future study.

All *Bacillus* isolates screened for aerobic denitrification activity showed the ability to convert a portion of the available NO_3^- in reactor flasks into NO_2^- under aerobic conditions. Several of these isolates were observed to remediate all of the NO_2^- produced within 24 hours. Because these assays were conducted in mineral medium where NO_3^- was the only N source, alternative sources of NO_2^- in reactor flasks are nonexistent. Although complete nitrogen balance on these reactors is an area of active research, the simultaneous disappearance of NO_3^- and appearance of NO_2^- compares favorably with known denitrification pathways and suggests a metabolic process capable of carrying out a redox reaction under aerobic conditions. Further studies are being conducted to determine the ultimate metabolic fate of NO_3^- -N, and thus care should be taken when interpreting these results. Again, these data support the hypothesis that the heterotrophic consortium of bacteria present in BiOWiSH™ Aqua is capable of carrying out a variation of the heterotrophic nitrification/denitrification pathway proposed by Kim et al (2005).

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. The elimination of $\text{NH}_4^+ - \text{N}$ and $\text{NO}_3^- - \text{N}$ from reactor flasks, combined with the reduction in Total N, observed during the present study make this bacterial consortium one of potential interest to wastewater professionals. Such a metabolic pathway could potentially be used to replace the current, two-step paradigm of aerobic nitrification and anaerobic denitrification with a single, aerobic treatment. Furthermore, the putative aerobic denitrification observed here could potentially be of use in applications where anaerobic reactors are cost-prohibitive or otherwise impractical by circumventing the need for an anaerobic treatment step. Formulations such as BiOWiSH™ Aqua 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.

ACKNOWLEDGEMENTS

Thanks to Nirupam Pal from the California Polytechnic University for providing editorial comments. Thanks also to Gary Jones of Mars™ Fishcare for providing information regarding the chemistry of commercially available colorimetric nitrogen test kits.

REFERENCES

- Berthelot, 1859, Neue Beobachtungen über den Schwefel. *Journal für Praktische Chemie*, 78 (1), 244–247.
- Bock, E.; Schmidt, I.; Stueven, R.; Zart, D., 1996, Verfahren zur biologischen Umsetzung von in Wasser gelöstem Ammonium unter Verwendung ammoniak-oxidierender Bakterien. *DE*, 196 (17), 331–341.
- Cataldo, D.; Haroon, L.; Schraeder, V. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* 6: 71–80.
- Dalsgaard, T.; Stewart, F. J.; Thamdrup, B.; De Brabandere, L.; Revsbech, N. P.; Ulloa, O.; Canfield, D. E.; DeLong, E. F., 2014, Oxygen at Nanomolar Levels Reversibly Suppresses Process Rates and Gene Expression in Anammox and Denitrification in the Oxygen Minimum Zone off Northern Chile. *mBio*, 5 (6).
- Griess, P., 1879, Bemerkungen zu der Abhandlung der HH. Weselsky und Benedikt Ueber einige Azoverbindungen". *Berichte der deutschen chemischen Gesellschaft*, 12 (1), 426–428.
- Hageman, J. H.; Shankweiler, G. W.; Wall, P. R.; Franich, K.; McCowan, G. W.; Cauble, S. M.; Grajeda, J.; Quinones, C., 1984, Single, chemically defined sporulation medium for *Bacillus subtilis*: growth, sporulation, and extracellular protease production. *Journal of Bacteriology*, 160 (1), 438–441.
- Hellinga, C.; Schellen, A. A. J. C.; Mulder, J. W.; van Loosdrecht, M. C. M.; Heijnen, J. J., 1998, The sharon process: An innovative method for nitrogen removal from ammonium-rich waste water. *Water Science and Technology*, 37 (9), 135–142.
- Hommers, N. G.; Sayavedra-Soto, L. A.; Arp, D. J., 2003, Chemolithoorganotrophic Growth of *Nitrosomonas europaea* on Fructose. *Journal of Bacteriology*, 185 (23), 6809–6814.
- Jetten, M. S. M.; Wagner, M.; Fuerst, J.; van Loosdrecht, M.; Kuenen, G.; Strous, M., 2001, Microbiology and application of the anaerobic ammonium oxidation ('anammox') process. *Current Opinion in Biotechnology*, 12 (3), 283–288.
- Kim, J. K.; Park, K. J.; Cho, K. S.; Nam, S.-W.; Park, T.-J.; Bajpai, R., 2005, Aerobic nitrification–denitrification by heterotrophic *Bacillus* strains. *Bioresource Technology*, 96 (17), 1897–1906.
- Koops, H.-P.; Pommerening-Röser, A., 2001, Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. *FEMS Microbiology Ecology*, 37 (1), 1–9.
- McDonald, J.H. 2014. *Handbook of Biological Statistics* (3rd ed.). Sparky House Publishing, Baltimore, Maryland.
- Prosser, J. I., 1989, Autotrophic nitrification in bacteria. *Advances in Microbial Physiology*, 30, 125–181.
- Seesuriyachan, P.; Kuntiya, A.; Sasaki, K.; Techapun, C., 2009, Biocoagulation of dairy wastewater by *Lactobacillus casei* TISTR 1500 for protein recovery using micro-aerobic sequencing batch reactor (micro-aerobic SBR). *Process Biochemistry*, 44 (4), 406–411.
- Shannon, M. A.; Bohn, P. W.; Elimelech, M.; Georgiadis, J. G.; Mariñas, B. J.; Mayes, A. M., 2008, Science and technology for water purification in the coming decades. *Nature*, 452 (7185), 301–310.
- Schmidt, I.; Sliemers, O.; Schmid, M.; Bock, E.; Fuerst, J.; Kuenen, J. G.; Jetten, M.S.M.; Strous, M., 2003, New concepts of microbial treatment processes for the nitrogen removal in wastewater. *FEMS Microbiology Reviews*, 481–492.

Schmidt, S. P.; Basolo, F.; Trogler, W. C., 1987, Reactions between dimanganese, dirhenium, and manganese–rhenium decacarbonyl and oxidants. *Inorg. Chim. Acta*, 131 (2), 181–189.

Schreiber, F., 2009, Detecting and Understanding Nitric Oxide Formation during Nitrogen Cycling in Microbial Biofilms. Universitaet Bremen, Brmen.

Shapleigh, J., 2006, The Denitrifying Prokaryotes. In *The Prokaryotes*, Dworkin, M.; Falkow, S.; Rosenberg, E.; Schleifer, K.-H.; Stackebrandt, E., Eds. Springer New York; 769–792.

Strous, M.; Fuerst, J. A.; Kramer, E. H.; Logemann, S.; Muyzer, G.; van de Pas-Schoonen, K. T.; Webb, R.; Kuenen, J. G.; Jetten, M. S., 1999, Missing lithotroph identified as new planctomycete. *Nature* 400 (6743), 446–449.

Third, K. A.; Sliemers, A. O.; Kuenen, J. G.; Jetten, M. S., 2001, The CANON system (Completely Autotrophic Nitrogen-removal Over Nitrite) under ammonium limitation: interaction and competition between three groups of bacteria. *Systematic and Applied Microbiology* 24 (4), 588–596.

Tramper, J.; Grootjen, D. R. J., 1986, Operating performance of *Nitrobacter agilis* immobilized in carrageenan. *Enzyme and Microbial Technology* 8 (8), 477–480.

Verbaendert, I.; Boon, N.; De Vos, P.; Heylen, K., 2011, Denitrification is a common feature among members of the genus *Bacillus*. *Syst Appl Microbiol* 34 (5).



www.biowishtech.com
BiOWiSH™ is a registered trademark of BiOWiSH Technologies, Inc. v2