Heliyon 6 (2020) e03711

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Aeromonas hydrophila, Bacillus thuringiensis, Escherichia coli and Pseudomonas aeruginosa utilization of Ammonium-N, Nitrate-N and Urea-N in culture

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ARTICLE INFO

Keywords:
Agriculture
Microbiology
Agricultural soil science
Agricultural water management
Bacteria
Nitrogen
Utilization
Ammonium
Nitrate
Urea

ABSTRACT

Agricultural drainage ditches represent a major source of nutrient pollution. Shifts in nitrogen source and use of animal manures have changed the bacterial composition both in species of bacteria and their abundance in agricultural ditches. This change affects how nitrogen is being cycled and potentially the final forms of available nutrients. In particular, animal manures often have bacteria such as *Escherichia coli* present, increasing the abundance of a bacterial species in ditches. Research has shown that the effect of different nitrogen sources is to change bacterial community composition (class, family). How this influences the role of an individual bacterial species is poorly understood. Thus, our question was how individual species would respond to different sources of nitrogen. We used *Aeromonas hydrophila, Bacillus thuringiensis*, *Escherichia coli* and *Pseudomonas aeruginosa* that are common in agricultural ditches and exposed them to different concentrations of nitrogen in cultures of 1×10^{0} and 1×10^{-1} dilutions from a stock solution of bacteria. Nitrogen sources were ammonium chloride, sodium nitrate and urea. The results showed *A. hydrophila* and *E. coli* have strong similarities particularly with nitrate-N and urea-N utilization and the response was often correlated with the amount of nutrient added. *P. aeruginosa* while similar did not show any strong correlation with amount of nutrient added. *B. thuringiensis* was different from the other three bacteria in utilization or production. Research has provided insight into the role of some bacteria in nitrogen cycling and may be valuable in the future to developing management strategies to reduce nutrients.

1. Introduction

Nitrogen as ammonium, nitrate or urea have been the major source of non-point pollution in aquatic ecosystems (Land, 2012; Yan et al., 2017). The principle route for nutrient removal and transformation into nitrogen gases is by way of bacteria, which represent the major constituent of the organisms in aquatic ecosystem (McLellan et al., 2015; Mendes et al., 2015; Zeng et al., 2016). Over the past three decades, there has been a shift from ammonia and nitrate based fertilizers to urea as a nitrogen source in agriculture. Urea has been implicated as a source for domoic acid (a potent neurotoxin) which is produced by *Pseudo-nitzschia* spp. (Glibert et al., 2014). This shift has been driven by regulatory and environmental concerns (Glibert et al., 2014; Cahill et al., 2017). There have been some concerns about animal manures, particularly poultry litter, which are the primary source of fertilizer on the Delmarva Peninsula (Howarth et al., 2002; Smil, 2002). This is particularly true in agricultural drainage systems where nitrogen inputs periodically exceed those of natural systems such as forest, grassland or wetland drainages (Campbell et al., 2017). It is important to know the capacity for individual bacteria to break down nitrogen in fertilizers as this may provide important clues for improving nutrient management.

Field studies have shown that when nitrogen sources are changed, microbial communities change in their composition (Yan et al., 2017) with the gain or loss of specific bacteria (Lv et al., 2017; Yan et al., 2017). The use of poultry litter and other forms of animal manures has added bacteria to agricultural soils and ditch sediments (Maeda et al., 2011; Kostadinova et al., 2014) and altered the dominance of specific bacteria in these systems (Xu et al., 2013). Such shifts in bacterial dominance will have an impact on the cycling of nutrients (Fields, 2004). The effect of the environment on bacterial community composition is known (Espenberg et al., 2018) however, more work is needed to understand the

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https://doi.org/10.1016/j.heliyon.2020.e03711
Received 13 January 2020; Received in revised form 10 February 2020; Accepted 26 March 2020
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role of individual bacterial species in nutrient cycling (Jacoby et al., 2017).

The bacteria (*Aeromonas hydrophila, Bacillus thuringiensis*, *Escherichia coli* and *Pseudomonas aeruginosa*) chosen for this study was because they have been routinely isolated from sediments of forest and agricultural drainages as well as tributaries of the Chesapeake Bay (unpublished data). *A. hydrophila* is a heterotrophic, gram negative, rod-shaped bacterium belonging to the Gammaproteobacteria. It is present in fresh or brackish water and can survive using nitrate under aerobic and anaerobic conditions (Tan et al., 2015; Talagrand-Reboul et al., 2017). *B. thuringiensis* is a gram-positive, rod-shaped soil bacterium belonging to the *Bacilli* and can survive in extreme environments (Slonczewski and Foster, 2011) and shown to utilize ammonium in the production of spores (Yusoff et al., 2003). *E. coli* is a rod-shaped, gram-negative bacterium belonging to the Gammaproteobacteria and is common in the environment, foods, and intestines of animals (Tenaillon et al., 2010). It is being continuously added to the soils by way of poultry litter, manures (Ishii and Sadowsky, 2008), and has been shown to use varying sources of nitrogen under aerobic and anaerobic conditions (Brown et al., 2014). *P. aeruginosa* is a gram-negative, rod-shaped bacterium belonging to the Gammaproteobacteria. It is nutritionally very versatile responding to different nitrogen sources (Geisseler et al., 2010) and can survive in nutritionally poor waters (Itah and Essien, 2005).

The objective of this study was to determine the response of the four bacteria noted above to ammonium-N, nitrate-N and urea-N under controlled conditions. The parameters examined were final concentration, change in concentration of added nitrogen and nitrogen metabolites as well as growth as a function of change in optical density (OD).

2. Methods

2.1. Bacteria selection

The bacteria chosen for this study were isolated from a private farm on the Delmarva Peninsula in the spring, summer and fall of 2016 and 2017. Grab samples from the sediment surface layer of ditches were taken and transferred to sterile bags on ice. Twenty-five gram subsamples were mixed with 100mL of buffered peptone water (BPW) and incubated overnight in a 37 °C shaker. Aliquots of the subsamples were then mixed with BPW (1:9, V:V) incubated overnight. One-μl aliquots streaked on nutrient agar, incubated overnight, with isolated colonies stained with the Gram's stain. All bacteria were presumptively identified using BIOLOG Gen III™ microplates and microbial identification systems software (Hayward, CA) following the manufacturer's instructions. To ensure that we are using well stablished strains of the species identified we obtained pure cultures from USDA that had been sequenced and these were compared with the isolated bacteria from the field.

2.2. Determination of growth

To avoid exogenous sources of dissolved organic nitrogen (DON) we chose to use 2% (w/v) maltose as the carbon source. In a previous study, we exposed each of the four bacteria to maltose, glucose and lactose at 2% (w/v) in distilled water from which it was determined that 2% (w/v) maltose exhibited the best growth rates which is consistent with the literature (unpublished data, Shu et al., 2014). To evaluate growth, we used OD as an approximate measure as have other authors (Takahashi, 2016; Taabodi et al., 2019). At the beginning and end of each test, OD was measured and the change in OD calculated by subtracting initial from final OD. Using 2% (w/v) maltose, we evaluated growth over 36 h and determined that all four bacteria were in their growth phase after 24 h, and this length of incubation was considered appropriate for this study. It was felt to be important that the bacteria were in the middle of their growth phase. As part of the study, we examined growth rates using four dilutions (10^0, 10^{-1}, 10^{-2} and 10^{-3}). From this, we determined that 10^0 and 10^{-1} had the highest percent change in OD after 24 h. The best growth performances were at 10^0 and 10^{-1} whereas 10^{-2} and 10^{-3} showed very poor growth performance and were not used for the study.

2.3. Nutrient response

Nitrogen sources used were ammonium-N as ammonium chloride, nitrate-N as sodium nitrate and urea-N as reagent grade urea, which are the most common sources of nitrogen used as fertilizers by agriculture (Zhang et al., 2016; USDA, 2017). For ammonium chloride we added 15.7, 31.4 and 62.8mg-N/L to the media which yielded concentrations of 5, 10 and 20mg-N/L ammonium-N; and sodium nitrate at 6.85, 13.7 and 27.4mg-N/L which yielded concentrations of 5, 10 and 20mg-N/L nitrate-N, respectively. Urea was added at 4.34, 8.68 and 17.36ug-N/L to yield concentrations of 0.1, 0.2 and 0.3mg-N/L urea-N, respectively. These concentrations are consistent with those observed in the field (Kibet et al., 2016; King et al., 2017). To each concentration of nutrient (5, 10 and 20; 0.1, 0.2 and 0.3mg-N/L), we added 1×10^6 bacteria/mL which is designated as 10^0 and 1×10^5 bacteria/mL which is designated as 10^{-1}. Cultures were incubated at 37 °C for 24 h. Following incubation cultures were filtered for urea-N using glass filters (0.45μm) and for ammonium-N and nitrate-N using paper filters (0.45μm). Filtered samples were stored at 4 °C and analyzed within 24 h following filtration. The concentrations of ammonium-N, nitrate-N and urea-N were determined using the LACHAT QuickChem™ (8000 series) method 10-107-04-1-A (Diamond, 1995). Controls for this study were cultures of each concentration of nutrient (ammonium-N, nitrate-N and urea-N) without bacteria in 2% maltose and incubated with corresponding test cultures. Changes in nutrient concentrations were calculated by subtracting final from control concentrations. It is important to note that mixed cultures were avoided, as it was uncertain about the proportion present in sediments and the growth rate between the bacteria. While this would have resulted in data, it would not provide any idea of which bacteria is responsible for the change in nutrient concentrations. This also applies to mixed nutrients as the study was focused on single nutrient source.

2.4. Statistical analysis

All data was recorded and exported to Excel files as mg-N/L. Data was analyzed as ammonium-N and nitrate-N (5, 10, and 20mg-N/L) and urea-N (0.1, 0.2 and 0.3mg-N/L), which resulted in $4 \times 3 \times 3$ matrix (4 bacteria, 3 nutrients and 3 concentrations). Analyses were performed using SAS version 9.4 (SAS Institute, 2012). Differences in nutrient utilization by bacteria data were assessed for normality to support testing with parametric statistics (Proc Univariate). Treatment differences were evaluated by Student's two sample *t* test (Proc TTest). Although most pairings had equal variances, the Satterhwaite option was used to detect differences in all comparisons. In reporting results, production of a nutrient will result in positive number and utilization negative. Treatment differences discussed in the text were considered significant at $p \leq 0.05$.

3. Results

3.1. Ammonium chloride

At 10^0 *A. hydrophila* showed significant utilization of ammonium-N at 5 ($p < 0.0403$) and 10mg-N/L ($p < 0.0493$) with differences of -0.974 ± 0.752 and -1.666 ± 0.867, respectively (Figure 1, Table 1). There was some indication that *A. hydrophila* at 10^{-1} did utilize ammonium-N at 10 and 20mg-N/L with differences of -0.574 ± 0.354 and -1.014 ± 0.730, but neither was considered significant (Figure 1, Table 2). At 20 mg-N/L, there was significant variation between repetitions and as such that data considered invalid. The ODs for *A. hydrophila* at 10^0 and 10^{-1} were different with 10^0 exhibiting higher growth, however between concentrations there was little difference in growth at each dilution (Tables 3

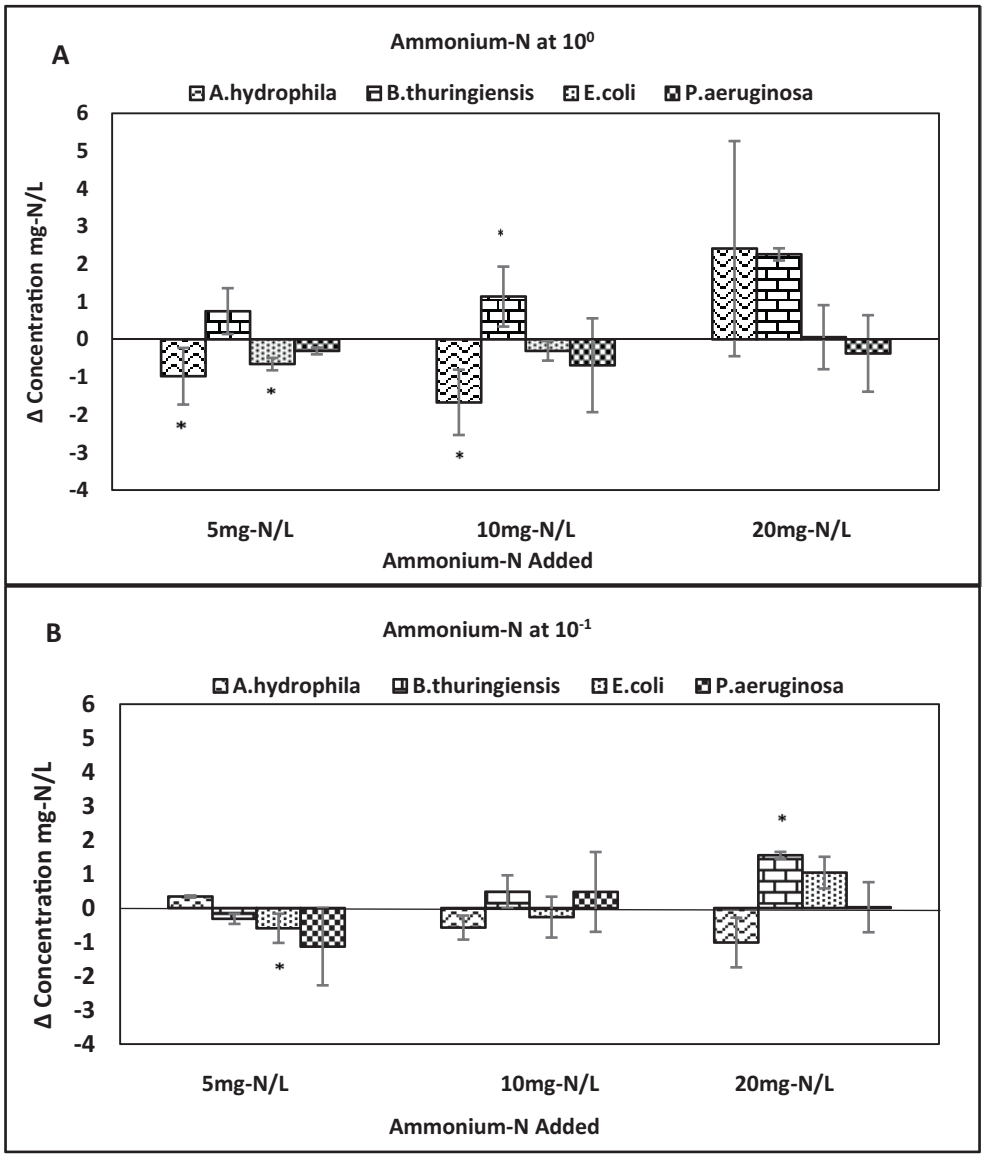

Figure 1. Change in ammonium-N concentrations (Δ) when ammonium chloride was added to the media at 5, 10 and 20mg-N/L at bacterial dilutions of 10^0 (A) and 10^{-1} (B). Values below zero indicates utilizations and values above zero production. The asterisks designate significant difference ($p < 0.05$) between final concentrations and initial control concentration.

and 4). At 10^0 *B. thuringiensis* consistently produced ammonium-N at all three concentrations with a trend of increasing production along with increased concentrations. Change in concentrations were 0.754 ± 0.609, 1.139 ± 0.795 and 2.258 ± 0.162 at 5, 10 and 20mg-N/L, respectively (Figure 1, Table 1). Only at 10mg-N/L was production significantly different from the control ($p < 0.0287$). At 10 and 20 mg-N/L *B. thuringiensis* at 10^{-1} also produced ammonium-N with differences of 0.479 ± 0.484 and 1.553 ± 0.100 at 10 and 20mg-N/L, respectively with 20mg-N/L considered significant ($p < 0.0002$) (Figure 1, Table 2). With *B. thuringiensis* at 10^0 ODs were not significantly different however at 10^{-1} there was a clear difference with *B. thuringiensis* growing best at 5mg-N/L with a changes in OD of 0.106 ± 0.009 as opposed to 0.067 ± 0.017 and 0.065 ± 0.010 at 10 and 20mg-N/L, respectively (Tables 3 and 4). At 10^0 and 10^{-1} at 5 and 10mg-N/L *E. coli* utilized ammonium-N with differences of -0.653 ± 0.164 and -0.301 ± 0.259 at 10^0; and -0.596 ± 0.429 and -0.267 ± 0.601 at 5 and 10mg-N/L, respectively. The difference was significant for both dilutions at 5mg-N/L ($p < 0.0016$; $p < 0.0460$) (Figure 1, Tables 1 and 2). For both 10^0 and 10^{-1} *E. coli* showed the greatest change in OD at 20mg-N/L with differences of 0.134 ± 0.088 and 0.073 ± 0.072, respectively (Tables 3 and 4). With *P.* aeruginosa at both 10^0 and 10^{-1} dilutions, there was significant variation between replicates and as such no trends could be seen and none of the differences were significant (Figure 1, Tables 1 and 2). At 10^0 for *P. aeruginosa* there was little difference in ODs between concentrations, at 10^{-1} they were also close however there was significant variation between replicates (Tables 3 and 4).

3.2. Sodium nitrate

At both dilutions and all three concentrations, *A. hydrophila* was shown to utilize nitrate-N. At 20mg-N/L there was significant variation between replicates for both dilutions. At 10^0 differences were -0.953 ± 0.212 and -1.586 ± 0.296 at 5 and 10mg-N/L, respectively, and both were statistically significant ($p < 0.0005$; $p < 0.0007$) (Figure 2, Table 1). At 10^{-1} there was utilization with differences of -1.151 ± 0.132 and -1.619 ± 0.416 at 5 and 10mg-N/L, respectively, however only 5mg-N/L was significant ($p < 0.0500$) (Figure 2, Table 2). At 20mg-N/L, like ammonium there was significant variation between replicates at both

Table 1. Response to each nutrient by all four bacteria at all three concentrations at 10^0.

Dilution 10^0				
	Ammonium-N	Nitrate-N	Urea-N	
A. hyrophila				
5mg-N/L	-0.974 ± 0.752*	-0.953 ± 0.212*	-0.038 ± 0.012	0.1mg-N/L
10mg-N/L	-1.666 ± 0.867*	-1.586 ± 0.296*	-0.065 ± 0.046	0.2mg-N/L
20mg-N/L	2.411 ± 2.853	-0.727 ± 1.505	-0.149 ± 0.004	0.3mg-N/L
B. thuringiensis				
5mg-N/L	0.754 ± 0.609	-0.030 ± 0.325	Zero control*	0.1mg-N/L
10mg-N/L	1.139 ± 0.795*	-0.111 ± 0.455	-0.072 ± 0.003*	0.2mg-N/L
20mg-N/L	2.258 ± 0.162	0.022 ± 0.959	-0.169 ± 0.019*	0.3mg-N/L
E. coli				
5mg-N/L	-0.653 ± 0.164*	-0.960 ± 0.186*	-0.030 ± 0.062	0.1mg-N/L
10mg-N/L	-0.301 ± 0.259	-0.809 ± 0.464*	-0.066 ± 0.024*	0.2mg-N/L
20mg-N/L	0.062 ± 0.850	0.039 ± 0.496	-0.133 ± 0.021*	0.3mg-N/L
P. aeruginosa				
5mg-N/L	-0.303 ± 0.091	-2.456 ± 0.312*	-0.080 ± 0.011*	0.1mg-N/L
10mg-N/L	-0.683 ± 1.244	-2.388 ± 0.409*	-0.181 ± 0.009*	0.2mg-N/L
20mg-N/L	-0.369 ± 1.014	-1.886 ± 0.462*	-0.267 ± 0.007*	0.3mg-N/L

The asterisks designate significant difference ($p < 0.05$) between final concentrations and initial control concentrations.

Table 2. Response to each nutrient by all four bacteria at all three concentrations at 10^{-1}.

Statistic Summary Table 10^{-1}				
	Ammonium-N	Nitrate-N	Urea-N	
A. hyrophila				
5mg-N/L	0.339 ± 0.032	-1.151 ± 0.132*	-0.062 ± 0.002	0.1mg-N/L
10mg-N/L	-0.574 ± 0.354	-1.619 ± 0.416	-0.146 ± 0.001*	0.2mg-N/L
20mg-N/L	-1.014 ± 0.730	-0.329 ± 1.704	-0.198 ± 0.024*	0.3mg-N/L
B. thuringiensis				
5mg-N/L	-0.317 ± 0.150	-0.192 ± 0.090	Zero control*	0.1mg-N/L
10mg-N/L	0.479 ± 0.484	0.144 ± 0.127	-0.020 ± 0.002	0.2mg-N/L
20mg-N/L	1.553 ± 0.100*	0.037 ± 0.375	-0.118 ± 0.023*	0.3mg-N/L
E. coli				
5mg-N/L	-0.596 ± 0.429*	-0.557 ± 0.141*	-0.077 ± 0.021*	0.1mg-N/L
10mg-N/L	-0.267 ± 0.601	-0.202 ± 0.121	-0.180 ± 0.001*	0.2mg-N/L
20mg-N/L	1.043 ± 0.465	-0.011 ± 0.112	-0.236 ± 0.003*	0.3mg-N/L
P. aeruginosa				
5mg-N/L	-1.135 ± 1.138	-0.298 ± 0.167	-0.072 ± 0.029*	0.1mg-N/L
10mg-N/L	0.475 ± 1.174	-0.494 ± 0.212	-0.170 ± 0.015*	0.2mg-N/L
20mg-N/L	0.024 ± 0.737	-0.162 ± 0.076	-0.121 ± 0.035*	0.3mg-N/L

The asterisks designate significant difference ($p < 0.05$) between final concentrations and initial control concentration.

dilutions and thus considered invalid. Growth was best for *A. hydrophila* at 20mg-N/L for 10^0 and 10^{-1} with changes in OD of 0.233 ± 0.310 and 0.173 ± 0.273, respectively (Tables 3 and 4). The differences in nitrate-N for *B. thuringiensis* were very small at both 10^0 and 10^{-1} for all three concentrations and none were significant (Figure 2, Tables 1 and 2). At both 10^0 and 10^{-1} *B. thuringiensis* did not show any significant differences in OD between concentrations and between dilutions at each concentration (Tables 3 and 4). For both dilutions *E. coli* utilized nitrate-N at 5 and 10mg-N/L, but no real change was seen at 20mg-N/L for either dilutions. At 10^0 utilization for both 5 and 10mg-N/L was significant with differences of -0.960 ± 0.186 and -0.809 ± 0.464, respectively ($p < 0.0039$; $p < 0.0286$) (Figure 2, Table 1). At 5mg-N/L for 10^{-1} there was a significant difference from the control ($p < 0.0020$) with a change of -0.557 ± 0.141 at 5mg-N/L (Figure 2, Table 2). No differences were seen at 10^0 and 10^{-1} in ODs for *E. coli* between concentrations and changes in OD between dilutions were also very similar (Tables 3 and 4). At all three concentrations there was a significant utilization of nitrate-N by *P. aeruginosa* at 10^0, even though there was little difference in amount of utilization between concentrations. The changes were -2.456 ± 0.312, -2.388 ± 0.409 and -2.390 ± 0.932 at 5, 10 and 20mg-N/L, respectively ($p < 0.0001$; $p < 0.0132$; $p < 0.0045$) (Figure 2, Table 1). The changes at 10^{-1} in nitrate-N were to utilize but none were significant (Figure 2, Table 2). Only at 10^{-1} was there a significant difference in growth for *P. aeruginosa* with the change in OD of 0.124 ± 0.165 (Tables 3 and 4).

3.3. Urea

At both 10^0 and 10^{-1}, *A. hydrophila* utilized urea-N and in both cases as concentrations of ammonium chloride increased as the amount of utilization of ammonium-N increased (Figure 3). Only at 10^{-1} for 0.2 and 0.3mg-N/L were changes significant ($p < 0.0244$; $p < 0.0001$) with values of.

-0.146 ± 0.001 and -0.198 ± 0.024, respectively (Figure 3, Table 2). While *A. hydrophila* grew best at all concentrations at 10^0 there was little

Table 3. Optical density response by all four bacteria at all three concentrations at 10^0.

Change in Optical Density at 10^0 (mean ± SD) in mg-N/L				
	Ammonium-N	Nitrate-N	Urea-N	
A. hyrophila				
5mg-N/L	0.057 ± 0.002	0.032 ± 0.004	0.055 ± 0.001	0.1mg-N/L
10mg-N/L	0.060 ± 0.006	0.046 ± 0.013	0.055 ± 0.005	0.2mg-N/L
20mg-N/L	0.050 ± 0.004	0.233 ± 0.310	0.042 ± 0.007	0.3mg-N/L
B. thuringiensis				
5mg-N/L	0.102 ± 0.010	0.085 ± 0.004	0.126 ± 0.007	0.1mg-N/L
10mg-N/L	0.094 ± 0.039	0.080 ± 0.005	0.125 ± 0.006	0.2mg-N/L
20mg-N/L	0.112 ± 0.009	0.084 ± 0.014	0.123 ± 0.011	0.3mg-N/L
E. coli				
5mg-N/L	0.040 ± 0.013	0.036 ± 0.008	0.053 ± 0.005	0.1mg-N/L
10mg-N/L	0.019 ± 0.005	0.037 ± 0.004	0.061 ± 0.015	0.2mg-N/L
20mg-N/L	0.134 ± 0.088	0.036 ± 0.007	0.065 ± 0.009	0.3mg-N/L
P. aeruginosa				
5mg-N/L	0.021 ± 0.003	0.031 ± 0.006	0.020 ± 0.003	0.1mg-N/L
10mg-N/L	0.020 ± 0.003	0.028 ± 0.003	0.026 ± 0.004	0.2mg-N/L
20mg-N/L	0.017 ± 0.005	0.038 ± 0.011	0.024 ± 0.002	0.3mg-N/L

Table 4. Optical density response by all four bacteria at all three concentrations at 10^{-1}.

Change in Optical Density 10^{-1} (mean ± SD) in mg-N/L				
	Ammonium-N	Nitrate-N	Urea-N	
A. hyrophila				
5mg-N/L	0.027 ± 0.005	-0.001 ± 0.002	0.011 ± 0.032	0.1mg-N/L
10mg-N/L	0.040 ± 0.001	0.047 ± 0.072	0.014 ± 0.042	0.2mg-N/L
20mg-N/L	0.034 ± 0.003	0.173 ± 0.273	0.012 ± 0.035	0.3mg-N/L
B.thuringiensis				
5mg-N/L	0.106 ± 0.009	0.104 ± 0.010	0.055 ± 0.164	0.1mg-N/L
10mg-N/L	0.067 ± 0.017	0.085 ± 0.007	0.048 ± 0.143	0.2mg-N/L
20mg-N/L	0.065 ± 0.010	0.098 ± 0.029	0.047 ± 0.140	0.3mg-N/L
E. coli				
5mg-N/L	0.040 ± 0.009	0.023 ± 0.003	0.013 ± 0.000	0.1mg-N/L
10mg-N/L	0.040 ± 0.001	0.020 ± 0.002	0.016 ± 0.003	0.2mg-N/L
20mg-N/L	0.073 ± 0.072	0.024 ± 0.010	0.027 ± 0.002	0.3mg-N/L
P. aeruginosa				
5mg-N/L	0.043 ± 0.129	0.124 ± 0.165	0.006 ± 0.002	0.1mg-N/L
10mg-N/L	0.042 ± 0.127	0.020 ± 0.005	0.014 ± 0.007	0.2mg-N/L
20mg-N/L	0.030 ± 0.089	0.037 ± 0.011	0.011 ± 0.006	0.3mg-N/L

difference between ODs at each concentration for each dilution (Tables 3 and 4). For *B. thuringiensis* changes in concentrations were significant only at 0.2 and 0.3mg-N/L for both dilutions ($p < 0.0001$; $p < 0.0001$). At 10^0 changes were -0.072 ± 0.003 and -0.169 ± 0.019; and at 10^{-1} -0.020 ± 0.002 and -0.118 ± 0.023 at 0.2 and 0.3 mg-N/L, respectively (Figure 3, Table 2) with only 0.3mg-N/L significant ($p < 0.0044$). Again, *B. thuringiensis* grew best at 10^0 but there was little difference in change of OD between concentrations for each dilution (Tables 3 and 4). Urea-N utilization was significant at 10^0 for E.*coli* at 0.2 and 0.3mg-N/L with changes of -0.066 ± 0.024 and -0.133 ± 0.021, respectively ($p < 0.0085$; $p < 0.0101$) (Figure 3, Table 1). At 10^0, 0.1mg-N/L there was significant variation between repetitions and thus this data was considered invalid. All three concentrations at 10^{-1} showed significant differences ($p < 0.0028$; $p < 0.0001$; $p < 0.0001$) with concentrations of -0.077 ± 0.021, -0.180 ± 0.001 and -0.236 ± 0.003 at 0.1, 0.2 and 0.3mg-N/L, respectively (Figure 3, Table 2). As previous, *E.coli* grew best at 10^0 with little difference between concentrations for both dilutions (Tables 3 and 4). There was significant utilization of urea-N by *P. aeruginosa* at both dilutions with differences of -0.080 ± 0.011, -0.181 ± 0.009 and -0.267 ± 0.007 at 0.1, 0.2 and 0.3mg-N/L, respectively ($p < 0.0003$; $p < 0.0001$; $p < 0.0001$) (Figure 3, Tables 1 and 2). At 10^{-1} differences were greater at -0.072 ± 0.029, -0.170 ± 0.015 and -0.121 ± 0.035 at 0.1, 0.2 and 0.3mg-N/L, respectively ($p < 0.0158$; $p < 0.0001$; $p < 0.0319$) (Figure 3, Table 2). As previous *P. aeruginosa* grew best at 10^0 with little differences between concentrations for both dilutions (Tables 3 and 4).

3.4. Comparison of responses to ammonium-N, nitrate-N and urea-N

In comparing individual species responses to ammonium-N, *A. hydrophila* and *E. coli* utilized, while *B. thuringiensis* produced ammonium-N. There was no trend seen with *P. aeruginosa* due to significant variation between replicates. Significant rates of utilization for *A. hydrophila* and *E. coli* at 10^0 occurred at 5 and 10mg-N/L and for 10^{-1} only for *E.coli* at 5mg-N/L. On the other hand, *B. thuringiensis* showed significant production at 10mg-N/L at 10^0 and 20mg-N/L at 10^{-1}. Both *B. thuringiensis* and *P. aeruginosa* did not show any differences in growth between dilutions. The greatest growth occurred for *A. hydrophila* at 10^0 but only at 5mg-N/L, while *B. thuringiensis* grew best at 5mg-N/L for both

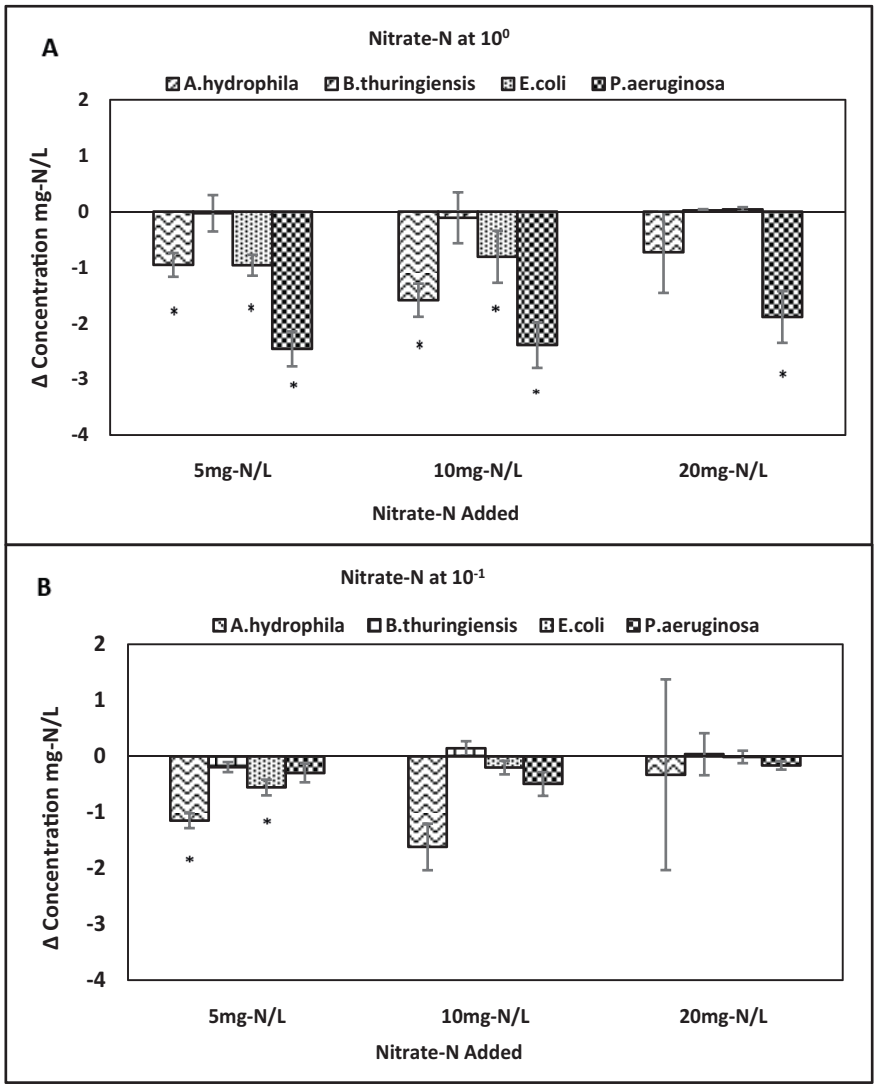

Figure 2. Change in nitrate-N concentrations (Δ) when sodium nitrate was added to the media at 5, 10 and 20mg-N/L at bacterial dilutions of 10^0 (A) and 10^{-1} (B). Values below zero indicates utilizations and values above zero production. The asterisks designate significant difference ($p < 0.05$) between final concentrations and initial control concentration.

dilutions and *E. coli* exhibited the greatest growth at 20mg-N/L for both dilutions. With nitrate-N, *A. hydrophila*, *E. coil* and *P. aeruginosa* utilized nitrate-N at significant levels for 5 and 10 mg-N/L at 10^0, while *P. aeruginosa* also at 20mg-N/L. A similar trend in utilization was seen at 10^{-1} but only to a lesser degree with only *A. hydrophila* and *E. coli* showing significant utilization. Changes in OD indicated that *A. hydrophila* grew best at 20mg-N/L while *P. aeruginosa* growth best at 5mg-N/L at 10^{-1} but not at 10^0. Neither *B. thuringiensis* nor *E. coli* appeared to respond in growth with ODs similar at both dilutions at all concentrations. The response to urea-N was most significant at 10^{-1} with all four bacteria showing significant utilization at all concentrations with the exception of *B. thuringiensis* at 0.1mg-N/L at 10^{-1}. There were fewer instances of significant utilization at 10^0 with only *B. thuringiensis*, *E. coli*, and *P. aeruginosa* showing significant utilization. Growth for all four bacteria was not different between concentrations and only *B. thuringiensis* and *P. aeruginosa* exhibited a greater growth at 10^0.

4. Discussion

Agricultural drainage ditches represents a major source of non-point pollution in water adjacent to open waters (Glibert et al., 2014). Our previous research focused on the presence of urea in the Chesapeake Bay, the result of which indicated the need to better understand how microbial communities influence nitrogen cycling (Kibet et al., 2016; Zhang et al., 2016; King et al., 2017). The use of animal manures and inorganic nitrogen has been shown to alter microbial communities both in agriculture soil and drainage ditches (Maeda et al., 2011; Xu et al., 2013; Kostadinova et al., 2014). Our surveys of sediments from ditches and tributaries has shown that *A. hydrophila, B. thuringiensis, E. coli* and *P. aeruginosa* were the most frequently found and thus ideal for this study.

Approaches to investigating the nitrogen cycle in agricultural systems include combinations of metagenomics and transcriptomics, mesocosms and constructed ditches; but all have failed to provide definitive information (Nayfach and Pollard, 2016; Zhang et al., 2018). Using individual bacteria and exposing them to common sources of fertilizer is the logical first. Ammonium-N, nitrate-N and urea-N are the constituents of most agricultural fertilizers (USDA, 2017; Zhang et al., 2018) and were coupled with the use of a simple low carbon medium, which is characteristic of most agricultural ditches (Zhang et al., 2016; Faust et al., 2018). The purpose of this study was to determine how each of the four selected bacteria would respond to inorganic sources of nitrogen and urea. Even from the early literature, it was difficult to determine how bacteria respond to sources of nitrogen when added to media, particularly when they are the sole source of nitrogen. Since 1970, the focus of

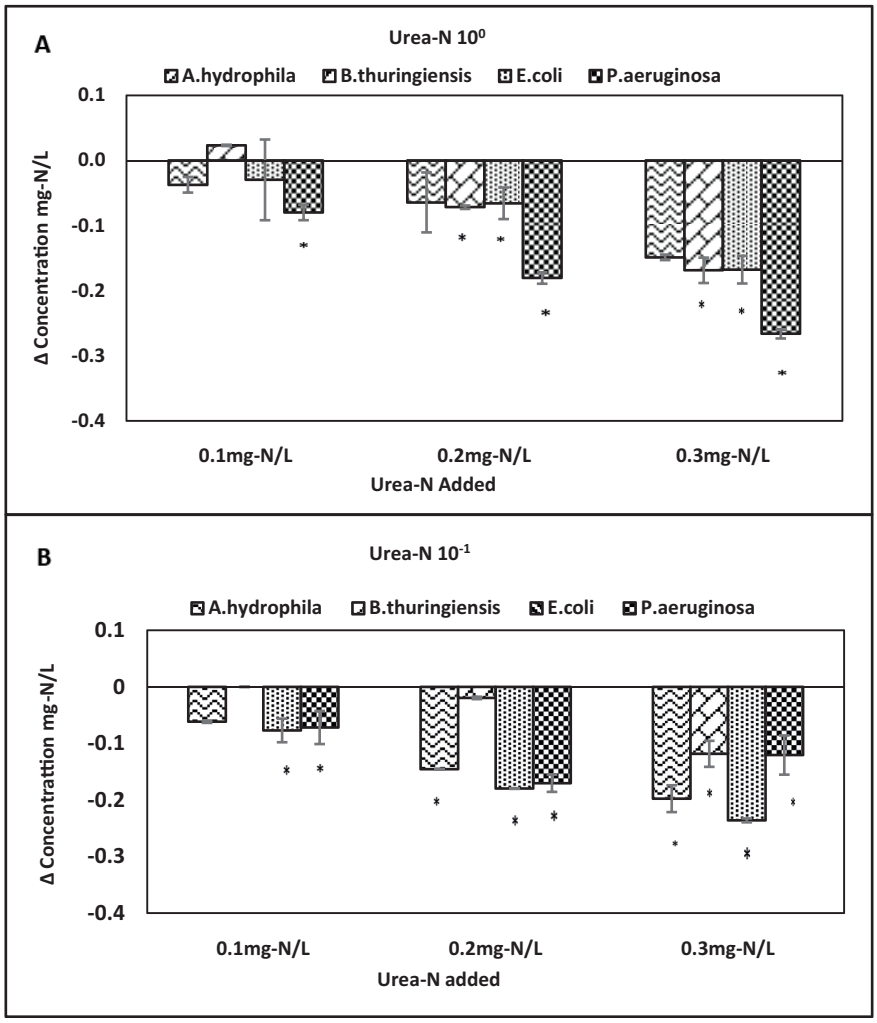

Figure 3. Change in urea-N concentrations (Δ) when reagent grade urea was added to the media at 0.1, 0.2 and 0.3mg-N/L at bacterial dilutions of 10^0 (A) and 10^{-1} (B). Values below zero indicates utilizations and values above zero production. The asterisks designate significant difference ($p < 0.05$) between final concentrations and initial control concentration.

research has been on genetic pathways through either genomics or transcriptomics, which has not provided specific information on response. Long term impact of this research may be that those bacteria found to exhibit significant utilization of a nutrient, such as nitrate or urea, they can either be promoted by manipulating environmental conditions or seeded into ditches to increase their relative numbers.

4.1. Ammonium-N

The Gammaproteobacteria are known to oxidize ammonium-N by way of ammonium monooxygenase (Abbott et al., 2003). At 100 there was consistent utilization of ammonium-N by A. *hydrophila*, *E. coli* and *P. aeruginosa* at 5 and 10mg-N/L with *A. hydrophila* and *E. coli* showing some significant utilization (Figure 1). Both *A. hydrophila* and *E. coli* have been shown to utilize ammonium-N (Messyasz et al., 2015; Taabodi et al., 2019). While *A. hydrophila* tends to oxidize ammonium-N anaerobically, there is evidence it can aerobically oxidize ammonium-N (Fernandez et al., 2009). It has been demonstrated to have glutamate synthase, which is involved in ammonium assimilation (Hasan et al., 2006). Likewise, *E.coli* has been shown to reduce ammonium-N (Brown et al., 2014; Shimizu, 2014) and is capable of assimilating ammonium-N (Nygaard et al., 2016). At exposures of 20mg-N/L, *A. hydrophila, E. coli* and *P. aeruginosa* varied significantly in their response suggesting that this concentration was too high. There is nothing in the literature to suggest how *B. thuringiensis* would respond to ammonium-N and we would have expected either no response or utilization. It exhibited production and since ammonium-N was the only nitrogen source we must assume that ammonium-N was produced as metabolic byproduct as dissolved organic nitrogen from dead bacteria. Changes in OD for *A. hydrophila* and *E. coli* increased as ammonium-N concentrations increased which supports the idea that they use ammonium-N as a nitrogen source. Poor growth was exhibited by *B. thuringiensis* which further supports that ammonium-N is not a preferred source of nitrogen.

4.2. Nitrate-N

Common to the Gammaproteobacteria is the presence of cytosolic nitrate reductase which is active under aerobic condition (Tiso and Schechter, 2015; Shao et al., 2016). All three Gammaproteobacteria had utilization of nitrate-N at 100 for 5 and 10mg-N/L and utilization at 10-1 (Figure 2). Nitrate is utilized by *A. hydrophila* through nitrate reductase (Gobi et al., 2018; Anburajan et al., 2019) and *E.coli* can assimilate nitrates (van Heeswijk et al., 2013) and possesses nitrate reductase (Tiso and Schechter, 2015). In addition, *P. aeruginosa* is an aerobic nitrate-reducing organism (Rodriguez et al., 2017). It has been shown that *B. thuringiensis* utilizes nitrate anaerobically (Nygaard et al., 2016) but under aerobic conditions of this study, there was minimal response. There was no significant difference in growth between *A. hydrophila*,

B. thuringiensis and *E.coli* but *P. aeruginosa* showed the greatest change in OD at 5mg-N/L.

4.3. Urea-N

Many of the Gammaproteobacteria have genes that allow for assimilation of urea (Solomon et al., 2010; Widner et al., 2018). There was significant utilization of urea at all concentrations for all four bacteria and the amount of utilization correlated with concentration with higher rates of utilization at higher concentrations. This is consistent with literature in that most bacteria, particularly the four studied, can utilize urea. Genomics has identified urease genes in *A. hydrophila* and *E.coli* (Seshadri et al., 2006). Environmental and gut microflora studies have shown these bacteria can assimilate urea (Sigurdarson et al., 2018). While, *Bacilli* species have been shown to assimilate urea it is uncertain whether they have urease (Gunka and Commichau, 2012).

4.4. Optical density (ODs)

Unexpectedly, all four bacteria exhibited lower growth when nitrogen was added than when on 2% maltose only. Environmental changes (Horz et al., 2004), nitrogen sources (Kuypers et al., 2018) and other factors such as forms of carbon (Shu et al., 2014) will affect growth. We may have shocked the bacteria by adding nitrogen, which would have had a negative effect on growth as has been shown (Amon et al., 2010; Shimizu, 2014). At higher concentrations of ammonium-N, *E. coli* exhibited higher ODs while *B. thuringiensis* exhibited lower. It has been reported that ammonium-N can be toxic (Muller et al., 2006) or be required for growth (Kuypers et al., 2018). Nitrate-N had a positive effect on the growth of *A. hydrophila* and *P. aeruginosa*, which was expected since they are nitrate-reducing bacteria. Growth, when urea-N was added to the media, varied between the bacteria and it has been suggested that urea-N can be toxic, which might be the case for *B. thuringiensis* but not the other three bacteria (Soman et al., 2017; Taabodi et al., 2019). In field studies, when the nitrogen source was changed the overall bacterial abundance did not change, making it difficult to explain all of the patterns of growth observed in this study (Avrahami et al., 2003; Horz et al., 2004).

5. Conclusions

In summary, we can suggest that *A. hydrophila* is active in the utilization of all three nitrogen sources, in particular nitrate-N and urea-N. However, *B. thuringiensis* produced ammonium-N, utilized urea-N but it did not respond to nitrate-N. Like *A. hydrophila*, *E. coli* utilized ammonium-N and nitrate-N at lower concentrations and utilized urea-N at all concentrations. It was different in response to ammonium-N in that utilized at lower concentrations but produced at higher concentrations. In response to nitrate-N and urea-N, *P. aeruginosa* showed significant utilization. Both *A. hydrophila* and *E.coli* grew best at higher concentrations of ammonium-N while *B. thuringiensis* at lower concentrations. In addition, *A. hydrophila* grew better at higher concentrations of nitrate-N. Growth of *P. aeruginosa* was better at lower concentration of nitrate-N but growth rates were very similar between all concentrations. This study does show that it is possible to expose bacterial isolates to various nitrogen sources in an effort to understand their roles in nitrogen cycling. Growth sometimes supported our observation of production or utilization however not consistently so. There is some evidence that higher concentrations of bacteria respond differently than lower concentrations of bacteria but for the most part tended to support our findings.

Declarations

Author contribution statement

Maryam Taabodi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Eric B. May: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ray B. Bryant: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Louis S. Saporito: Performed the experiments; Analyzed and interpreted the data; Olivia K. Skeen: Performed the experiments.

Fawzy M. Hashem: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Arthur L. Allen: Conceived and designed the experiments.

Funding statement

This work was supported by the urea project funded under the USDA-NIFA, United States. Grant # 2015-38821-24380 for the financial support for this study.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

We thank USDA-ARS in State College, Pennsylvania for laboratory support and statistical analysis.

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