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Experimental investigation of potential biological nitrogen provisioning by freshwater insect gut microbiomes using ^{15}N isotope analysis

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Abstract

Biological nitrogen (N) provisioning is a seminal function of the gut microbes in several terrestrial insects, given the unbalanced carbon (C) and N ratios of their diets. Although freshwater insects face comparable dietary N limitations like terrestrial insects, little is known about this function by their gut microbiomes. In this study, we investigated microbial nitrate reduction to ammonium pathways as possible routes of biological N provisioning in two freshwater insects; filter-feeding Hydropsychidae and grazers/collectors Baetidae. After incubation in filtered (microbe-free) artificial stream water (ASW) containing dissolved ^{15}N-labeled nitrate (treatment) or standard nitrate (control), bulk δ^{15}N values of treatment samples (Baetidae = 100.62 ± 10.23, mean ± S.E.; Hydropsychidae = 76.82 ± 7.20) were significantly higher than controls (Baetidae = 10.14 ± 0.12 ; Hydropsychidae = 9.03 ± 0.20) in both functional feeding groups ($F_{(3, 13)} = 296$, $P < 0.0001$). The treatment δ^{15}N values are cautiously interpreted as reflecting uptake and incorporation of microbe-derived ^{15}N-metabolites ($^{15}NH_4$ or ^{15}N-amino acids) into host tissues following nitrate reduction to ammonium pathways in the gut lumen. Microbial nitrate reduction to ammonium activities was assessed via the quantification of dissimilatory (*nrfA*) and assimilatory (*nasA*) nitrate reduction to ammonium gene transcripts. There were no significant differences between control and treatment groups within each insect groups. Overall, this study provides a demonstration of the feasibility of applying ^{15}N-stable isotope analysis for investigating, potential symbiotic functions of freshwater insect gut microbiomes, despite the preliminary nature of the results.

Keywords: Freshwater insects, gut microbiome, nitrogen provisioning, nitrate reduction.

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Introduction

The relevance of stable isotope techniques in advancing our insights into the functions and contributions of insect gut microbial associates to host nutritional ecologies cannot be overstated. Stable isotope analyses (SIA), particularly of the heavy nitrogen isotope ^{15}N, have been instrumental in providing empirical evidence of biological nitrogen provisioning through nitrogen fixation in termites [1–3], ants [4], beetles [5], and cockroaches [6], as well as via nitrogen recycling in wood-feeding beetles [5,7], termites [8] and ants [9]. These insect systems are overwhelmingly terrestrial, with little known about the occurrence and importance of biological nitrogen provisioning in freshwater insects, despite the fact that freshwater insects face comparable dietary N-limitations as their terrestrial ecological counterparts [10–12].

We sought, in this communication, to investigate biological nitrogen provisioning by the gut microbiomes of freshwater insects to bridge this gap in knowledge regarding gut microbial functions in freshwater insects. The main sources of microbial nitrogen provisioning in terrestrial insects are atmospheric nitrogen fixation and recycling of nitrogenous waste products. Nitrogen fixation has been proposed as a possible pathway for biological nitrogen provisioning in freshwater insects [12,13], with little evidence to corroborate this. This may be attributed to the technical difficulties associated with generating ^{15}N gas-enriched water systems to study this process, in a manner like exposing terrestrial insects to ^{15}N gas -enriched atmospheres to investigate nitrogen fixation. Additionally, little is known about nitrogen recycling in freshwater insects. Given their habitat, excretory systems, and nature of excretory products (ammonia), nitrogen recycling is possibly an unlikely route for biological nitrogen provisioning, in comparison to terrestrial insects, but this remains to be investigated. However, potential pathways for biological nitrogen provisioning in freshwater insects are microbe-mediated

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assimilatory nitrate reduction to ammonium (ANRA) and dissimilatory nitrate reduction to ammonium (DNRA) processes [14]. This is based on reported work demonstrating the occurrence of other microbe-mediated nitrate reducing processes, in particular, incomplete denitrification, in freshwater insect guts [15]. Unlike incomplete denitrification, which converts nitrate to N_2O, microbial ANRA and DNRA immobilize the nitrogen (from nitrate) into ammonium. The ammonium formed in the case of ANRA is released into bacterial cytoplasm and directly used for microbial biosynthesis, whereas the ammonium formed from DNRA, is secreted out across the bacterial periplasm and used for redox balancing [14]. We hypothesized in this study that both microbial nitrate reduction to ammonium processes (ANRA and DNRA) may be occurring in freshwater insect guts, serving as a source of biological nitrogen for associated hosts and that these processes would be amenable to investigation using ^{15}N stable isotope analysis.

To investigate this, freshwater insects were incubated in microcosms containing microbe-free artificial stream water (ASW) with ^{15}N-labeled nitrate (treatment) or standard/normal ^{14}N-labeled nitrate (control) without feeding for approximately ~39 hours. We anticipated higher $\delta^{15}N$ values in treatment insects relative to control insects. Within the gut lumen of treatment insects, ^{15}N-ammonium produced through DNRA by gut microbial assemblage would be directly available for host uptake (Fig. 1). Direct assimilation of ^{15}N-ammonium in the gut lumen pool by the freshwater insect host most likely proceeds via the coupled glutamine synthetase (GS) and glutamine-2-oxoglutarate amidotransferase (GOGAT) enzyme system [16,17], which is responsible for the assimilation of symbiotic ammonium from nitrogen fixation and nitrogen recycling in terrestrial insects [5,16,18,19]. In contrast, ^{15}N-ammonium produced through ANRA in the cytoplasm of gut bacteria in the treatment insect gut lumen will first be used by the

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bacterial assemblage in the biosynthesis of microbial ^{15}N-metabolites (amino acids, proteins). Subsequently, these ^{15}N-metabolites become available in the gut lumen for insect host uptake upon bacterial cell lysis (Fig. 1). Overall, we anticipated higher δ^{15}N values in treatment insects (incubated in ^{15}N-nitrate) which may be attributed to both direct and indirect uptake and incorporation of labeled nitrogen (either as ^{15}N-ammonium, or ^{15}N-metabolites) by the insect host following microbial reduction of ^{15}N-nitrate in the gut lumen.

There were three main guiding premises of the study outlined below. First, freshwater insects, as well as other freshwater macroinvertebrates and vertebrates do not have the genetic ability to metabolize (reduce) nitrate. This capability is limited to prokaryotes, fungi, and plants [14,20–23]. Furthermore, nitrate concentrations above 10 mg/L (161 mM) and 100 mg/L (16100 mM), respectively, have been demonstrated to adversely impact freshwater macroinvertebrate [23,24] and vertebrate [23,25,26] growth, physiology, and survival, further highlighting the inability to metabolize nitrate. Second, there is no change in the feeding behavior (filtering, scraping, gathering) of insects used in this study in the absence of food. This premise is based on observations that external factors, such as noise, light, and vibration initiated feeding behaviors in the mayfly *Rhithrogena pellucida* in the absence of food, which were the same as behaviors observed in the presence of food [27]. Finally, there is water intake into the digestive tract even in the absence of food due to the feeding behaviors mentioned above in freshwater insects [27]. Although food was not provided, suspended particulate matter in incubating waters was observed. These are most likely from materials/debris associated with insects during sample collection, as well as feces from insect during the incubation in macrocosm setups. These suspended materials may further promote feeding behaviors, providing a pathway for dissolved ^{15}N-nitrate into the insect gut lumen.

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Materials and Methods

Sample collection and study design

Freshwater insect sampling took place from several sites in the West Branch of the Mahoning River, in Northeast Ohio with a Surber sampler [28] in June of 2017. Insect samples were transported to the laboratory in a cooler with ice bags (within 45 minutes -1 hour after collection). In the laboratory, insects were acclimated to room temperature and identified to families using taxonomic keys [28]. One relatively abundant insect family, Hydropsychidae (filter-feeder) from this sampling effort was selected for further study with two analytical replicates for Hydropsychidae (n= ~ 9 -15 individuals per replicate) for treatment and control groups. In the interest of having larger sample sizes and higher replicates, live Brown Drake mayfly (Baetidae) (*Ephemera simulans* Walker) nymphs were obtained from a commercial supplier (http://www.thereelthingbait.com/, Green Bay, WI). These nymphs are burrowers with a grazing/collector feeding mode [29,30]. Artificial stream water (ASW) made as in Olapade & Leff (2005). Approximately 0.25 g each of $K^{15}NO_3$ (98 atom % ^{15}N, 2 atom % ^{14}N) or $K^{14}NO_3$ (99.6 atom % ^{14}N, 0.4 atom % ^{15}N) were added to 1 liter of ASW to a final concentration of 2.5 mM (0.04 mg/L) nitrate for ^{15}N-labeled nitrate treatment solution and ^{14}N-labeled nitrate control solution, respectively. Final ASW solutions were filtered through a 0.2 µm membrane filters (EMD Millipore, Billerica MA, USA) to remove bacteria. For the sake of clarity, controls are insect samples incubated in standard ^{14}N-labelled nitrate solution and treatments are insect samples incubated in ^{15}N-labeled solution. The final nitrate concentrations (2.5 mM or 0.04 mg/L) were considerably below reported lethal nitrate concentrations for a variety of freshwater insects (10 mg/L or 161 mM) [23]. Control and treatment insects were incubated in 250-300 ml of ASW under constant aeration for ~39 h in triplicates (n = 10-15 nymphs per replicate, N = 3)

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for Baetidae nymphs and duplicate (n = ~ 9 -15 small larvae per replicate, N = 2) for Hydropsychidae larvae

Sample processing and preparation for stable isotope analysis

After incubation, insects were cleaned once in a 1:1 ratio solution mixture of 0.1 % bleach and 0.1 % detergent solution mix and rinsed twice in deionized water to remove residual or attached $^{15}NO_3^-$ or $^{14}NO_3^-$ on insect carcasses. Alimentary/digestive tracts of all insects were removed and placed in 2 ml Eppendorf tubes containing Trizol™ Reagent (Thermo Fisher Scientific, Waltham, MA) and stored at -80 °C for RNA extraction, with control samples processed first. The remaining head capsules and carcasses were rinsed again as above to eliminate any additional nitrate residues as a result of the dissection process. All instruments used were washed and rinsed as above between sample groups to eliminate any residual $^{15}NO_3$ transfer as recommended [32]. Samples were stored at -80 °C for stable isotope analysis. Samples were dried under vacuum at -120 °C for 48 hours, and freeze-dried samples milled using individual sterile glass pestles in 1.5 ml Eppendorf tubes stable isotope analysis. Analytical replicates of each sample (0.03-0.22 g) were weighed, placed in tin capsules and folded into balls. Samples were analyzed using a Thermo Delta V Mass spectrometer (Delta Plus, ThermoFisher Scientific, Germany) coupled to a Costech 4010 Elemental Analyzer (Costech Analytical Technologies Inc., Valencia, CA, USA). The IRMS quantifies the atom percent abundance of ^{14}N and ^{15}N in each sample compared with known standards (air, atmospheric N_2) on scales normalized to known internal standards (urea $\delta^{15}N$ values). Analytical precision, based on multiple standard runs of urea was ± 0.37 ‰ (1σ, n = 9). Bulk $\delta^{15}N$ values ($^{15}N/^{14}N$) of samples were then calculated as $[(R_{smpl}/R_{stnd}) - 1] \times 1{,}000$ ‰, where R_{smpl} is the ratio of heavy to

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light isotope in each sample and R_{stnd} is the ratio of the heavy to light isotope in the standard [33].

RNA extraction and RT-qPCR

Insect gut samples in trizol were thawed and vortexed with beads to homogenize intact guts. Following this, 500 µl of the homogenized mixtures were added to supplied bead tubes in the RNeasy PowerMicrobiome Kit (Qiagen Inc., Germantown, MD, USA), and RNA extracted following manufacturer's protocol. RNA quantity and quality were assessed via absorbance using a Nanodrop (Thermo Fisher Scientific, Waltham, MA). cDNA was generated using the qScript cDNA Synthesis Kit (Quanta bio, Beverly, MA) according to the manufacturer's protocol. cDNAs were equilibrated to approximate concentrations via dilutions (~1:20) before quantitative PCR (qPCR). Universal primers for the periplasmic dissimilatory nitrate reduction to ammonium (DNRA), *nrfA* [34] and the cytoplasmic assimilatory nitrate reduction to ammonium (ANRA) *nasA* [35] genes, respectively, amplified 270 bp and ~750 bp gene fragments of both genes. Each 20 µl qPCR reaction mixture contained template cDNA, PerfecTa SYBR Green FastMix (Quanta bio, Beverly, MA, USA), water, and primers (0.2 µM each). qPCR reactions were carried out with a Stratagene MX3005P Real-time PCR System (Agilent Technologies, Santa Clara, CA, USA) with an initial denaturation condition of 95 °C for 5 min, and 40 cycles of 95 °C for 60 s, 51°C for 90 s, and 72 °C for 90 s for *nrfA*, and 40 cycles of 95 °C for 60 s, 61.5 °C for 90 s, and 72 °C for 30 s for *nasA*. All runs were followed by a melt/disassociation curve comprised of 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. Standard curves for runs were generated using serial dilutions of single insert plasmids acquired following the cloning of *nrfA* and *nasA* genes from *Pseudomonas aeruginosa* (ATCC number BAA-47; GenBank accession number AE004091) using the TOPO TA Cloning kit (Thermo Fisher Scientific, Waltham, MA,

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USA). There was no internal RNA control used in this study, and transcript copy numbers were normalized to no template controls. Transcript numbers were reported as copy number/per reaction µl.

Statistical analyses

All $\delta^{15}N$ and transcript copy number data were log-transformed for normality and used in a one-way analysis of variance, with treatment and control groups as the dependent variables in JMP 13 (JMP, SAS Inc. NC, USA). Reported values are non-transformed data.

Results and discussion

In this communication, we sought to demonstrate, as a proof of concept, the feasibility of ^{15}N stable isotope analysis in investigating biological nitrogen provisioning via nitrate reduction to ammonium in freshwater insects. Overall, there was a significant effect of incubation in ^{15}N-labeled nitrate relative to controls on bulk $\delta^{15}N$ values ($F_{(3, 13)} = 296$, $P < 0.0001$). Treatment $\delta^{15}N$ values for the two insect functional feeding groups (Baetidae + ^{15}N = 100.62 ± 10.23 ‰, mean ± S.E.; Hydropsychidae + ^{15}N = 76.82 ± 7.20 ‰) were significantly higher than corresponding control values (Baetidae = 10.14 ± 0.12 ‰; Hydropsychidae = 9.03 ± 0.20 ‰) ($P < 0.0001$)(Fig. 2A). Although limited in scope, this report suggests potential biological nitrogen provisioning by the gut microbiota of freshwater insects via nitrate reduction to ammonium using ^{15}N stable isotope analysis, since insects do not have the ability to utilize nitrate. The higher $\delta^{15}N$ values of treatment insects can be attributed to the reduction of ^{15}N-nitrate to ^{15}N-ammonium in the gut lumen via the combined direct and indirect pathways outlined in figure 1, and the subsequent incorporation of ^{15}N-ammonium by the insect hosts. These results represent the first

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attempt to empirically investigate this long-standing hypothetical gut microbial function in freshwater insects [13,36,37].

These results suggest the possibility that freshwater insects may be overcoming dietary N limitations through biological nitrogen provisioning by associated gut microbes through nitrate reduction to ammonium. Ammonium is an important end-product of both these nitrate reducing processes because ammonium is also the common intermediate metabolite from nitrogen fixation and nitrogen (urea) recycling processes by gut microbiota in a variety of terrestrial insects. This makes the current approach similar to other approaches used to determine biological ^{15}N-nitrogen provisioning and subsequent insect host uptake and routing into metabolites in a variety of terrestrial insects [5,18,38]. The determination of δ^{15}N values in freshwater insect host metabolites, such as amino acids, is an important addition to the bulk ^{15}N isotope analysis approach outlined here, that needs to be pursued in order to provide evidence of insect host ^{15}N uptake and routing of microbe-derived ^{15}N metabolites following microbial ^{15}N-nitrate reduction.

Our assumption that feeding behaviors would not be affected during incubation held up. This assumption is corroborated by the formation of external sheaths (coverings) in both control and treatment Hydropsychidae experimental groups (a member of the order Trichoptera known to form external protective casings) during incubation. Additionally, the higher δ^{15}N values in treated samples relative to controls further support our assumption that feeding behavior would be accompanied by the intake of water containing dissolved ^{15}N-labeled nitrate into insect guts. Concerns about ^{15}N-labeled nitrate adhering to insect cuticles and inflating measured δ^{15}N value were minimized (and possibly eliminated) by washing insects twice; once before dissection and rewashing the head capsules and carcasses after dissection. A modification of the current method should include quantification of bulk δ^{15}N values from the incubating solutions in order to

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control for such concerns. Overall, the current results, concerns notwithstanding, are indicative of potential biological nitrogen provisioning by freshwater insect gut microbiomes.

It remains to be determined if biological nitrogen provisioning via nitrate reduction to ammonium varies among freshwater insect functional feeding groups. Such differences, if any, will most likely be mediated by the composition of gut microbiomes among functional feeding groups, and not necessarily attributable to any particular microbiome taxa. In a previous study, significant differences in community composition were determined among the gut microbiomes of filter feeding (Hydropsychidae) and grazing/scraping (Baetidae) functional feeding groups from the same stream [39]. In the referenced study, *Halomonadaceae, Shewanellaceae, Ruminococcaceae, Clostridiaceae,* and *Enterobacteriaceae,* were significantly more abundant in filter feeders, whereas *Comamonadaceae, Acidimicrobiaceae, Flavobacteriaceae,* and *Intrasporangiaceae* were significantly more abundant in grazer/scrapers. Whether these differences result in different gut microbial functions, and in particular, nitrate reduction to ammonium functions remains unclear. This is because several of these bacterial families belong to well-known bacterial phyla and classes that have representatives capable of both DNRA and ANRA since nitrate reduction capabilities are widely distributed among phylogenetically distinct bacterial taxa, and not limited to certain taxa [20,40,41]. In this study, although $\delta^{15}N$ values from ^{15}N treatment samples from both functional feeding groups were comparable, we uncovered significant differences in transcript copy numbers of the DNRA-related *nrfA* gene ($F_{(3, 13)} = 9.04$, $P = 0.0014$) (Fig. 2B) and ANRA-related *nasA* gene ($F_{(3, 15)} = 4.96$, $P = 0.014$)(Fig. 2C) between Baetidae and Hydropsychidae ^{15}N-treatment groups. Further characterization of nitrate reduction to ammonium coupled to gut microbiome analysis needs to be carried out for various freshwater functional feeding groups to determine if there are group-specific differences in both aspects as

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have been determined for among terrestrial insects. In contrast, there were relatively minimal differences in *nrfA* and *nasA* transcript copy numbers between treatment and control samples in each functional feeding groups (Fig. 2B and 2C). This is to be expected as microbial *nrfA* and *nasA* gene expressions are expected to occur within the gut lumen of both treatment and control insects under sublethal nitrate concentrations, as was the case in this study. However, further studies requiring an internal RNA control in the RT-qPCR assay are required to definitively establish this. The $\delta^{15}N$ values and gene transcript numbers are presented in Table S1.

In conclusion, we present a proof-of-concept approach to investigating biological nitrogen provisioning by freshwater insect gut microbiomes through nitrate reduction to ammonium pathways using ^{15}N stable isotope analysis. The conceptual framework for this biological provisioning is presented in figure 1. Further verifications of this approach in freshwater insects are required and will ultimately enable comparison of biological nitrogen provisioning by the gut microbiota of closely related terrestrial and aquatic insect taxa from both evolutionary and ecological perspectives.

Author contributions

PAA and LGL conceived and designed the study. PAA and SB executed the study. JW provided IRMS capabilities, and TEA performed the isotope ratio mass spectrometry analyses. PAA, JW, TEA, SB, and LGL wrote the manuscript.

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Conflicts interests

The authors declare no conflict of interest.

Ethical Statement

No animal rights were violated in the execution of this study. Guidelines of the Kent State University's Office of Research Compliance and Kent State Institutional Animal Care and Use Committee (IACUC) did not apply to the use of insects.

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414 **Figure 1**. **Proposed conceptual scheme of potential nitrogen provisioning by freshwater**
415 **insect gut microbiomes through nitrate reduction**. ^{15}N-Ammonium produced from DNRA
416 contributes directly to the lumen ammonium and metabolite pool and is taken up directly by host
417 insect. In contrast, microbial ^{15}N-microbial metabolites produced following ANRA in bacterial
418 cytoplasm become available for host uptake only after bacterial cell lysis.

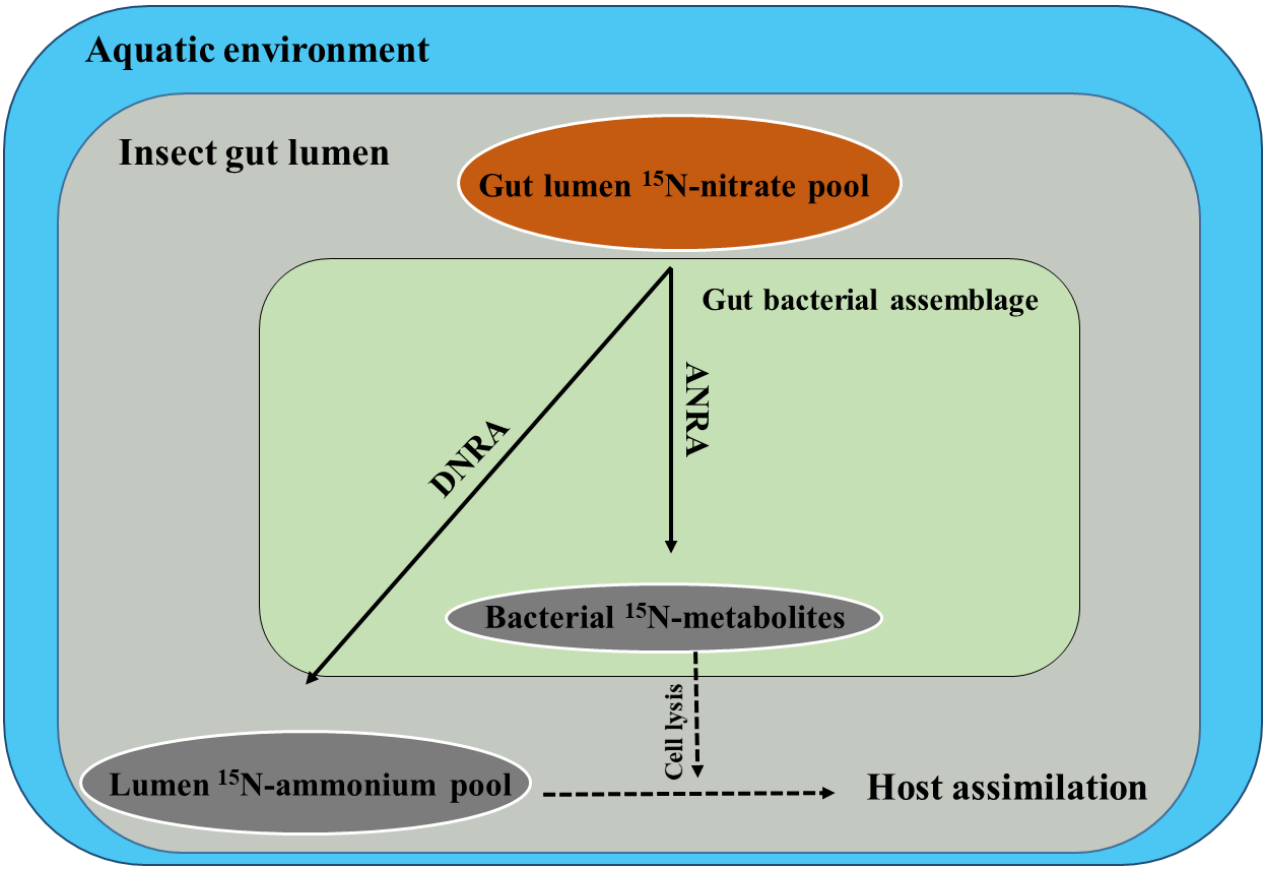

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Figure 2. $\delta^{15}N$ values from control and treatment samples. The (**A**) $\delta^{15}N$ values (‰), (**B**) *nrfA* transcripts, and (**C**) *nasA* transcripts (copy numbers/µl x 10^4) of control (incubated in ^{14}N-labeled nitrate solution) and treatment (incubated in ^{15}N-labeled nitrate solution) Hydropsychidae (filter feeding) and Baetidae (grazers/scrapers) after 39 h incubation. Significant differences among samples were determined at $P = 0.05$.

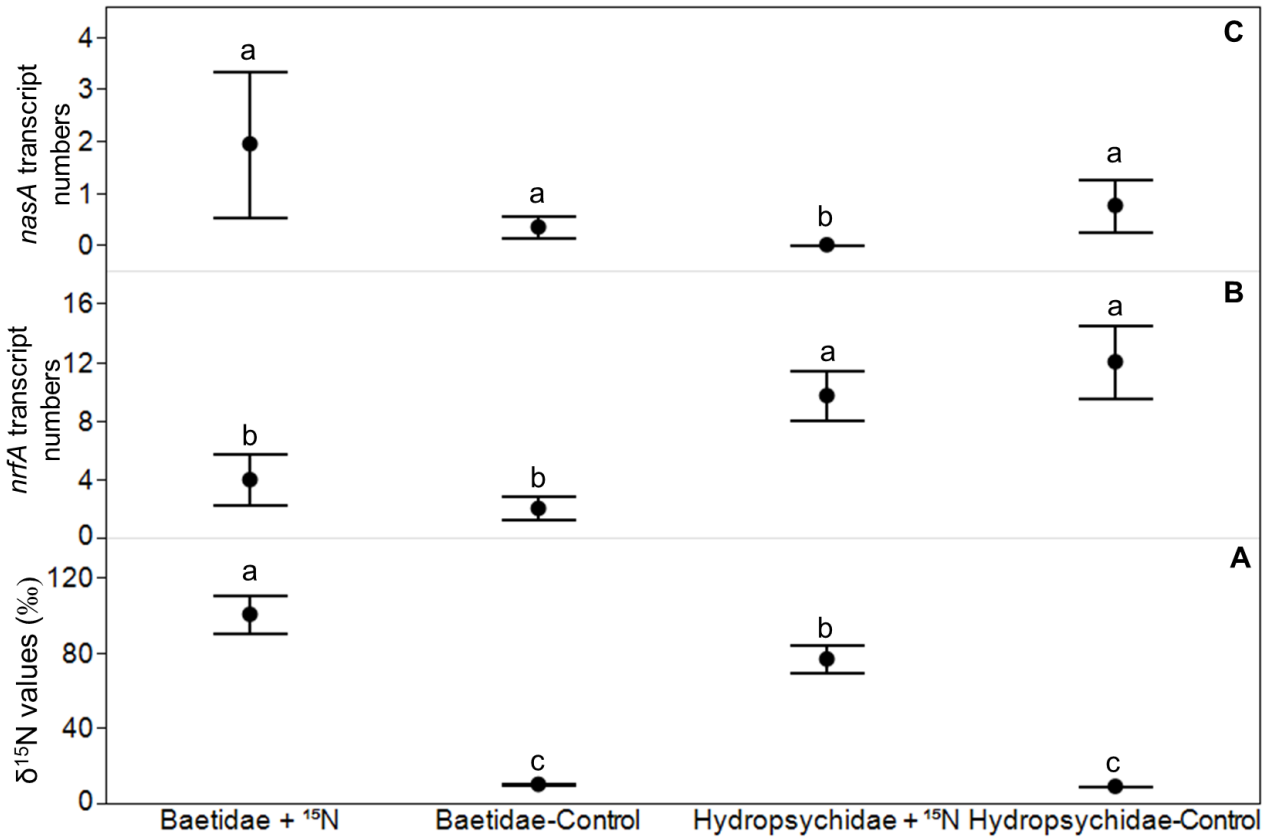

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Table S1. Sample information and $\delta^{15}N$ data from experiments one and two.

Sample	Water type	Groups	$\delta^{15}N$ vAir	log ^{15}N	*nrfA* transcript copies	Log *nrfA*	*nasA* transcript	Log *nasA*	Categories
Hyd-AS-1-c-aHyac1	ASW	Hydropsychidae-Cont.	9.266	0.966892	75610	4.878579	2858	3.456062224	Control
Hyd-AS-1-c-aHyac2	ASW	Hydropsychidae-Cont	9.477	0.976671	103300	5.0141	22410	4.350441857	Control
HydAc2-a	ASW	Hydropsychidae-Cont	8.767	0.942851	111700	5.048053	258.4	2.412292509	Control
HydAc2-b	ASW	Hydropsychidae-Cont	8.627	0.93586	190200	5.279211	4795	3.680788612	Control
Hydan1-a	ASW	Hydropsychidae + ^{15}N	68.275	1.834262	131700	5.119586	N/A	N/A	Treatment
Hydan1-b	ASW	Hydropsychidae + ^{15}N	61.189	1.786673	118700	5.074451	15.07	1.178113252	Treatment
Hydan2-a	ASW	Hydropsychidae + ^{15}N	91.25	1.960233	60890	4.784546	8.96	0.95230801	Treatment
Hydan2-b	ASW	Hydropsychidae + ^{15}N	86.572	1.937377	77160	4.887392	1.14	0.056904851	Treatment
ASW-X1	ASW	Baeidae + ^{15}N	105.743	2.024252	N/A	N/A	88520	4.947041405	Treatment
ASW-X1	ASW	Baeidae + ^{15}N	97.358	1.988372	N/A	N/A	16630	4.220892249	Treatment
ASW-X2	ASW	Baeidae + ^{15}N	119.719	2.078163	92230	4.964872	616.8	2.790144365	Treatment
ASW-X2	ASW	Baeidae + ^{15}N	135.211	2.131012	21810	4.338656	190.3	2.279438788	Treatment
ASW-X3	ASW	Baeidae + ^{15}N	72.163	1.858315	20710	4.31618	155.9	2.192846115	Treatment
ASW-X3	ASW	Baeidae + ^{15}N	73.559	1.866636	24110	4.382197	4910	3.691081492	Treatment
ASW-Y1	ASW	Baetidae-Control	10.665	1.027961	N/A	N/A	378.8	2.57840997	Control
ASW-Y1	ASW	Baetidae-Control	10.078	1.003374	7479	3.873844	6.99	0.844477176	Control
ASW-Y2	ASW	Baetidae-Control	10.157	1.006765	9482	3.9769	13430	4.128076013	Control
ASW-Y2	ASW	Baetidae-Control	9.958	0.998172	5788	3.762529	105.3	2.022428371	Control
ASW-Y3	ASW	Baetidae-Control	9.763	0.989583	44270	4.64611	6412	3.806993514	Control
ASW-Y3	ASW	Baetidae-Control	10.234	1.010045	33320	4.522705	3257	3.512817759	Control

Note: FSW and ASW stand for filtered stream water and artificial stream water, respectively.