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The Ecological Consequences of Horizontally Transferred Nitrogen Fixation Genes in Cyanobacterium Acaryochloris marina

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Acaryochloris marina, a marine cyanobacterium, commonly lives attached to red algae or colonial ascidians, or sea squirts, in shallow marine environments. Known for its use of chlorophyll *d* as its primary photosynthetic pigment, *A. marina* absorbs light in the far-red range of the spectrum, while most other photosynthetic organisms produce chlorophyll *a* and absorb light maximally at blue and red wavelengths. Nitrogen (N) fixation involves the reduction of atmospheric N_2 to biologically available ammonia (NH_3) and is performed by a wide variety of prokaryotic microorganisms. Nitrogen is often a limiting nutrient in aquatic ecosystems, so nitrogen fixing bacteria provide an essential nutrient in the environment. *A. marina* is typically not regarded as a cyanobacterium capable of nitrogen fixation, despite the presence of nitrogen fixation (*nif*) genes in close cyanobacterial relatives.

Recently through comparative genomics, the Miller Lab at the University of Montana discovered an *A. marina* clade consisting of three strains – GR1, MU08, and MU09 – that possess the ~20 *nif* genes required to perform N fixation (Miller et al, 2022). The three primary *nif* genes are *nifHDK*, which encode the nitrogenase reductase and maturation protein, the nitrogenase alpha chain, and the nitrogenase beta chain, respectively. When these three genes are expressed, they combine to form nitrogenase, the enzyme that performs nitrogen fixation. After the discovery that novel genes are present, the first two points of inquiry that follow are: (1) how did the strains acquire these genes?; and (2) are the genes functional in these strains? For the first question, there are two ways that the genes may have been acquired: they were either vertically inherited from the common ancestor of *A. marina* (with concomitant gene loss in other *A. marina* strains) or obtained by horizontal transfer, likely from another cyanobacterial species. Horizontal

gene transfer is observed frequently in cyanobacteria, so this is a likely source of novel genes (Chen et al, 2005).

The investigation into the origin of *nif* genes in these strains of *A. marina* was conducted using a phylogenetic approach, designing two trees. One tree is based on *nifHDK* sequences from *A. marina* strains GR1, MU08, and MU09, as well as those from closely related cyanobacterial species. The other tree, which represents the species tree, is reconstructed from 16S rRNA sequences from these same strains of *A. marina* and other species. When the two trees are compared, if the topology between *A. marina* branches and the nearest cyanobacterial relatives is the same in both trees, then this is evidence that *nif* genes were vertically inherited. If the topologies do not match, this is evidence of horizontal transfer, since the genes are more closely related to a different species than that which contains the most similar *nifHDK* sequences to those in the *A. marina* strains.

To test whether these strains of *A. marina* can express their *nif* genes to produce nitrogenase, this study was conducted using a physiological approach, growing each strain in a flask without essential fixed nitrogen (e.g., NH_3). If the strains with *nif* genes continue to grow and/or survive, this is evidence that *A. marina* strains GR1, MU08, and MU09 are capable of reducing N_2 to NH_3, which is used by the cell for growth and cell division. Strains in media with and without NH_3 will be compared, showing the difference, if any, between growth rates. A strain not containing *nif* genes – *A. marina* CCMEE 5410 – will also be grown in media with and without NH_3 to act as a control.

Literature review

Acaryochloris marina is not heavily researched; however, since its discovery, it has drawn the attention of marine ecologists and microbiologists due to its unique nature and widespread presence throughout the Earth's oceans as well as its fresh bodies of water. While the vast majority of photosynthetic organisms utilize chlorophyll *a* to absorb light energy, the cyanobacterium *Acaryochloris marina* is distinctive in its use of chlorophyll *d* as its major photosynthetic pigment (Miyashita et al, 1996). This brought field-wide fame to the species, with scientists questioning what else has evolved with this pigment (Swingley et al, 2008). Discovered in 1996 by researchers in Japan, the cyanobacterium uses chl *d* to absorb light at a longer wavelength of light in the far-red region (Miyashita et al, 1996). "Photosynthesis at the far-red region of the spectrum in *Acaryochloris marina*" by researchers in Pakistan and Saudi Arabia displays the structural differences between chl *a* and chl *d* as well as the resulting differences in wavelength absorption (Badshah et al, 2017). The genetics of *A. marina*, the physiological and structural mechanisms of each photosystem, and the light harvesting system are also discussed within Badshah et al. (2017). A review of chl *d* and *A. marina* by researchers at the University of Sydney in Australia details the evolution and physiological uses of chl *d* in this cyanobacterium (Loughlin et al, 2013). The review also discusses the genomic compositions of *Acaryochloris* species, such as the presence of nitrogen fixation genes in *Acaryochloris* sp. HICR111A.

Nitrogen fixation, one of the most complex physiological processes in bacteria, is a major contributor to nitrogen available for primary production (Montoya et al, 2004). Genes responsible for nitrogen fixation had not been recognized as present within the *Acaryochloris* genus until 2012 when a cooperative effort between labs across Europe published an annotated

genome of the previously mentioned *Acaryochloris* sp. HICR111A (Pfreundt et al, 2012). After performing physiological tests, the researchers found evidence that this strain expresses these genes to form nitrogenase, the enzyme that performs nitrogen fixation. The most common method to quantify the activity of nitrogenase, as used by the aforementioned researchers, is an acetylene reduction assay (Yoon et al, 2017; Tsujimoto et al, 2014). In this test, acetylene gas is injected into the cellular environment, and the amount of resulting ethylene gas is measured after a certain amount of time (Stewart et al, 1967). This method is effective, because nitrogenase reduces acetylene to ethylene just like it reduces atmospheric nitrogen.

Acaryochloris and *Thermoleptolyngbya* are referred to as non-heterocystous cyanobacteria, meaning they do not produce a differentiated cell known as a heterocyst. The purpose of the heterocyst is to provide a cellular environment where nitrogen fixation can occur. Thus, in non-heterocystous cyanobacteria, nitrogen fixation must occur within a single cell along with all other metabolic processes (Bergman et al, 1997). The enzyme nitrogenase is inhibited by the presence of oxygen – heterocysts provide nitrogenase with a microaerobic environment – so unicellular cyanobacteria must separate oxygen producing processes from nitrogenase (Haselkorn, 1978). To solve this problem, they have tightly regulated diurnal rhythms that perform photosynthesis during the day and nitrogen fixation at night (Sherman et al, 1998).

This regulation is controlled by differential gene expression in response to stimuli such as concentrations of oxygen and fixed nitrogen, as well as light intensity. Recent research has found two transcriptional regulators that, when absent from mutant cyanobacteria, nitrogenase is either expressed abnormally or not at all (Tsujimoto et al, 2014). The researchers concluded that one of the transcriptional regulators is induced by low concentrations of oxygen and the other is induced by low concentrations of fixed nitrogen. Induction of either regulator results in the expression of

nitrogen fixation (*nif*) genes (Tsujimoto et al, 2014). The ~20 *nif* genes in the genome produce peptides that combine to form nitrogenase. Researchers in a Canadian lab have obtained DNA sequences of the regions containing *nif* genes in several species and found that while the amino acid sequences of nitrogenase are highly conserved across species of cyanobacteria, intergenic regions have low similarity (Jackman & Mulligan, 1995). This implies that transcriptional regulator sequences can vary between species.

The difference in *nif* arrangement varies between cyanobacteria with and without heterocysts. The main structural genes, *nifHDK*, are grouped together in non-heterocystous cyanobacteria, but have a space between *nifHD* and *nifK* in heterocystous cyanobacteria (Bolhuis et al, 2010; Kallas et al, 1985). Despite this, the same nitrogenase is expressed in both types of cyanobacteria; expression is just regulated differently (Thiel & Pratte, 2014). In higher concentrations of oxygen, cyanobacteria have other approaches in addition to their diurnal rhythms to separate nitrogenase from oxygen, preventing or slowing inhibition. Tests were performed on isolated nitrogenase and intracellular nitrogenase, showing that intracellular nitrogenase takes six times as long to be inhibited as isolated nitrogenase in an environment with a concentration of oxygen greater than that of air (Klipp et al, 2004). From the observation by Klipp et al. (2004), one can infer that the cell has mechanisms to replace inhibited nitrogenase and/or limit contact between nitrogenase and oxygen other than temporally.

One of the components of nitrogenase is the electron-transfer iron protein (Hoffman et al, 2014). Iron reacts with oxygen, so this is likely the mechanism by which nitrogenase inhibition occurs, a large contributor to the evolved regulation of nitrogenase. In work from the Miller Lab at the University of Montana, nitrogen fixation genes have been discovered in the genomes of some strains of *A. marina* closely related to *Acaryochloris* sp. HICR111A (Miller et al, 2022).

The research detailed within this paper will verify whether these *A. marina* strains can produce a functional nitrogenase and will determine from where only some strains have acquired these genes.

Methods

Phylogenetic analyses

Genomes of *Acaryochloris marina* strains GR1, MU08, and MU09 have yet to be publicly available, other sequences were obtained from Cyanobase and NCBI. Publicly available assemblies for *Acaryochloris sp.* HICR111A (NCBI accession JN585763.1), *Cyanothece* sp. PCC 7425 (Cyanobase assembly ID GCA_000022045.1), *Halothece sp.* PCC 7418 (Cyanobase assembly ID GCA_000317635.1), *Microcoleus* sp. PCC 7113 (Cyanobase assembly ID GCA_000317515.1), *Leptolyngbya sp.* PCC 7375 (Cyanobase assembly ID GCA_000316115.1), *Leptolyngbya* sp. Heron Island J (Cyanobase assembly ID GCA_000482245.1), *Synechococcus* sp. PCC 7335 (Cyanobase assembly ID GCA_000155595.1), *Synechococcus sp.* JA-3-3Ab (Cyanobase assembly ID GCA_000013205.1), *Synechococcus* sp. JA-2-3Ba (Cyanobase assembly ID GCA_000013225.1) were included in phylogenetic reconstructions. 16S rRNA and *nifHDK* sequences were aligned into respective files using the program ClustalW (Berman-Frank, 2003). Two maximum likelihood trees were constructed from these alignments with 100 bootstrap replicates using PAUP* version 4.0 according to the GTR+I+G model of sequence evolution selected by MODELTEST.

Culture conditions

Controls were grown in ASN-III medium which was made to the specifications stated in "Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria" by

Rippka et al. (1979). Slight changes were made to this recipe, resulting in a modified saltwater medium consisting of: 25.0 g NaCl; 3.5 g $MgSO_4$ x $7H_2O$; 2.0 g $MgCl_2$ x $6H_2O$; 0.75 g $NaNO_3$; 0.75 g K_2HPO_4 x $3H_2O$; 0.5 g $CaCl_2$ x $2H_2O$; 0.5 g KCl; 0.02 g $NaCO_3$; 3 mg citric acid; 3 mg ferric ammonium citrate; 0.5 mg EDTA; and 1 mL micronutrient solution (2.86 g H_3BO_3, 1.81 g $MnCl_2$ x $4H_2O$, 0.222 $ZnSO_4$ x $7H_2O$, 0.391 g Na_2Mo_4 x $2H_2O$, 0.079 g $CuSO_4$ x $5H_2O$, and 0.0494 g $Co(NO_3)_2$ x $6H_2O$ dissolved in 1 L distilled H_2O) dissolved in 1 L and adjusted to pH 8.2 (Ulrich et al, 2021). Experimental cultures were grown in ASN-III -N medium, consisting of the same recipe as ASN-III, but lacking $NaNO_3$. All cultures were kept in an incubator at 25° C for the duration of the experiment. To simulate a natural environment, the lights in the incubator were on a 12-hour cycle.

Check for contaminants

Prior to the growth experiment, to confirm the absence of nitrogen fixing contaminants, each culture was plated on IO BG-11 agar lacking fixed nitrogen, consisting of the following dissolved in 1 L of distilled water: 25 g Instant Ocean; 0.0036 g $CaCl_2$-H_2O; 0.012 g $FeNH_4$Citrate; 0.001 g Na_2EDTA; 0.04 g K_2HPO_4; 0.075 g $MgSO_4$ x $7H_2O$; 0.02 g Na_2CO_3; and 1 mL of micronutrient solution (2.86 g H_3BO_3, 1.81 g $MnCl_2$ x $4H_2O$, 0.222 $ZnSO_4$ x $7H_2O$, 0.391 g Na_2Mo_4 x $2H_2O$, 0.079 g $CuSO_4$ x $5H_2O$, and 0.0494 g $Co(NO_3)_2$ x $6H_2O$ dissolved in 1 L distilled H_2O). IO BG-11 media was buffered with 10 mM HEPES at pH 8.0 (Ulrich et al, 2021). After incubating at room temperature in an environment conducive to growth, contaminants did not appear, allowing the conclusion to be made that contaminating diazotrophs were not present in the cultures.

Growth experiment

Duplicates of each of the four *A. marina* strains in ASN-III media were inoculated, as well as duplicates of each strain in ASN-III -N media, lacking a source of fixed N (sodium nitrate). Optical densities (ODs) were taken for four of our existing purified lab strains of *A. marina* (GR1, MU08, MU09, and 5410) and the equation $OD_1 * V_1 = OD_2 * V_2$ was used to determine how many milliliters of each original culture were needed to begin the experiment with an OD of 0.01 in each new culture. Homogenization of each culture for accurate ODs consisted of scraping the bottom of each flask with a sterile wooden scraper 20 times in each direction, horizontally and vertically, then scraping the bottom edges in a circular motion, as well as the sides of the flask, and giving a vigorous swirl before transferring 2 mL to the cuvette.

To inoculate the +N controls, each calculated amount from the original culture was transferred to fresh ASN-III media. To inoculate the -N flasks, each culture was first rinsed with fresh ASN-III -N media to remove excess fixed nitrogen. To perform the -N rinse, the previously calculated amounts from each original culture were pipetted into 2 mL tubes and each tube was labeled with its strain name. The tubes were centrifuged for seven minutes on maximum speed (12,700 rpm) and the supernatant was pipetted out. One milliliter of ASN-III -N media was pipetted into each tube, tubes were vortexed to reconstitute the culture, and the process was repeated two more times, until the cultures had been rinsed three times. Each tube was then poured into its respective flask of fresh ASN-III -N media. Optical densities were taken at T_0 after vigorously swirling each flask, and then about every 48 hours thereafter, homogenizing the cultures as before.

Results and Discussion

Horizontal Gene Transfer of Nitrogen Fixation Genes in A. marina

A. marina strains GR1, MU08, and MU09 have the coding potential to convert atmospheric nitrogen (N_2) to fixed nitrogen to be assimilated. Due to the rarity of this within *A. marina*, our lab has investigated the evolutionary origins of these nitrogen fixation (*nif*) genes. The construction of phylogenies for *nifHDK* and 16S rRNA genes has clarified our understanding of the relatedness of cyanobacteria containing nitrogen fixation genes (Figure 1). The trees provide evidence of horizontal transfer, since the topologies of the two trees do not match, so the *nif* genes must have a different evolutionary history. If the topology between *A. marina* branches and the nearest cyanobacterial relatives were the same in both trees, that would have been evidence that *nifHDK* genes were vertically inherited from the closest related species. However, this was not the case; therefore, *A. marina* has likely acquired its nitrogen fixation genes from the a donor related to the distantly related cyanobacterium, *Leptolyngbya* sp. PCC 7375. This is an example of how multigenic, physiologically complex functions can be transferred.

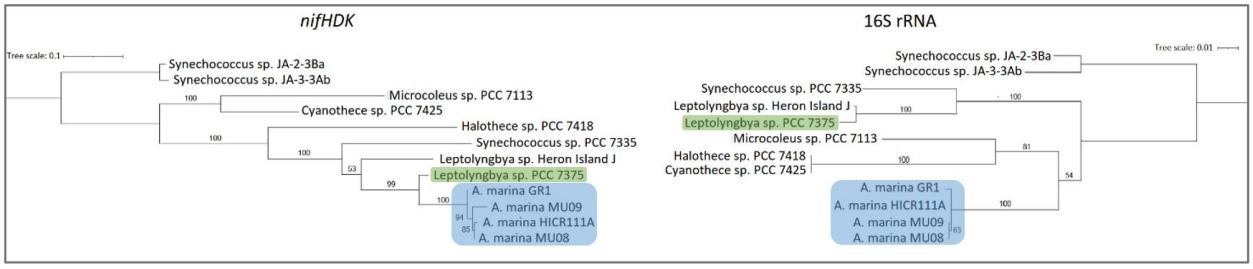

Figure 1. Maximum likelihood phylogenies for *nifHDK* and 16S rRNA genes reconstructed with a GTR+I+G model. Bootstrap support values greater than 50% are shown for 1000 bootstrap replicates. Branch lengths are in units of expected

number of nucleotide substitutions per site. The two topologies were significantly different by an SH test ($p < 0.0001$). Figure and legend included in Miller et al. (2022).

Assimilation of Atmospheric N_2 by A. marina

After finding that *A. marina* has likely acquired its nitrogen fixation genes by HGT, it must then be determined whether *nif*-containing *A. marina* strains are able to perform nitrogen fixation. By performing a growth experiment with and without fixed nitrogen in the media, we found that *nif*-containing *A. marina* strains MU08 and MU09 grow at very similar rates in both conditions. As expected, *A. marina* strain CCMEE 5410 does not grow in media lacking fixed nitrogen, because it does not contain the genes required for nitrogen fixation. This is evidence that *nif*-containing strains of *A. marina* can produce a functional nitrogenase that has little to no impact on growth rate as compared to strains grown in the presence of nitrogen. It is not yet known whether nitrogenase is being produced in nitrogen replete conditions; more research must be performed to determine the regulation and expression of this enzyme in *A. marina*.

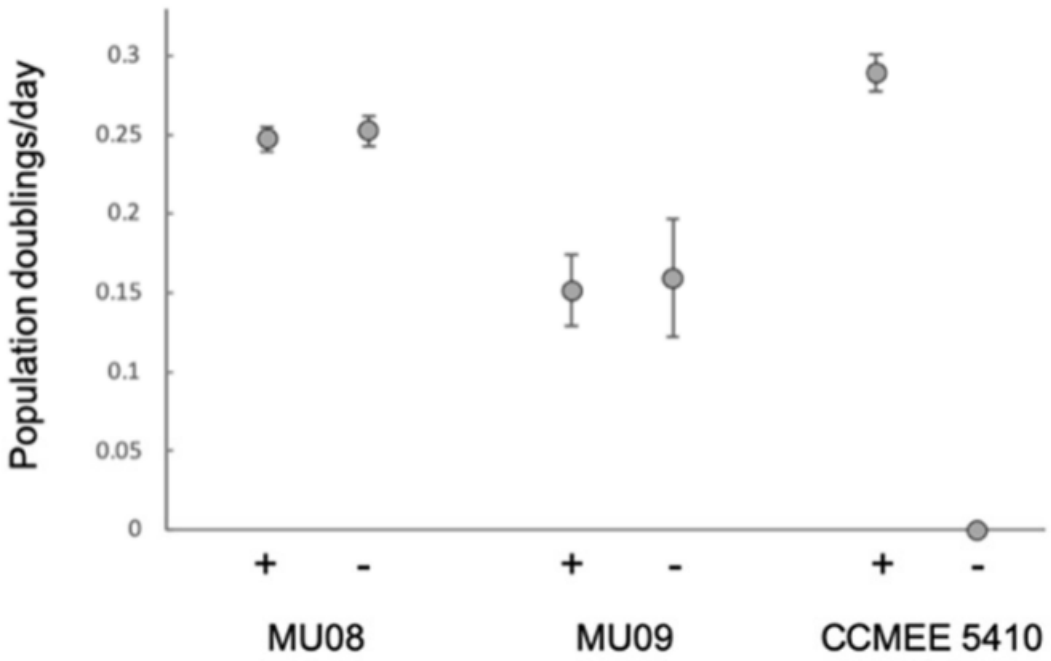

Figure 2. Population growth rate in the presence (+) or absence (−) of combined nitrogen for nif-containing strains MU08 and MU09 and for nif-lacking strain CCMEE 5410. Error bars are standard errors. Figure and legend included in Miller et al. (2022).

Conclusions and future research directions

It has now been determined from where recently discovered *nif* genes in strains of cyanobacterium *Acaryochloris marina* have come. By following the methods of phylogenetic analysis stated in this research, the likely source of *nif* genes in strains GR1, MU08, and MU09 of *A. marina* is the distantly related cyanobacterium, *Leptolyngbya* PCC 7375. These strains of *A. marina* have acquired the *nif* genes through horizontal transfer. Prior to confirming this through our phylogenies, we predicted this, since there are very few strains that contain genes for nitrogen fixation relative to the entire genus *Acaryochloris*, and vertically inherited genes would

have been subsequently lost in all other strains of *A. marina* after acquisition, which is much less likely.

There is now evidence to support that *nif* genes in strains GR1, MU08, and MU09 of *A. marina* are expressed to form a fully functional nitrogenase. We hypothesized that if nitrogen fixation is occurring in a system in which only *A. marina* is present, *nif*-containing strains of *A. marina* should be able to produce a functional nitrogenase. Inoculation of *A. marina* strains MU08 and MU09 in media lacking fixed nitrogen, and subsequent growth of the strains provides evidence that *A. marina* is performing nitrogen fixation, and thus expresses *nif* genes to produce a functional nitrogenase. Growth rates of strains with and without *nif* genes inoculated in N-replete media and N-deficient media were then compared to identify whether strains with *nif* genes are growing at similar rates with and without fixed nitrogen in the media. Growth rates of strains with and without *nif* genes were also compared to each other to exhibit the difference between the growth of *nif* containing strains and the death of strains lacking *nif* genes in N-deficient media. This provides evidence that strains with *nif* genes are effectively reducing atmospheric nitrogen to ammonia by expressing their *nif* genes and producing nitrogenase.

This research informs the field of the ability to transfer large, physiologically complex groups of genes from one cyanobacterium to another through horizontal transfer. Now that strains of *A. marina* are found to produce nitrogenase, future research related to this should be directed toward a physiological experiment determining whether nitrogenase in *A. marina* is regulated similarly to other unicellular nitrogen-fixing cyanobacteria. This could be done by performing an acetylene reduction assay to quantify the amount of nitrogen fixation occurring at different times in a 24-hour period. If nitrogen fixation in *A. marina* is regulated similarly to that of other unicellular cyanobacteria, the expected outcome would be more nitrogen fixation

occurring during the night, and less during the day to account for the temporal separation of nitrogenase and oxygen common in non-heterocystous cyanobacteria.

This work, performed in the Miller Lab at the University of Montana, has been published in part within a peer-reviewed paper titled, "Genomic and Functional Variation of the Chlorophyll d-Producing Cyanobacterium *Acaryochloris marina*" (Miller et al, 2022).

Ethical considerations

Research ethics are often difficult to navigate in life science-focused fields. This is especially the case in non-medical research not involving human subjects since they are not discussed as often. Human and large mammal research is typically at the center of ethical discussion, leaving other forms of scientific research with unspecified ethics. There is also a clear lack of diversity in all scientific fields. Types of ethics in science that must be addressed include those related to the environment and cultural diversity in research.

Environmental concerns pertaining to ethics of lab-based research that do not seem to be addressed often, include the large amounts of plastic waste and energy used to facilitate research. Types of plastic waste include the large amount of single-use pipette tips, petri dishes, containers in which media is made, and centrifuge tubes. While some forms of plastic waste cannot be avoided due to the necessity of sterility, many can be replaced with other materials. The laboratory in which the following research was conducted is conscious of the plastic waste produced, so use of glassware and reusable plastics is very common; however, this is not the case with all laboratories, due to the lack of universal regulation of plastic use.

Energy consumption – another environmental impact – also must be considered in biological research labs. Large, energy use-heavy apparatuses and appliances are common in the

laboratory, such as incubators, autoclaves, and centrifuges. Incubators, for example, are most often running 24 hours a day, sometimes at very high temperatures, using electric heat – not the most efficient form of energy, running fans, and powering light bulbs. The lab in which this research was conducted has four incubators – each the size of a refrigerator – one of which is maintaining bacterial strains at a very high temperature of 75°C (167°F). One possible solution to the issue of energy overuse is to balance a lab's carbon footprint in other ways, such as purchasing carbon offsets (e.g., paying to plant trees), recycling, composting, and using less electricity elsewhere in the lab.

Another potential ethical concern pertaining to the environment is from where samples of bacteria are collected, and whether this is done without impacting the ecosystem. Many of the samples in the lab in which this research was conducted are from Yellowstone National Park or the Oregon Coast. When collecting samples, this lab is very careful in gathering water samples and not contaminating waters with other collected samples. An ecosystem has the potential to be changed dramatically if a species of bacterium collected from one creek is accidentally transferred to another creek in which the bacterium was formerly absent. An ecosystem can be even more impacted in other types of ecological research in which an invasive species, such as a mollusk or a fish, is unintentionally relocated to a different body of water. Environmental impact is one of the many factors that must be taken into account when considering biological research ethics.

An aspect of ethics more applicable to the field of science as a whole is that of cultural diversity in literature as well as accommodations for diversity in the laboratory setting. This same issue is shown by Cronin et al, a paper featuring a diverse group of authors that found 60% of all ecology and evolution PhDs awarded in the US in 2018 were to recipients who identified

as White (Cronin et al, 2021). There was not a single Black or African American author in the bibliography of this paper. This will be fixed in the future by searching for authors with diverse backgrounds, possibly by looking at labs that are recognized for being diverse, in each of my papers now and in the future. The lack of diversity in this bibliography without actively searching for diversity is representative of how the field as a whole is lacking.

While there are many other ideas as to how to approach this situation – which should undoubtedly be implemented as soon as possible – the issue is that lack of diversity in science is systemic, so it will take time before change is truly seen. An extensive history of racism and exclusion exists in ecology and evolution, and racist scientists are still taught about in scientific courses (Cronin et al, 2021). While avoiding the mentioning of the scientist responsible for a particular discovery would be helpful in remediating this issue, scientists from diverse backgrounds that assisted – or even did more work – could be acknowledged instead. Dr. Pascale Guiton, an African woman who has risen though the field of parasitology believes that one way to accommodate those from diverse backgrounds is to change hypothetical math problems, for example, to situations that all students can imagine (Guiton, 2021). She discusses a problem in which a train to Paris is referenced, even though she has never "been on a train nor to France" (Guiton, 2021). There are an increasing number of resources describing how and why to create an anti-racist environment in both the classroom and the laboratory, so there are no more excuses as to why anti-racist environments can't be established (Guiton, 2021; Cronin et al, 2021; Chaudhary & Berhe, 2020).

The principal investigator of the lab in which this research was conducted reached out to assist another professor at the University of Montana who has led science programs on campus in the summer, directed toward American Indians from the surrounding reservations. These

students are invited – if in good academic standing – back to campus year after year. This is an efficient way to improve representation of certain groups in scientific fields, while providing an incentive for maintaining their grades; he visits reservations – many of their homes – and gives them the resources to get interested in science and have scientific experiences on a campus known for their research. This same approach should be taken by universities near areas of large African American populations. Representation of all racial and cultural groups is lacking noticeably in ecology and evolution, in addition to all other fields within science.

References

Badshah, S. L., Mabkhot, Y., & Al-Showiman, S. S. (2017). Photosynthesis at the far-red region of the spectrum in Acaryochloris marina. *Biological Research*, *50*(1), 1–9. https://doi.org/10.1186/s40659-017-0120-0

Bergman, B., Gallon, J. R., Rai, A. N., & Stal, L. J. (1997). N 2 Fixation by non-heterocystous cyanobacteria. *FEMS Microbiology Reviews*, *19*(November), 139–185.

Berman-Frank, I., Lundgren, P., & Falkowski, P. (2003). Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. *Research in Microbiology*, *154*(3), 157–164. https://doi.org/10.1016/S0923-2508(03)00029-9

Bolhuis, H., Severin, I., Confurius-Guns, V., Wollenzien, U. I. A., & Stal, L. J. (2010). Horizontal transfer of the nitrogen fixation gene cluster in the cyanobacterium Microcoleus chthonoplastes. *ISME Journal*, *4*(1), 121–130. https://doi.org/10.1038/ismej.2009.99

Chaudhary, B., & Berhe, A. (2020). Ten simple rules for building an anti-racist lab. *PLOS Computational Biology*, *16*(10), 1–9. https://doi.org/10.32942/osf.io/4a9p8

Chen, M., Hiller, R. G., Howe, C. J., & Larkum, A. W. D. (2005). Unique origin and lateral transfer of prokaryotic chlorophyll-b and chlorophyll-d light-harvesting systems. *Molecular Biology and Evolution*, *22*(1), 21–28. https://doi.org/10.1093/molbev/msh250

Cronin, M. R., Alonzo, S. H., Adamczak, S. K., Baker, D. N., Beltran, R. S., Borker, A. L., Favilla, A. B., Gatins, R., Goetz, L. C., Hack, N., Harenčár, J. G., Howard, E. A., Kustra, M. C., Maguiña, R., Martinez-Estevez, L., Mehta, R. S., Parker, I. M., Reid, K., Roberts,

M. B., … Zavaleta, E. S. (2021). Anti-racist interventions to transform ecology, evolution, and conservation biology departments. *Nature Ecology and Evolution*, *5*(9), 1213–1223. https://doi.org/10.1038/s41559-021-01522-z

Guiton, P. S. (2021). mSphere of Influence: No More Excuses—Addressing Race, Racism, and Socioeconomic Issues in the Science Classroom and Laboratory. *Msphere*, *6*(1), 10-21. https://doi.org/10.1128/mSphere.00010-21

Haselkorn, R. (1978). Heterocysts. *Ann. Rev. Plant Physiol.*, *29*, 319–344.

Hoffman, B. M., Lukoyanov, D., Yang, Z. Y., Dean, D. R., & Seefeldt, L. C. (2014). Mechanism of nitrogen fixation by nitrogenase: The next stage. *Chemical Reviews*, *114*(8), 4041–4062. https://doi.org/10.1021/cr400641x

Jackman, D. M., & Mulligan, M. E. (1995). Characterization of a nitrogen-fixation (nif) gene cluster from Anabaena azollae 1a shows that closely related cyanobacteria have highly variable but structured intergenic regions. *Microbiology*, *141*(9), 2235–2244. https://doi.org/10.1099/13500872-141-9-2235

Kallas, T., Coursin, T., & Rippka, R. (1985). Different organization of nif genes in nonheterocystous and heterocystous cyanobacteria. *Plant Molecular Biology*, *5*(5), 321–329. https://doi.org/10.1007/BF00020630

Klipp, W., Masepohl, B., Gallon, J. R., & Newton, W. E. (2004). Genetics and Regulation of Nitrogen Fixation in Free-Living Bacteria. *Kluwer Academic Publishers*, 111–139. https://doi.org/10.1007/1-4020-2179-8_6

Kutschera, U., & Schauer, S. (2012). Prokaryotic biodiversity in marine versus terrestrial ecosystems: Methylobacteria and research ethics. *J Marine Sci Res Dev, 2*(4), 1-2. http://dx.doi.org/10.4172/2155-9910.1000e113

Loughlin, P., Lin, Y., & Chen, M. (2013). Chlorophyll d and Acaryochloris marina: Current status. *Photosynthesis Research, 116*(2–3), 277–293. https://doi.org/10.1007/s11120-013-9829-y

Miller, S. R., Abresch, H. E., Baroch, J. J., Fishman Miller, C. K., Garber, A. I., Oman, A. R., Ulrich, N. J. (2022). Genomic and Functional Variation of the Chlorophyll d-Producing Cyanobacterium *Acaryochloris marina. Microorganisms, 10*(3), 569. https://doi.org/10.3390/microorganisms10030569

Miyashita, H., Ikemoto, H., Kurano, N., Adachi, K., Chihara, M., & Miyachi, S. (1996). Chlorophyll d as a major pigment. *Nature, 383*(6599), 402. https://doi.org/10.1038/383402a0

Montoya, J. P., Holl, C. M., Zehr, J. P., Hansen, A., Villareal, T. A., & Capone, D. G. (2004). High rates of N2 fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean. *Nature, 430*(7003), 1027–1031. https://doi.org/10.1038/nature02824

Pfreundt, U., Stal, L. J., Voß, B., & Hess, W. R. (2012). Dinitrogen fixation in a unicellular chlorophyll d-containing cyanobacterium. *ISME Journal, 6*(7), 1367–1377. https://doi.org/10.1038/ismej.2011.199

Rippka, R., Deruelles, J., & Waterbury, J. B. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology*, *111*(1), 1–61. https://doi.org/10.1099/00221287-111-1-1

Sherman, L. A., Meunier, P., & Colón-López, M. S. (1998). Diurnal rhythms in metabolism: A Day in the life of a unicellular, diazotrophic cyanobacterium. *Photosynthesis Research*, *58*(1), 25–42. https://doi.org/10.1023/A:1006137605802

Stewart, W. D. P., Fitzgerald, G. P., & Burris, R. H. (1967). In situ studies on N2 fixation using the acetylene reduction technique. *Proceedings of the National Academy of Sciences of the United States of America*, *58*, 2071–2078.

Tsujimoto, R., Kamiya, N., & Fujita, Y. (2014). Transcriptional regulators ChlR and CnfR are essential for diazotrophic growth in nonheterocystous cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(18), 6762–6767. https://doi.org/10.1073/pnas.1323570111

Ulrich, N. J., Uchida, H., Kanesaki, Y., Hirose, E., Murakami, A., & Miller, S. R. (2021). Reacquisition of light-harvesting genes in a marine cyanobacterium confers a broader solar niche. *Current Biology*, *31*(7), 1539-1546.e4. https://doi.org/10.1016/j.cub.2021.01.047

Swingley, W. D., Chen, M., Cheung, P. C., Conrad, A. L., Dejesa, L. C., Hao, J., Honchak, B. M., Karbach, L. E., Kurdoglu, A., Lahiri, S., Mastrian, S. D., Miyashita, H., Page, L., Ramakrishna, P., Satoh, S., Sattley, W. M., Shimada, Y., Taylor, H. L., Tomo, T., … Touchman, J. W. (2008). Niche adaptation and genome expansion in the chlorophyll d-producing cyanobacterium Acaryochloris marina. *Proceedings of the National Academy of*

Sciences of the United States of America, *105*(6), 2005–2010. https://doi.org/10.1073/pnas.0709772105

Yoon, K.-S., Nguyen, N. T., Tran, K. T., Tsuji, K., & Ogo, S. (2017). Nitrogen Fixation Genes and Nitrogenase Activity of the Non-Heterocystous Cyanobacterium *Thermoleptolyngbya* sp. O-77. *Microbes and Environments*, *32*(4), 324–329. https://doi.org/10.1264/jsme2.ME17015