Metagenomic evaluation of the caddisfly-associated microbiome and its implications for nutrient cycling in montane streams

A Thesis

by

Chloe Pryor

Approved by:

Dr. Frank Stewart, Advisor
School of Biological Sciences
Georgia Institute of Technology

Signature: 

Date Approved: May 4, 2022

Dr. Mark Hay
School of Biological Sciences
Georgia Institute of Technology

Signature: 

Date Approved: May 4, 2022

Introduction

Rivers and streams play an essential role in mediating the excess supply of nitrogen in the environment created through human activities via the processes of nutrient transport and cycling. Therefore, research on stream biogeochemistry is necessary in supporting efforts to protect water quality and downstream ecosystems, as well as to better understand the impacts of nitrogen on the environment (*Schlesinger et al.*, 2006).

This project is part of a larger study on the influence of macroinvertebrates as ecosystem engineers in streams. The study specifically focuses on caddisfly larvae (*Hydropsychidae*) as an example due to their ubiquity and silk retreat-building behavior. Conducted by a team of scientists at Montana State University over the past couple years, the research involves lab experiments with stream analog mesocosms, field experiments, and modeling. The researchers have two separate lines of investigation to explore how caddisflies impact stream biogeochemistry, particularly nitrate uptake. First, they hypothesize that the nets and retreats constructed by caddisflies obstruct boundary layer flow in streambeds and increase residence times of metabolites in stream sediments. Second, they hypothesize that the same obstruction of flow allows for increased growth of biofilms and development of novel anoxic microhabitats, which contributes to a higher nitrate uptake by microbes.

This project supplements the larger aims of the caddisfly study by extending the discussion to include the microbiome associated with caddisflies, including microbes associated with the outer surface of the caddisflies as well as microbes found in the gut. We suggest that caddisflies not only allow for increased biomass and subsequent microbial activity through their net- and retreat-building activities, but also serve as microbial habitats themselves for a unique insect-associated microbiome.

This research contributes to the field of stream ecology in further exploring the caddisfly-associated microbiome. It bolsters the overall research study in furthering scientific knowledge of the regulation of stream metabolism by macroinvertebrates as ecosystem engineers. On a broader scale, it contributes to the goals of understanding and mitigating the impact of excess nitrogen on environmental and human health.

Literature Review

Human activities, particularly the use of fertilizers in agriculture, have resulted in a heightened supply of nitrogen to the environment. Vitousek et al. (1997) noted that human activities roughly doubled the amount of nitrogen added to the terrestrial nitrogen cycle and that this impact was growing. Anthropogenic modifications to the nitrogen cycle occur through the use of fertilizers, cultivation of nitrogen-fixing crops, burning of fossil fuels, and land development. Excess nitrogen has many negative consequences for environmental and human health including eutrophication, creation of low oxygen zones, pollution of drinking water, air pollution, disruption of food web structure, loss of biodiversity, and even impact on climate (*Davidson et al.*, 2011). River systems play a crucial role in mediating this phenomenon via transport of nutrients from terrestrial environments to marine environments in addition to nutrient cycling through biogeochemical processes including denitrification and biological uptake. Considering the importance of rivers and streams in environmental and human health, as well as the limited scientific understanding that exists surrounding processes in stream ecology, more research is needed on the biogeochemistry of streams (*Schlesinger et al.*, 2006; *Wymore et al.*, 2016; *Gomez-Velez et al.,* 2015; *Kim et al.*, 2016).

Organisms in streams act as ecosystem engineers by altering their own environments through overlapping physical and chemical means (*Nogaro et al.,* 2009). In 2015, Albertson and Allen performed a meta-analysis of existing research on the ways that various organisms in streams affect the stability or erosion of their habitats. They found a significantly high impact by aquatic insects, although this group was generally understudied in comparison to other taxa like fish and crustaceans. While insects have been studied comprehensively as pollinators because of the large agricultural and economic importance of their interactions with plants, they are often understudied and undervalued in other areas of ecology (*Eisenhauer et al.*, 2019).

For instance, caddisflies are an order of insects characterized by moth-like terrestrial adult forms and aquatic larvae, which are known as “nature’s underwater architects” (*Frandsen et al*., 2019). There are over 16,000 extant species of caddisflies found in freshwater streams on every continent except Antarctica. As larvae, they serve as macroinvertebrate ecosystem engineers by structurally altering the boundary layer flow of water through streams. This is done via bioturbation or disturbance of the sediments as well as the construction of their silk retreats

and nets in the streambeds (*Nogaro et al.*, 2009; *Albertson et al.,* 2019). Through this alteration, they interact with the biogeochemistry of streams. By obstructing the interstitial spaces in the sediments of the streambed, it is hypothesized that they prolong flow, increase residence time of metabolites, and allow for more time for biochemical processes. Moreover, obstruction of flow in the stream may also allow for stronger growth of biofilms and therefore increased microbial biomass and creation of unique anoxic environments for microbial communities, thus contributing to higher nitrate uptake by microbes (*Albertson and Poole*, 2020).

As well as emphasizing the role of aquatic insects, Albertson and Allen (2015) also call attention to the impact of microbes, specifically biofilms, on stream ecology. Microbes play a very important role in ecosystems especially in the context of nutrient cycling; for instance, they perform many reactions involved in nitrogen cycling (*Kim et al.*, 2016; *Ren et al.*, 2017). While many impressive advances have been made in the study of microbiology, further research is needed to elucidate the connections between composition of microbiomes and function in ecosystems (*Findlay*, 2010; *Battin et al.*, 2016). To date, much of the research on biofilms has primarily focused on medical environments; however, it is essential we also study them in natural environments like streams (*Battin et al.*, 2016).

Studying biofilms with regard to carbon cycling, Singer et al. (2010) found that heterogeneity in stream mesocosms resulted in higher microbial diversity, carbon uptake, and diversity of carbon use. By extension, they discuss how loss of variety in stream habitats can reduce biodiversity and metabolic diversity. This corresponds with the behavior of caddisflies as ecosystem engineers via modifying environments and providing unique microhabitats for microbes, thereby altering the biology of streams by allowing for greater microbial biodiversity and associated biochemistry.

In addition to creating novel microhabitats and allowing for thicker biofilms, caddisflies themselves can be seen as environments for insect-associated microbes. The gut provides an ideal habitat for microbes with its relative stability and availability of nutrients compared to the external environment, potentially supporting higher rates of microbial activity compared to exterior surface-attached biofilms. As caddisflies have been observed at densities of up to 2500 insects per square meter in montane streams and are ubiquitous across the globe, it is probable that the metabolic processes mediated by their gut microbes are consequential for bulk chemical cycling in streams (*Albertson and Poole,* 2020).

The conditions of the gut also determine the chemistry that takes place therein. Most animal intestinal tracts are oxygen-poor, resulting in predominantly anaerobic microbial metabolisms which include processes such as fermentation, denitrification, and methanogenesis. While the oxygen availability and other physicochemical conditions of insect guts vary, aquatic insects tend to have a higher relative abundance of anaerobic bacterial groups in their gut microbiomes compared to insects in other environments (*Yun et al.*, 2014). Therefore, in this research, we expect to see the caddisfly gut as a niche for anaerobic metabolisms and associated chemical transformations.

Much of the research on microbial stream ecology has focused on benthic biofilms, while fewer studies have analyzed aquatic insect-associated microbiomes (*Ren et al.*, 2017; *Receveur et al.*, 2020). While researchers have studied the impact of leaf litter on the community composition of the gut microbiome of caddisflies, the metabolic potential of microbes associated with caddisflies has not been thoroughly explored (*Fritz*, 2019). This research seeks to expand scientific understanding on insect-associated microbiomes, aquatic insects as ecosystem engineers, and the overall biogeochemistry of montane streams.

Methods

Sample Collection

The team at Montana State University collected samples for this study from Cherry Creek, Montana (45.612318, -111.516994) on April 7th, 2021. At the time of sampling, the water level of the mountain creek was less than 0.3 meters. The river was visually inspected for riffle patterns on the surface that indicate rock formations. These submerged rocks were then inspected for caddisfly larvae as well as their silk nets and retreats (n=25 of each). Sterile steel tweezers were used to place the larvae, nets, and retreats in separate cryovials in 200 µL of preservation buffer each (25 mM sodium citrate, 10 mM EDTA, 5.3 M ammonium sulfate, pH 5.2). Sterile cotton swabs were used to take samples of areas of rock approximately 20 cm away from caddisfly retreats using a swiping motion for approximately 20 seconds or until coloration was visible. The tips of the cotton swabs were then broken off and placed in cryovials with 200 µL of preservation buffer. Samples were then transported in a cooler back and kept at -80 ºC at Montana State University.

DNA Extraction and Sequencing

In the laboratory at Montana State University, DNA was extracted from the samples using the MO BIO PowerSoil DNA Isolation Kit and libraries were prepared for sequencing. For the preserved caddisflies, whole flies were homogenized using a mortar pestle before the DNA extraction protocol. Samples were then sent off for shotgun sequencing to be performed at the Georgia Genomics and Bioinformatics Core at the University of Georgia using Illumina NextSeq. This resulted in a collection of four metagenomes describing the four distinct microbiomes of caddisfly larvae (n=4), their nets (n=4), retreats (n=4), and nearby rocks (n=4).

Comparative Metagenomics by Sample Type

Reference-independent or de novo comparative metagenomics was performed to assess differences in composition of the metagenomes from the four different sample types (larvae, nets, retreats, rocks) based solely on the content of the metagenomes rather than the use of a reference database. The tool SimkaMin was used to create a Bray-Curtis distance matrix indicating the dissimilarity between metagenomes based on kmer frequency profiling of the sequenced reads (*Benoit et al.*, 2020). This strategy incorporates not only presence or absence data, but is also weighted by abundance. The distance matrix was then visualized via non-metric multidimensional scaling (NMDS). Analysis of similarity (ANOSIM) multivariate testing was used to evaluate dissimilarity between the four groups as well using the vegan package for community ecology in R. The ANOSIM test reports an R value between 0 and 1, where values closer to 1 suggest greater differences between groups.

Reference-dependent comparative metagenomics was also performed to assess differences between metagenomes at the protein level. First, the sequenced reads in each of the 16 metagenomes were assembled into contiguous sequences or contigs using the assembly tool MEGAHIT (*Li et al.,* 2015). The gene calling tool Prodigal was then used to identify individual genes from the assembled contigs (*Hyatt et al*., 2010). The nucleotide sequences of these genes were translated into amino acid sequences and run against the Universal Protein Resource reference database using the tool BLASTX (*The Universal Protein Resource*, 2008). These results were then incorporated into a matrix to be used for multivariate analyses visualized on an NMDS plot. ANOSIM testing was also used to evaluate dissimilarity between the proteomes.

Results

Comparative Metagenomics by Sample Type

The NMDS plot in Figure 1 visualizes dissimilarity between the metagenomes of the four sample types (larvae, nets, retreats, and rocks). The clustering of the samples in each group demonstrates differences in community structure of the distinct microbiomes. The ANOSIM test also indicated dissimilarity between the groups with reported values of R=0.95 and p=0.001.

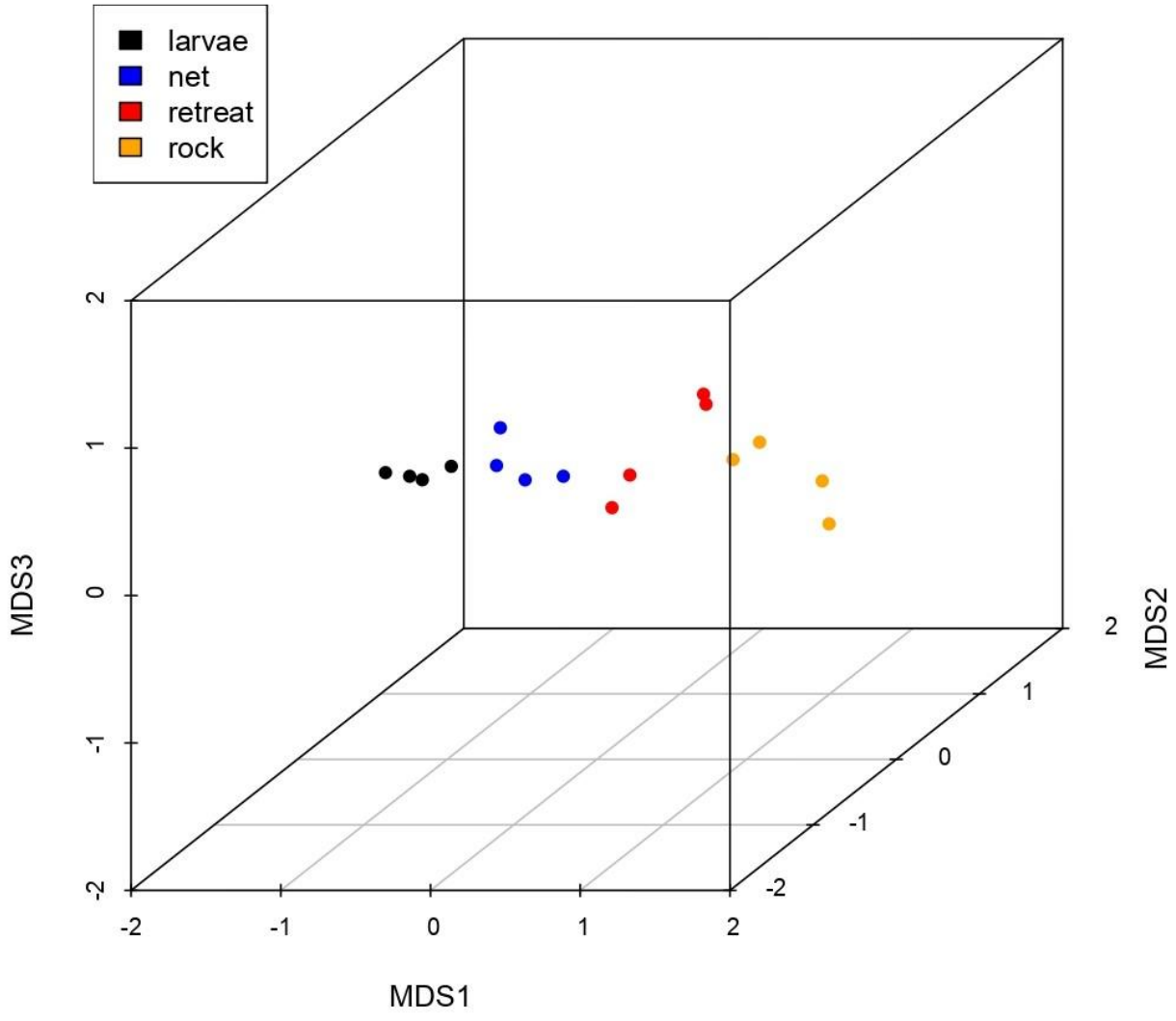

Figure 1: This NMDS plot shows the dissimilarity between the four metagenomes (larvae, nets, retreats, and rocks) using a multidimensional visualization of the Bray-Curtis distances generated by the tool SimkaMin. The points represent the four individual metagenomes for each of the four sample types, with black representing larvae, blue representing the nets, red representing the retreats, and yellow representing the background rocks.

The NMDS plot in Figure 2 visualizes dissimilarity in the proteomes of the metagenomes. While there is less obvious clustering between each of the sample types in this figure, the larvae metagenomes are still distinct from the rest of the samples. ANOSIM testing further supports this visual evidence of dissimilarity with reported values of R=0.48 and p=0.001.

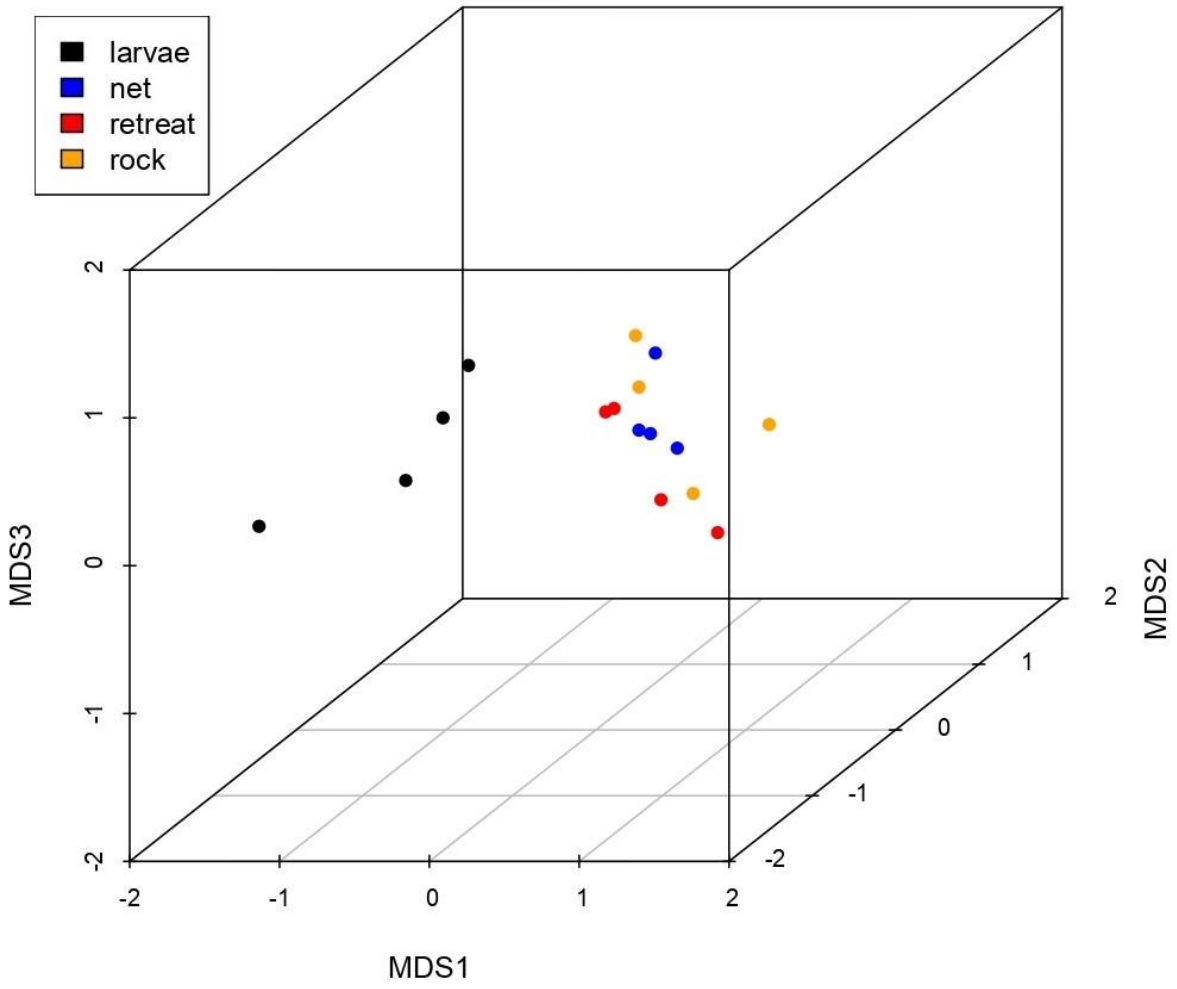

Figure 2: This NMDS plot shows the dissimilarity between the proteomes of the four metagenomes (larvae, nets, retreats, and rocks) using a multidimensional visualization of the matrix generated using the results of the BLASTX search against the UniProt reference database. The points represent the four individual metagenomes for each of the four sample types, with black representing larvae, blue representing the nets, red representing the retreats, and yellow representing the background rocks.

Discussion

The non-metric multidimensional scaling visualizations or NMDS plots demonstrated clustering of the metagenomes by sample type (larvae, nets, retreats, and background rocks) based on community structure as well as proteomes or predicted functional genes. Along with the results of the ANOSIM testing, this supports our hypothesis that the caddisfly serves as a habitat for a unique community of microbes in freshwater streams.

It is worth noting that the degree of dissimilarity or clustering by sample type was less strong for the proteome comparison. This could potentially be attributed to loss of informational resolution when the nucleotide sequences were translated to protein sequences. It could also be a result of limitations in the bioinformatics analysis process. Further research could be undertaken in order to replicate these findings or to more comprehensively address the question.

The dissimilarity between the caddisfly-associated microbiome and the microbiomes of caddisfly-created structures and nearby surfaces aligns with the results of other studies that have examined the dissimilarity between microbiomes of different aquatic macroinvertebrate species in mountain streams. For example, Receveur et al. (2020) performed a comparison of the microbiomes of mayflies, crane flies, stoneflies, and caddisflies in an alpine stream in Italy and found distinct community structures and functional composition between the various insect species.

While this study demonstrates that the caddisfly-associated microbiome is taxonomically and functionally distinct from the other stream microbiomes, it does not describe the taxa or functions that account for these differences. Next steps include investigating the metabolic processes that might potentially be enriched in the microbiome in order to better understand how this microbial niche contributes to stream biogeochemistry.

Several stressors currently threaten the ecology of the mountain west, including loss of snow cover, widespread drought, increased water temperature, forest fires, and nutrient loading from agriculture and developed lands. These phenomena, alongside other disturbances such as changes in temperature, atmospheric composition, or precipitation, may alter the habitats of montane streams (*Findlay,* 2010). If these changes affect the abundance or behavior of important species like caddisflies, we may see corresponding alterations in nutrient cycling and stream

metabolism. These possibilities highlight the need for research predicting ecological responses to changes in climate and other disturbances.

This study furthers scientific knowledge of the caddisfly-associated microbiome, the role of aquatic macroinvertebrates as ecosystem engineers in streams, and stream metabolism in the context of excess anthropogenic nitrogen and its impacts on the planet.

Conclusion

Anthropogenic excess nitrogen in the environment has many negative impacts on environmental and human health, including eutrophication, pollution, and loss of biodiversity. River systems carry the burden of regulating this phenomenon via nutrient transport and cycling processes that are not well understood. An ongoing project at Montana State University explores the impact of caddisflies in stream biogeochemistry by obstructing interstitial spaces with the silk structures that they create. This obstruction allows for increased time for metabolic processes as well as stronger growth of biofilms. We hypothesize that caddisflies also influence stream chemistry by serving as habitats for a unique microbiome. To explore this, we collected samples of caddisflies, their silk nets and retreats, and swabs of nearby rocks from a mountain stream in Montana, sequenced the microbial genetic material to generate metagenomes, and performed comparative metagenomics. Comparisons of the community structure and the functional genes of the metagenomes indicated clustering by sample type, supporting the hypothesis that the caddisfly-associated microbiome was dissimilar from the other stream microbiomes. Future steps could include studies that replicate these findings, explorations of possibly enriched metabolic processes in the caddisfly-associated microbiome, and investigations of possible changes in stream metabolism due to current and future environmental stressors.

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References

Albertson, L. K., and D. C. Allen (2015), Meta-analysis: Abundance, behavior, and hydraulic energy shape biotic effects on sediment transport in streams, *Ecology, 96*(5), 1329–1339.

Albertson, L., and J. Poole (2020), Macroinvertebrate ecosystem engineers mediate whole-stream metabolism and nutrient uptake.

Albertson, L. K., L. S. Sklar, S. D. Cooper, and B. J. Cardinale (2019), Aquatic macroinvertebrates stabilize gravel bed sediment: A test using silk net-spinning caddisflies in semi-natural river channels, *PLoS ONE, 14*(1), e0209,087.

Battin, T. J., K. Besemer, M. M. Bengtsson, A. M. Romani, and A. I. Packmann (2016), The ecology and biogeochemistry of stream biofilms, *Nature Reviews Microbiology, 14*(4), 251–263, doi:10.1038/nrmicro.2016.15.

Benoit, G., M. Mariadassou, S. Robin, S. Schbath, P. Peterlongo, and C. Lemaitre (2020), SimkaMin: fast and resource frugal de novo comparative metagenomics, *Bioinformatics 36*(4), 1275-1276, doi:10.1093/bioinformatics/btz685.

Davidson, E. A., M. B. David, J. N. Galloway, C. L. Goodale, R. Haeuber, J. A. Harrison, R. W. Howarth, D. B. Jaynes, R. R. Lowrance, B. T. Nolan, J. L. Peel, R. W. Pinder, E. Porter, C. S. Snyder, A. R. Townsend, and M. H. Ward (2011) Excess nitrogen in the U.S. environment: Trends, risks, and solutions, *Issues in Ecology*, *15*.

Eisenhauer, N., A. Bonn, and C. A. Guerra (2019), Recognizing the quiet extinction of invertebrates, *Nature Communications, 10*(1), 50, doi:10.1038/s41467-018-07916-1.

Findlay, S. (2010), Stream microbial ecology, *Journal of the North American Benthological Society, 29*(1), 170–181, doi:10.1899/09-023.1.

Frandsen, P. B., M. G. Bursell, A. M. Taylor, S. B. Wilson, A. Steeneck, and R. J. Stewart (2019), Exploring the underwater silken architectures of caddisworms: comparative silkomics across two caddisfly suborders, *Philosophical Transactions of the Royal Society*, doi:10.1098/rstb.2019.0206.

Fritz, R. (2019), Leaf litter effects on the active gut microbiome of a freshwater shredding caddisfly, M. S. Thesis, Northern Arizona University, *ProQuest Dissertations & Theses*, https://www.proquest.com/docview/2278078939/fulltextPDF/E5F632BDD94543D4PQ/.

Gomez-Velez, J. D., J. W. Harvey, M. B. Cardenas, and B. Kiel (2015), Denitrification in the Mississippi River network controlled by flow through river bedforms, *Nature Geoscience*, *8*, 941 EP –.

Hyatt, D., G. Chen, P. F. LoCascio, M. L. Land, F. W. Larimer, and L. J. Hauser (2010), Prodigal: prokaryotic gene recognition and translation initiation site identification, *BMC Bioinformatics, 11*, 119, doi:10.1186/1471-2105-11-119.

Kim, H., H. Bae, K. R. Reddy, and A. Ogram (2016), Distributions, abundances, and activities of microbes associated with the nitrogen cycle in riparian and stream sediments of a river tributary, *Water Research*, *106*, 51-61, doi:10.1016/j.watres.2016.09.048.

Li, D., C. Liu, R. Luo, K. Sadakane, and T. Lam (2015), MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct *de Bruijn* graph, *Bioinformatics 31*(10), 1674-1676, doi:10.1093/bioinformatics/btv033.

Liu, N., L. Zhang, H. Zhou, M. Zhang, X. Yan, Q. Wang, Y. Long, L. Xie, S. Wang, Y. Huang, and Z. Zhou (2013), Metagenomic insights into metabolic capacities of the gut microbiota in a fungus-cultivating termite (Odontotermes yunnanensis), *PLoS ONE, 8*(7), e69184, doi:10.1371/journal.pone.0069184.

Nogaro, G., F. Mermillod-Blondin, M. Valett, F. Franois-Carcaillet, J.-P. Gaudet, M. Lafont, and J. Gibert (2009), Ecosystem engineering at the sedimentwater interface: bioturbation and consumer-substrate interaction, *Oecologia, 161*(1), 125–138.

Receveur, J. P., S. Fenoglio, M. E. Benbow (2020), Insect-associated bacterial communities in an alpine stream, *Hydrobiologia, 847,* 331-344, doi:10.1007/s10750-019-04097-w.

Ren, Z., H. Gao, J. J. Elser, and Q. Zhao (2017), Microbial functional genes elucidate environmental drivers of biofilm metabolism in glacier-fed streams, *Scientific Reports*, *7*, 12668, doi:10.1038/s41598-017-13086-9.

Schlesinger, W. H., K. H. Reckhow, and E. S. Bernhardt (2006), Global change: The nitrogen cycle and rivers, *Water Resources Research, 42*(3), doi:10.1029/2005WR004300.

Singer, G., K. Besemer, P. Schmitt-Kopplin, I. H¨odl, and T. J. Battin (2010), Physical heterogeneity increases biofilm resource use and its molecular diversity in stream mesocosms, *PLoS ONE, 5*(4), e9988, doi:10.1371/journal.pone.0009988.

The UniProt Consortium (2008), The Universal Protein Resource (UniProt), *Nucleic Acids Research*, *36*, doi:10.1093/nar/gkm895.

Vitousek, P. M., J. D. Aber, R. W. Howarth, G. E. Likens, P. A. Matson, D. W. Schindler, W. H. Schlesinger, and D. G. Tilman (1997), Human alteration of the global nitrogen cycle: Sources and consequences, *Ecological Applications*, *7*(3), 737-750.

Wymore, A. S., A. A. Coble, B. Rodriguez-Cardona, and W. H. McDowell (2016), Nitrate uptake across biomes and the influence of elemental stoichiometry: A new look at linx ii, *Global Biogeochemical Cycles, 30*(8), 1183–1191, doi:10.1002/2016GB005468.

Yun, J., S. W. Roh, T. W. Whon, M. Jung, M. Kim, D. Park, C. Yoon, Y. Nam, Y. Kim, J. Choi, J. Kim, N. Shin, S. Kim, W. Lee, and J. Bae (2014), Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host, *Applied and Environmental Microbiology*, *80*(17), 5254-5264, doi:10.1128/AEM.01226-14.