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ORIGINAL ARTICLE

Nitrogen fixation rates in forested mountain streams: Are sediment microbes more important than previously thought?

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Abstract

1. Biological nitrogen (N) fixation, the microbial conversion of N_2 gas to ammonia, makes N available to food webs. Low-N streams often have a high relative abundance of N-fixing taxa, suggesting that N fixation is an important N source in these systems. Despite this potential, stream N fixation has not been well-characterised, particularly compared to lakes and marine environments. One unknown is the relative contributions of various N-fixing organisms, particularly heterotrophic microbes.
2. In low-N streams in the Cascade Mountains (Washington, USA), three groups of N-fixers predominate: cyanobacteria (*Nostoc paramelioides*) colonies that house a midge symbiont (*Cricotopus* spp.), cyanobacteria without a midge symbiont, and heterotrophic sediment microbes. In seven streams, we measured N fixation rates in each group with the acetylene reduction assay and a $^{15}N_2$ calibration.
3. Cyanobacteria N fixation rates were relatively low (7.9 ± 8.9 $\mu g\,N\,m^{-2}\,hr^{-1}$, mean $\pm$ *SD*) compared to other mountain streams. Although rates were comparable among types of N-fixers, our sediment conversion ratio (moles of ethylene produced:moles of N fixed) was 0.16:1, much lower than our cyanobacteria conversion ratio of 1.72:1 and the commonly used theoretical ratio of 3:1. Sediment N fixation rates (5.7 ± 4.0 $\mu g\,N\,m^{-2}\,hr^{-1}$) were higher than previously reported rates measured only with acetylene reduction.
4. The midge symbiosis did not greatly impact N fixation rates; however, owing to their prevalence, colonies with the midge probably contributed more total N to streams than colonies without the midge. Additionally, N fixation by sediment heterotrophs was comparable to that of cyanobacteria colonies on an areal basis.
5. Our study demonstrated that the contribution of sediment heterotrophs previously may have been underestimated in streams, especially considering that sediment heterotrophs are probably present for a longer portion of the growing season than cyanobacteria in temperate and boreal ecosystems.

KEYWORDS
$^{15}N_2$, acetylene reduction assay, *Cricotopus*, cyanobacteria, heterotrophic N-fixers, *Nostoc paramelioides*

1 | INTRODUCTION

Food webs in forested mountain streams usually rely on allochthonous inputs of nutrients (Vannote et al., 1980); however, these inputs are frequently seasonal. For example, in snow-pack fed streams, leaf litter and spawning salmon contributions peak in the autumn, wet atmospheric deposition peaks in winter, and soil runoff is highest during the spring melt. Determining the role of *in situ* nutrient sources may help to complete the picture of stream food webs when allochthonous inputs are low. One potential *in situ* nutrient source is biological nitrogen (N) fixation, a microbial process that converts N_2 gas into a biologically accessible form of N (i.e. ammonia). Although in-stream N fixation often has been viewed as less important than other N inputs (e.g. fertiliser runoff, marine-derived N) to streams (Howarth et al., 1988), N fixation can be the main N source to low-N streams (Carmiggelt & Horne, 1975; Grimm & Petrone, 1997). Specifically, stream N fixation could be important in the Cascade Mountains of the Pacific Northwest, which contain many sections of older volcanic rock with low N to phosphorus (P) ratios (Leland, 1995). Many Pacific Northwest streams, which formerly supported healthy salmon runs, probably receive only 6–7% of the marine-derived N and P that they historically received from salmon (Gresh et al., 2000). In addition, increased tree biomass as a consequence of fire suppression results in more stored terrestrial N, further reducing stream N inputs (Bernal et al., 2012). These conditions increase the likelihood that Cascade streams are N-depleted and therefore more heavily reliant on N fixation.

The primary groups of N-fixers in the Cascades include heterotrophic microbes in benthic sediments and aquatic cyanobacteria, predominantly *Nostoc paramelioides* (family *Nostocaceae*), which is filamentous and forms colonies (Dodds et al., 1995). *Nostoc paramelioides* colonies are present in two types: (1) those that form a symbiotic relationship with the chironomid (midge) larva *Cricotopus nostocicola* or *fuscata* (Brock, 1960), hereafter referred to as "cyano-midge", and (2) those without a midge symbiont, hereafter referred to as "cyano-only." The midge larva tunnels into the *Nostoc* colony and uses the colony as food until pupation (Brock, 1960). In return for food and protection from predators, the midge benefits the *Nostoc* colony by attaching it firmly to the rocks, changing its shape to improve gas exchange, and increasing *Nostoc* dispersal via the spread of reproductive filaments during midge emergence (Dodds & Marra, 1989). The relationship between *N. paramelioides* and the midge may impact N fixation rates by changing the shape and surface area of the colony to increase diffusion and add stability, especially in currents faster than 10 $cm\,s^{-1}$ (Dodds, 1989), conditions which are common in high-gradient Cascade streams.

In addition to possible impacts from biological factors, many physical and chemical factors influence N fixation, which is a high-energy process, theoretically requiring 16 ATP to fix one N_2 molecule (Kim & Rees, 1994). It is catalysed by the nitrogenase enzyme, which contains P and micronutrients. For autotrophic cyanobacteria, N fixation tends to increase with light (Berrendero et al., 2016; Carmiggelt & Horne, 1975; Grimm & Petrone, 1997), P (Marcarelli & Wurtsbaugh, 2006, 2007, Kunza & Hall, 2013, but see Scott et al., 2009), temperature (Marcarelli & Wurtsbaugh, 2006; Welter et al., 2015) and substrate stability (Marcarelli & Wurtsbaugh, 2009). Dissolved inorganic N (DIN) frequently inhibits N fixation in cyanobacteria, because they can meet their nutritional needs with less energy (Eberhard et al., 2018; Hiatt et al., 2017; Kunza & Hall, 2013, 2014; Marcarelli & Wurtsbaugh, 2006, 2007; Scott et al., 2009). Likewise, heterotrophic N fixation rates increase with carbon (C; energy) availability, such as that provided by leaf litter (Tam et al., 1981) and fine sediment (Francis et al., 1985). Heterotrophic N fixation also increases with P (Romero et al., 2012) and often is inhibited by DIN (Caton et al., 2018; Eberhard et al., 2018, but see Knapp, 2012). Although cyanobacterial and heterotrophic N fixation are influenced by similar physicochemical drivers, the midge symbiosis may cause distinct patterns in cyano-only and cyano-midge fixation rates.

Drivers of N fixation are well-established in general, but they have not been thoroughly explored in streams (Marcarelli et al., 2008). In particular, the relative contributions of different taxa have not been examined. Notably, very few studies have measured N fixation rates in sediment heterotrophs, which recently have been identified as important N-fixers in marine environments (Aoki & McGlathery, 2019; Newell et al., 2016; Rao & Charette, 2012), suggesting that they also could be important in streams.

We measured N fixation rates, along with possible explanatory variables, in seven forested streams in the Cascade Mountains, Washington, USA, during the summer and autumn of 2019. Our objectives were to (a) determine the relative importance of the major groups of N-fixers: cyano-midge, cyano-only and sediment microbes, and (b) establish rates and physicochemical drivers of N fixation. We hypothesised that light would be the primary predictor of cyanobacteria N fixation. Likewise, we predicted that cyanobacteria would fix more N than sediment microbes in streams with higher light availability, whereas sediment heterotrophs would fix more N in shaded streams. Finally, we hypothesised that the presence of a midge symbiont would increase cyanobacteria N fixation rates in our high-gradient streams.

2 | METHODS

We measured N fixation rates for cyano-midge, cyano-only and sediment heterotrophs using the acetylene reduction assay with a $^{15}N_2$ calibration. Cyanobacteria N fixation rates were measured *in situ* in four streams that developed sufficient colonies, and sediment N fixation rates were measured in the laboratory with samples collected from seven streams. We compared N fixation rates between groups and analysed physicochemical drivers of N fixation. Finally, we scaled our rates to whole-stream N fixation rates, based on the percentage coverage of each type of N-fixer.

2.1 | Study area

We selected seven streams in the Okanogan-Wenatchee National Forest on the eastern slopes of the Cascade Mountains in Washington State (Figure 1). Average annual precipitation for this area (1899–2016) is 571.8 mm with generally dry summers (July average, 9.1 mm) and most precipitation occurring in the winter (December average, 104.6 mm; Western Regional Climate Center, https://wrcc.dri.edu/cgi-bin/cliMAIN.pl?wa1504). Streams in this area are fed largely by snow melt, causing highest flows during the early spring followed by lower base flows in late summer. The area is characterised by cold, wet winters and hot, dry summers, with average lows of −6.8°C in January and average highs of 27.4°C in July. The region's vegetation consists of mixed conifer such as Douglas fir (*Pseudotsuga menziesii*), Ponderosa pine (*Pinus ponderosa*), grand fir (*Abies grandis*) and subalpine fir (*Abies lasiocarpa*; Lillybridge et al., 1995) in the uplands and deciduous species such as red alder (*Alnus rubra*), a known N-fixer (Hibbs et al., 1994), in riparian zones.

We expected similarly low nutrient concentrations in all streams, so sites were selected for accessibility and expected presence of cyanobacteria, and to achieve a gradient of light availability. One site was located in the Taneum Creek watershed south of Cle Elum, Washington, and six were located in the Teanaway Forest north of Cle Elum (Figure 1). All sites are located on lands historically managed and used by the Yakama Nation. Early Euro-American colonists' use included mining and timber harvest. Current human use consists mainly of recreational hiking and camping as well as some grazing and logging, and stream restoration activities, to improve habitat for migratory salmonids, are ongoing. Three of the sites did not develop *Nostoc* colonies in 2019, so we measured only sediment N fixation at those sites (Figure 1).

2.2 | Study design

We measured rates of N fixation in the main types of N-fixers present in the streams—cyano-midge, cyano-only and sediment microbes—using the acetylene reduction assay. Nitrogenase, the enzyme used to fix N_2, also converts acetylene into ethylene such that ethylene accumulation can be used as a proxy for N fixation (Flett et al., 1976; Hardy et al., 1968; Stewart et al., 1967). Estimating the moles of N fixed for every mole of ethylene produced requires a conversion ratio and 3:1 (ethylene:N) is standard (Stewart et al., 1967). Because this conversion ratio can vary by environment and microbial community (Graham et al., 1980; Howarth et al., 1988; Paerl, 1982), we calibrated our acetylene reduction assay with $^{15}N_2$ in one stream.

We sampled each stream site once during 2019 after sufficient cyanobacteria colonies had developed in the stream (late July to early September). We measured cyanobacteria N fixation rates in the field, but we completed sediment N fixation assays in the laboratory because preliminary measurements indicated that sediment rates required more time to be detectable. Samples of each type of N-fixer were placed in gas-tight containers to create three different chamber environments: (1) control (stream water only) to account for background ethylene production, (2) ethylene control (stream water and an ethylene-spiked atmosphere) to detect ethylene consumption, and (3) acetylene treatment (acetylene-saturated stream water). To determine the change in ethylene over time, we measured

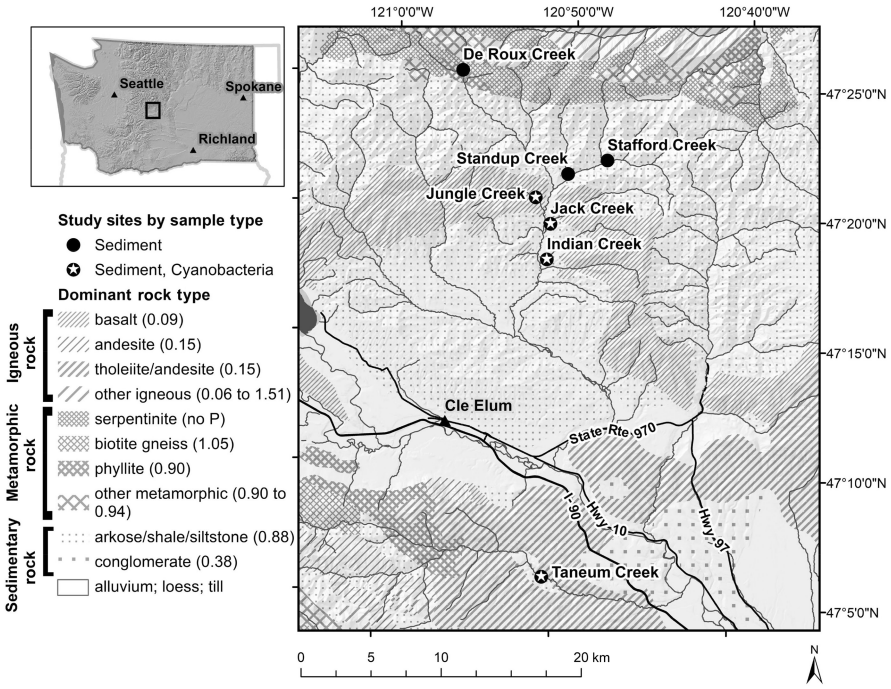

FIGURE 1 Location of the seven study streams in the Washington cascades, USA. The map denotes which type of N-fixing organisms were sampled at each study location and the geology of the area. Inset map shows the location of the study area within Washington state. In the legend, numbers in parentheses indicate the N:P ratio as calculated from Morford et al. (2016), except for serpentinite (Porder & Ramachandran, 2013). Data sources for map: USGS National Hydrography Dataset, USGS digital elevation models, USGS geologic database, US Census Bureau

ethylene concentrations via gas chromatography in gas samples collected from chambers before and after incubation.

2.2.1 | Sediment and cyanobacteria sampling

At each site, we established one stream reach of c. 150 m with five evenly spaced transects. At each transect we collected a composite sample of rocks with cyanobacteria colonies, choosing a representative rock from the left, middle and right of the stream for both cyano-midge and cyano-only colonies, resulting in five replicate samples for each type of cyanobacteria in each stream. Collecting composite samples allowed us to account for variability across each transect. *Nostoc* colonies were easily differentiated by their shape: cyano-midge colonies were larger and flat, shaped like an ear, with the colony extending out from the rock, whereas cyano-only colonies were much smaller and spherical. If a selected rock had both types of colonies present, we gently removed colonies of one type before incubating. If there were insufficient colonies, we reduced the number of replicates and combined rocks from different transects as needed (Table 1). We also recorded colony type and substrate type every 10 cm along evenly spaced transects (15 per reach) to quantify the percent coverage of each colony and substrate type. In general, cyano-midge colonies were much more abundant than cyano-only colonies.

At each of the five sampling transects, we also collected sediment from the left, middle and right to create composite samples, resulting in five replicate sediment samples per stream. To gather sediment samples, we plunged a 60-ml syringe with the tapered end cut off into the sediment to collect 10–30 ml of sample per core (c. 3 cm deep), gathering ≥80 ml of sediment per transect with approximately equal volumes from the left, middle and right. We also collected ≥2 L of unfiltered stream water, which was placed on ice along with the sediment cups and returned to the laboratory for the N fixation incubation.

2.2.2 | Cyanobacteria N fixation incubations

Nostoc N fixation was measured following the methods of Arango et al. (2009) and Kunza and Hall (2013). Specifically, we had five control, five acetylene treatment and three ethylene control chambers per stream. To prevent contamination with acetylene, control chambers were assembled and sealed before acetylene treatment chambers. Sampled rocks with colonies were placed into 2.125-L clear polyvinyl chloride (PVC) chambers (Cambro) equipped with a Swagelok septum port in the lid. Rocks were covered with 1 L of unfiltered stream water, then sealed with a lid made gas-tight with vacuum grease and a rubber O-ring. For ethylene controls, we replaced 30 ml of the chamber headspace with 30 ml of 1,000 ppm ethylene (GASCO) to achieve a concentration between 8.6 and 30.1 ppm (concentrations varied due to incubated rock volume). Acetylene treatment chambers were created by covering samples with 10% acetylene-saturated stream water.

Acetylene was generated by adding deionised water to calcium carbide (Alfa Aesar) in a sealed Wheaton bottle with a septum cap. Aquarium tubing was attached to the bottle by piercing the septum with a needle. An air stone was attached to the other end of the

TABLE 1 Physicochemical characteristics of the seven study sites

Study site	Latitude And longitude	Elev. (m)	T (°C)	Mean width (m)	Mean PAR (µmol photons m^{-2} s^{-1})	Mean Canopy Cover %	Alder Cover %	CM %	n
De Roux Creek	**47° 25′ 14″ N, 120° 56′ 28″ W**	**1,158**	**13.22**	**5.7**	**NA**	**52.8**	**19.8**	**0**	**0**
Stafford Creek	47° 22′ 1″ N, 120° 48′ 3″ W	945	12.14	3.1	NA	84.3	19.9	0	0
Standup Creek	47° 21′ 24″ N, 120° 50′ 9″ W	878	14.63	2.6	NA	79.5	7.4	0	0
Indian Creek	47° 18′ 8″ N, 120° 51′ 9″ W	768	14.04	1.6	476.08	66.4	32.5	9.9	5
Jack Creek	47° 19′ 30″ N, 120°51′1″ W	792	18.10	1.7	318.29	75.3	14.4	13.8	5
Jungle Creek	47°20′28″ N, 120° 51′ 53″ W	817	17.02	2.6	503.79	62.0	32.3	5.2	5
Taneum Creek	47° 5′ 53″ N, 120° 50′ 33″ W	732	17.07	7.2	424.98	59.4	31.7	26	5

Note: Values collected on multiple days were averaged. Elev., elevation; T, stream temperature; Mean PAR, photosynthetically active radiation calculated by multiplying percentage open sky per transect by average light logged in full sun. PAR was collected only if cyanobacteria were present; alder cover %, percentage of riparian trees within 3 m of the stream; CM, cyanobacteria with a midge (%, percentage coverage and *n*, number of replicates); C, cyanobacteria without a midge; S, sediment; Tb, turbidity; DO, dissolved oxygen; Con., conductivity; Disch., discharge; NO_3^-, nitrate; NO_2^-, nitrite; DIN, dissolved inorganic nitrogen; TP, total phosphorus. At Indian Creek there were insufficient quantities of cyanobacteria without the midge to perform any replicates.

aquarium tubing and placed into a container of unfiltered stream water. Acetylene then was bubbled through the tubing into the stream water for ≥10 min with gentle swirling to ensure that the water was saturated with dissolved acetylene.

All chambers were returned to the appropriate transect for at least a 4-hr incubation. At the start and end of the incubation, we swirled chambers gently for 3 min to allow gases to equilibrate and then took a 5-ml gas sample, which was stored underwater at room temperature in a pre-vacuumed 3-ml exetainer. To maintain chamber pressure after the initial sampling, we added 5 ml of air to control chambers, 5 ml of prepared 246.7 ppm ethylene to ethylene control chambers, and 5 ml of prepared 10% acetylene and air mixture to the acetylene treatment chambers. Because this addition minimally altered the chambers' composition, we corrected our data for any changes in ethylene concentration caused by the initial sampling and gas replacement.

At the end of the incubation, we placed the rocks from the chambers into 1-gallon Ziploc bags and transported them back to the laboratory on ice. Rocks were stored in the refrigerator at 5°C and processed within one week of collection. Specifically, cyanobacteria colonies were counted, removed, dried and ashed (see Appendix S1 for calculation of ash-free dry mass [AFDM]) to allow a mass-normalised comparison between cyano-midge and cyano-only rates. We also estimated rock surface area in each chamber.

2.2.3 | Sediment incubations

We conducted sediment assays for five controls, five ethylene controls and five acetylene treatment bottles per stream. Samples collected in the field were stored in the refrigerator at 5°C for no longer than 4 days to ensure that the microbial community was unchanged (Gupta et al., 2014) before setting up incubations. We used 24-hr laboratory incubations for sediment samples, which allowed time to detect N fixation while minimising artefacts. Our sediment incubations were performed near the laboratory window at ambient light and temperature (ranging from 23.1 to 24.5°C) conditions instead of at stream temperature (ranging from 13.2 to 18.0°C) as we did not have access to a temperature-controlled incubator. The higher temperatures may have inflated our sediment N fixation rates and so we also calculated temperature-adjusted rates using the Van't Hoff-Arrhenius equation and activation energy for N fixation (Welter et al., 2015).

Sediment samples within each transect were mixed well, and 20 ml of sediment were added to a 125-ml Wheaton bottle. Exact sediment volume varied owing to the presence of small pebbles in some samples. To control and ethylene control bottles, we added 70 ml of unfiltered stream water and sealed the bottle. For ethylene control bottles, 16 ml of headspace air was removed through the septum and replaced with 16 ml of 1,000 ppm ethylene, achieving an actual concentration of 93 to 276 ppm ethylene. The range in concentrations resulted from variation in sediment volume and flow rate from the ethylene tank valve, which we replaced before making standards. For the treatment bottles, 63 ml of unfiltered stream water and 7 ml of acetylene-saturated water were added to the samples and sealed. At the start and end of the 24-hr incubation, all bottles were swirled and sampled like those for the cyanobacterial assay. After the initial gas sampling, gas pressure was maintained, and ethylene concentrations were corrected as detailed in the section "Cyanobacteria N fixation incubations" above.

C %	n	S %	n	Tb (NTU)	DO (mg/L)	Con. (mS/cm)	pH	Disch. (m^3/s)	NO_3^--N, NO_2^--N (mg N/L)	NH_4^+-N (mg N/L)	DIN (mg N/L)	TP (mg P/L)
0	**0**	**7.9**	**5**	**0.4**	**8.81**	**0.091**	**7.99**	**0.088**	**0.051**	**0.034**	**0.085**	**0.049**
0	0	6.1	5	0.4	9.21	0.122	7.79	0.047	0.038	0.006	0.044	0.043
0	0	4.9	5	0.4	8.59	0.107	7.75	0.023	0.042	0.005	0.047	0.050
1.1	0	21.2	5	8.4	4.69	0.247	7.27	0.000	0.045	0.012	0.057	0.212
2	2	11.8	5	1.2	8.22	0.237	7.91	0.001	0.030	0.011	0.041	0.060
2.1	4	8.6	5	1.3	8.36	0.192	7.94	0.009	0.052	0.011	0.063	0.060
4.1	5	9.4	5	0.4	8.68	0.155	8.07	0.495	0.034	0.010	0.045	0.077

TABLE 2 Variable meanings for Bunsen coefficient and ideal gas law calculations

Variable	Meaning	Units
α	Bunsen's Coefficient	Dimensionless
T	Temperature at time of measurement	Kelvin
n_{aq}	Moles ethylene found in the aqueous phase	Moles
n_g	Moles ethylene found in the gaseous phase	Moles
P_{gT}	Partial pressure of the headspace gas at the temperature of measurement	Standard atmosphere
V_{aq}	Volume of the aqueous phase	Litres
V_g	Volume of the gaseous phase	Litres
R	Gas constant, 0.08206	atm L mol^{-1} K^{-1}

TABLE 3 Variable meanings for nitrogen isotope calculations

Variable	Meaning
AE_i	Atom excess in the sample, calculated by subtracting the atom % of the control sample from the atom % of the treatment sample.
Atom %	The number of ^{15}N atoms in the sample divided by the total number of N atoms multiplied by 100
TN_i	Total amount of N in the sample, calculated by multiplying the percentage N values obtained from the isotopic analysis by the total dry weight of the sample.
AE_{atm}	Atom excess in the chamber or bottle, calculated by subtracting the atom % of the control chamber or bottle from the atom % of the treatment chamber or bottle
t	Incubation time (hr)

2.3 | Gas chromatography and N fixation rate calculations

All gas samples were analysed via gas chromatography within 3 weeks of sample collection. We injected 2 ml of each gas sample into a Shimadzu GC-2014 Greenhouse Gas Analyser gas chromatograph equipped with a flame ionisation detector and a Hayesep T 80/100 column (GC settings: injector 100°C, column 80°C, detector 250°C, N_2 carrier gas 49.9 ml/min).

We used the Bunsen Coefficient and the Ideal Gas Law to calculate moles of ethylene produced per sealed container (i.e. bottles and chambers) in the aqueous and gas phases according to the following equations (Breitbarth et al., 2004, see Table 2).

$$\alpha = e^{-189.757+\frac{10092.7}{T}+(26.979\ \ln T)} \quad (1)$$

$$n_{aq} = \frac{P_{gT} \times \alpha \times V_{aq}}{R \times T} \quad (2)$$

$$n_g = \frac{P_{gT} \times V_g}{R \times T} \quad (3)$$

Measured concentrations of ethylene in the headspace samples were converted from ppm to partial pressures by multiplying by 10^{-6}, assuming 1 atm of pressure at the time of sample injection into the gas chromatograph. We then calculated the total moles of ethylene produced per chamber by adding the results of Equations 2 and 3 together. We converted ethylene produced into moles of N fixed using a 1.72:1 ratio for cyanobacteria and a 0.16:1 ratio for sediment microbes as calculated from our calibration procedure (see next section). To account for ethylene production not resulting from acetylene reduction, positive values from controls were subtracted in a pairwise fashion from treatment values. Substrate area and sample incubation time were then used to produce normalised N fixation rates (μg N m^{-2} hr^{-1} for both cyanobacteria and sediment microbes). AFDM also was used to produce normalised N fixation rates for cyanobacteria (μg N g AFDM^{-1} hr^{-1}). Finally, we estimated reach-scale N fixation rates by multiplying reach area by the proportion of the stream with a particular type of N-fixer and then multiplying by the average N fixation rate for that reach and N-fixer. Scaling bottle and chamber incubations to a whole-stream rate introduces variability, both because of spatial variability in N fixation rates and in areal coverage. With these whole-stream estimates, we must assume that the transects are representative of areal coverage in the reach and that our composite samples across these transects also are representative.

2.4 | $^{15}N_2$ Calibration

As acetylene reduction conversion ratios can be highly variable (Howarth et al., 1988), we performed a $^{15}N_2$ calibration procedure following methods similar to those of Montoya et al. (1996). We ran both acetylene reduction and $^{15}N_2$ assays for cyanobacteria (both cyano-midge and cyano-only) at Jungle Creek in September

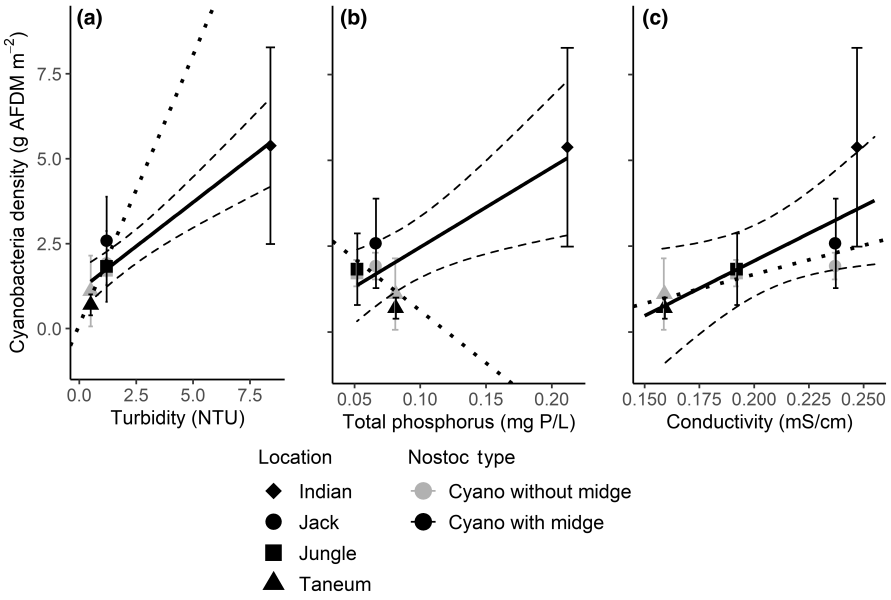

FIGURE 2 Simple linear regressions of cyanobacteria density with (a) turbidity, (b) total phosphorus and (c) conductivity. Points represent the mean of all replicates for each *Nostoc* type for each stream, and error bars represent *SD*. Only the mean value for each stream was used to generate the regression. Black lines represent a line of best fit with 95% confidence regions bounded by dashed lines. The dotted lines represent a line of best fit if Indian Creek is removed from the regression

(Figure 1). This stream was selected for its intermediate flow conditions and a sufficient density of cyano-only colonies. Sediment samples were collected from Jungle Creek at the same time and incubated in the laboratory. For $^{15}N_2$ measurements, at least four control and five treatment replicates were incubated for each type of N-fixer.

In order to perform $^{15}N_2$ incubations for cyanobacteria and sediment, samples were placed into chambers or bottles, respectively, filled to overflowing with unfiltered stream water, and sealed underwater. For cyanobacteria treatment chambers, we added $^{15}N_2$ gas by piercing the septum with an empty syringe, then using another syringe to add 10 ml of $^{15}N_2$ gas (98%, Cambridge Isotope Labs) to the chamber, and allowing the empty syringe to fill with water to equalise the pressure in the chamber. For sediment treatment bottles, we added 2 ml of $^{15}N_2$ gas to each. After swirling for 3 min, we incubated the chambers in the stream for 6 hr and the bottles in the laboratory for 24 hr.

All cyanobacteria and sediment samples were dried, weighed, ground, and packaged into tin capsules for isotopic analysis. We collected the fine sediment by swirling each bottle and pouring the mixture through a sieve into a pre-weighed drying pan. All isotopic samples were run at the WSU Stable Isotope Core Laboratory (Pullman, Washington, USA) on an ECS 4010 elemental analyser (Costech) and a Delta Plus XP isotopic ratio mass spectrometer equipped with a Conflo III (Thermo Finnigan).

In order to calculate the N fixation rates in each sample, we used the following equation (Knowles & Blackburn, 1993, see Table 3):

$$\text{nitrogen fixed} = \frac{AE_i \times TN_i}{AE_{atm} \times t} \quad (4)$$

In order to calculate the amount of $^{15}N_2$ dissolved in the water, we used a Bunsen coefficient to estimate the amount of dissolved N_2 from the air (Weiss, 1970) and the known proportion (0.0036765) of ^{15}N atoms to ^{14}N atoms in the atmosphere (Robinson, 2001) plus any $^{15}N_2$ added.

2.5 | Explanatory variables

We measured light, red alder percentage coverage, substrate type, discharge, temperature, turbidity, conductivity, pH, dissolved oxygen and nutrient concentrations (see Appendix S1). Light levels were measured at each transect using densiometer readings that were multiplied by photosynthetically active radiation measurements at a full-sun location for each stream. Red alder was only found directly next to the stream and percentage coverage was quantified by counting the number of alder trees and non-alder trees within 3 m on both sides of the stream reach. In addition, substrate type and percentage cover of cyanobacteria colonies were characterised at each transect. Stream physicochemical factors (discharge, temperature, turbidity, conductivity, pH and dissolved oxygen) were measured at one downstream location per stream. Water samples also were collected at this location for nitrate, ammonium, phosphate and total P (TP) analysis.

2.6 | Data analysis

In order to compare N-fixer types, we used analysis of variance (ANOVA) with N fixation rate as the dependent variable, N-fixer type as a fixed factor, and stream as a random factor. We also compared cyanobacteria types, where rates were normalised per g AFDM to eliminate potential differences resulting from colony sizes. To compare cyanobacteria and sediment heterotrophs, rates were normalised to area. To test the hypothesis that light is a good predictor of cyanobacteria N fixation, we performed a linear regression using light and N fixation values at the transect level,

while controlling for stream as a random factor. We also performed linear regressions at the stream level to examine the influence of other explanatory variables (TP, DIN, alder coverage, conductivity, discharge and turbidity) on N fixation rates by N-fixer type. Turbidity and TP were collinear ($r > 0.83$), so we included TP only because P is a component of nitrogenase. Additionally, we analysed predictors of cyanobacteria density (g AFDM m^{-2} of stream) and cyanobacteria colony size (g AFDM per colony) for cyano-only and cyano-midge at the stream level. For the linear regression analysis of each response variable, we used a false discovery rate to control for false positives (Glickman et al., 2014). This method uses the distribution of *p*-values and the number of tests to determine a false discovery rate rather than simply lowering α according to the number of tests as in the Bonferroni correction. We set our maximum false discovery rate to 0.05. To determine the threshold (α) for each response variable, the maximum false discovery rate (0.05) was multiplied by the rank of result and divided by the total number of tests performed (5), such that α ranges from 0.01 to 0.05. All data were checked for normality and heteroscedasticity. If assumptions were not met, we log-transformed the data to meet assumptions.

All statistical tests were performed in R (v3.5.1, R Foundation for Statistical Computing, 2018). Graphs were generated using the packages "ggplot2" (Wickham, 2016) and "ggpattern" (Mike & Davis, 2020). *Post hoc* tests used the packages "emmeans" (Lenth, 2020) and "multcompView" (Graves et al., 2019). Linear regressions for variables analysed with stream as a random factor used the packages "nlme" (Pinheiro et al., 2020) and "MuMIn" (Barton, 2020).

3 | RESULTS

3.1 | Distribution of cyanobacteria

Of the seven sites selected, only four developed cyanobacteria colonies by the end of the summer (Jungle Creek, Jack Creek, Indian Creek and Taneum Creek). In streams that developed colonies, conductivity was higher (0.207 ± 0.043 mS cm^{-1}, mean ± *SD*) compared to streams without colonies (0.107 ± 0.016 mS cm^{-1}), and elevation was lower (777 ± 36 m vs. 994 ± 146 m). In all streams with cyanobacteria colonies, cyano-midge colonies were 2.5- to 9-fold more abundant than cyano-only colonies (Table 1).

Cyanobacteria density increased with conductivity ($y = 32x - 4.3$, $SE = 11.6$, $r^2 = 0.52$, $p = 0.040$; Figure 2). Cyanobacteria density also increased with TP ($y = 23x + 0.15$, $SE = 6.5$, $r^2 = 0.66$, $p = 0.016$) and turbidity ($y = 0.52x + 1.1$, $SE = 0.07$, $r^2 = 0.89$, $p < 0.001$), but TP and turbidity were collinear ($r = 0.98$, $p = 0.0001$) and TP was just above the false discovery threshold ($p = 0.016$ vs. $\alpha = 0.01$). In addition, the linear regressions for turbidity and TP may be driven by unique conditions at Indian Creek (Figure 2), where summertime evaporation had reduced the velocity to 0 m s^{-1}, causing higher turbidity and TP compared to other streams (Table 1). Conductivity also slightly exceeded the false positive threshold ($p = 0.04$ vs. $\alpha = 0.02$), but the relationship was not driven by an outlier stream (Indian Creek), so we concluded that conductivity most reliably explained differences in cyanobacteria colony distribution and density.

3.2 | $^{15}N_2$ Calibration and ethylene controls

The calibration procedure of the $^{15}N_2$ and acetylene reduction methods yielded ratios used to convert moles of ethylene into moles of N fixed for each N-fixer type. The conversion ratio for cyano-only colonies was highly inconsistent (CV = 115) most probably as a result of their small size, so the conversion ratio obtained for cyano-midge (1.72 ± 0.14 moles ethylene, mean ± *SE*, to 1 mole of N fixed) was used for all cyanobacteria rates. Calibration of sediment rates yielded a ratio of 0.16 ± 0.06:1.

Forty-three of 53 ethylene control chambers consumed ethylene (i.e., negative production) with an overall average of -2.5 ± 2.6 μmol ethylene m^{-2} hr^{-1} (mean ± *SD*; Table 4; Figure 3). Ethylene consumption probably is dependent on ethylene concentrations (De Heyder et al., 1997; Elsgaard, 1998) so we were unable to use these controls to directly correct N fixation rates, but we note that the $^{15}N_2$ calibration procedure accounts for background ethylene consumption. Our cyanobacteria values obtained with the calibration conversion ratio are 1.74-fold higher than the values we would have obtained using a 3:1 conversion ratio, and our sediment values were 19.2-fold higher.

3.3 | N fixation rates

3.3.1 | Differences in fixation rates by N-fixer type

Areal N fixation rates did not differ among N-fixing groups ($F_{2,11} = 0.68$, $p = 0.55$). Rates were highest in cyano-midge (10.0 ± 10.9 μg N m^{-2} hr^{-1}; mean ± *SD*), followed by sediment microbes (5.6 ± 4.0 μg N m^{-2} hr^{-1}) and lowest in cyano-only (5.1 ± 6.1 μg N m^{-2} hr^{-1}; Table 4; Figures 4, S1), although the ranges of these measurements make them comparable. Rates expressed per g AFDM also were not different between cyano-midge (4.8 ± 5.9 μg N g^{-1} hr^{-1}) and cyano-only (2.8 ± 2.3 μg N g^{-1} hr^{-1}, $F_{1,5} = 1.11$, $p = 0.40$).

Mean sediment N fixation rates ranged from 1.9 in De Roux to 12.5 μg N m^{-2} hr^{-1} in Indian (Table 4). If we account for incubation temperatures exceeding those of the streams with a Van't Hoff–Arrhenius correction, the rates ranged from 0.13 in Stafford to 1.72 μg N m^{-2} hr^{-1} in Taneum but were still not different from cyano-only or cyano-midge rates ($F_{2,11} = 3.54$, $p = 0.11$). Although the temperature correction introduces some uncertainty to the rate estimates, sediment microbes are actively fixing N in these streams at rates that probably are comparable to cyanobacteria.

TABLE 4 Nitrogen fixation and ethylene control production rates by fixer type and location

Sample type	Location	Areal N fixation ± *SD* $\left(\frac{\mu g\ N}{m^2\ hr}\right)$	Areal N fixation temperature-corrected ± *SD* $\left(\frac{\mu g\ N}{m^2\ hr}\right)$	N fixation per biomass ± *SD* $\left(\frac{\mu g\ N}{g\ AFDM\ hr}\right)$	Nitrogenase activity ± *SD* $\left(\frac{\mu mol\ C_2H_4}{m^2\ hr}\right)$	Ethylene control ± *SD* $\left(\frac{\mu mol\ C_2H_4}{m^2\ hr}\right)$
Cyano with Midge	Indian	6.27 ± 4.76	NA	1.25 ± 0.59	0.77 ± 0.59	3.54 ± 1.13
	Jack	6.45 ± 4.69		2.36 ± 1.60	0.79 ± 0.58	−3.59 ± 3.72
	Jungle	25.92 ± 20.30		13.61 ± 9.12	3.19 ± 2.50	−2.64 ± 2.80
	Taneum	1.16 ± 0.47		1.80 ± 0.63	0.14 ± 0.06	−0.17 ± 0.09
Cyano without Midge	Jack	1.84 ± 2.16	NA	0.86 ± 0.95	0.23 ± 0.27	NA
	Jungle	12.15 ± 9.56		5.38 ± 8.37	1.50 ± 1.18	−2.62 ± 1.00
	Taneum	1.30 ± 0.88		2.12 ± 2.14	0.16 ± 0.11	−0.60 ± 0.57
Sediment	De Roux	1.85 ± 0.91	0.16 ± 0.08	NA	0.02 ± 0.01	−5.62 ± 1.14
	Stafford	1.98 ± 2.04	0.13 ± 0.13	NA	0.02 ± 0.02	−2.05 ± 0.49
	Standup	3.67 ± 4.17	0.46 ± 0.42	NA	0.04 ± 0.04	0.35 ± 0.15
	Indian	12.25 ± 22.37	0.96 ± 1.76	NA	0.14 ± 0.25	−4.53 ± 1.96
	Jack	3.86 ± 4.17	0.87 ± 0.94	NA	0.04 ± 0.05	−2.86 ± 0.61[a]
	Jungle	6.58 ± 4.29	1.30 ± 1.00	NA	0.06 ± 0.05	−3.74 ± 0.40
	Taneum	9.03 ± 5.22	1.65 ± 0.96	NA	0.10 ± 0.06	−4.29 ± 0.64

Note: Numbers represent mean ± *SD*. N fixation is reported on an areal basis as well as on a per unit mass basis for cyanobacteria only. For the purpose of comparing with the ethylene controls, the pre-conversion ratio values of ethylene measured are also shown in column five. Ethylene controls were created by replacing 30 ml of headspace with 1,000 ppm ethylene for cyanobacteria incubations and by replacing 16 ml of headspace with 1,000 ppm ethylene for sediment incubations. Negative ethylene control values indicate ethylene consumption, and positive ethylene control values indicate ethylene production.

[a]Owing to depletion of our ethylene supply, this control was run with 80% less ethylene.

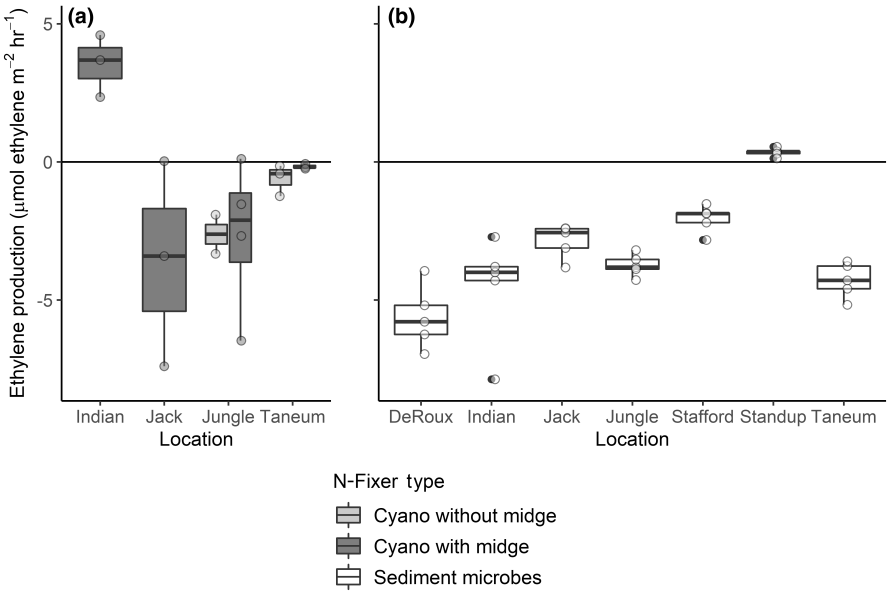

FIGURE 3 Box plot of ethylene production for (a) cyanobacteria and (b) sediment microbes with negative values indicating ethylene consumption. Note that sediment ethylene controls had higher starting values (92.6–276.3 ppm) than cyanobacteria ethylene controls (8.6–30.1 ppm); also note that Jack Creek sediment ethylene controls received less ethylene than the other streams (0.01–0.07 ppm). Box plot hinges show the first and third quartiles and whiskers extend from the hinge to the largest value that is no further than 1.5-fold larger than the interquartile range. The horizontal line shows the median. Each point overlaying the boxplots shows the ethylene production or consumption value measured at a single transect within the stream

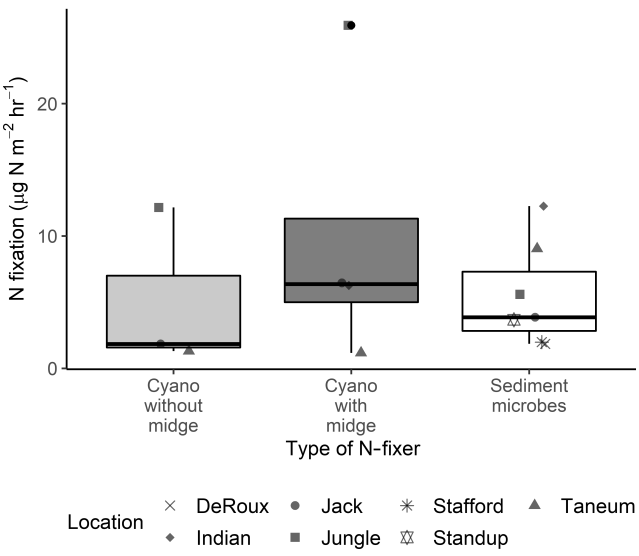

FIGURE 4 Box plot comparing N fixation rates among different types of N-fixers across all streams. Box plot hinges show the first and third quartiles and whiskers extend from the hinge to the largest value that is no further than 1.5-fold larger than the interquartile range. The horizontal line shows the median. Groups were statistically comparable to each other. Each point overlaying the boxplots shows the mean N fixation rate for one stream. Note that the black dot in the cyano with midge plot is part of the box plot (Jungle Creek exceeded the interquartile range by 1.5-fold)

3.4 | Factors associated with N fixation

The N fixation rates for cyanobacteria and sediment were driven by different variables. In the sediment, N fixation increased with TP ($y = 57x + 1.2$, $SE = 15$, $r^2 = 0.68$, $p = 0.014$; Figure 5), which was slightly above our false discovery threshold of $\alpha = 0.01$. Although Indian Creek appears to drive this relationship, the positive trend is stronger without this point ($y = 227x - 8.2$, $SE = 40.7$, $r^2 = 0.86$, $p = 0.005$).

Contrary to our hypothesis, light did not drive cyanobacteria N fixation rates ($y = -0.002x + 8.5$, $SE = 0.01$, $r^2 = -0.036$, $p = 0.82$; Figure S2). We observed a broad range of light values (64–988 µmol photons m^{-2} s^{-1}), which we expected to allow us to detect an effect of light. Likewise, we did not observe an effect of red alder coverage.

4 | DISCUSSION

We estimated the importance of three types of N-fixers to stream N cycling and highlighted important physicochemical drivers in the Washington Cascades. Cyanobacteria were found in streams with higher conductivity and lower elevation, and their biomass increased with conductivity and TP. N fixation rates were comparable among the three groups of N-fixers, with sediment rates being higher than expected. Cyano-only and sediment N fixation rates did not differ among

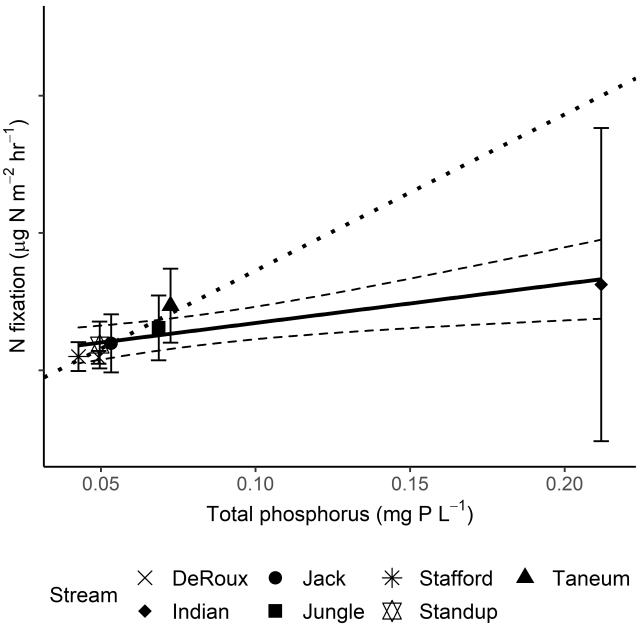

FIGURE 5 The relationship between sediment N fixation rates and TP. Each point represents the mean of all transect values in a stream and error bars are *SD*. The solid lines represent a line of best fit with a 95% confidence region bounded by the dashed lines. The dotted line represents a line of best fit if Indian Creek were removed from the analysis

study locations, yet those for cyano-midge N-fixers were notably higher in Jungle Creek. Contrary to our hypothesis, light did not drive cyanobacteria N fixation rates; however, sediment rates were driven by TP.

4.1 | N fixation rates in context

4.1.1 | Cyanobacteria rates

Nostoc N fixation rates were low across our streams. In a review of 22 stream studies of various locations and climates, Marcarelli et al. (2008) reported 10 µg N m^{-2} hr^{-1} as the median N fixation rate for cyanobacteria, whereas our median of all cyanobacteria rates was 2.7 µg N m^{-2} hr^{-1}. The highest cyanobacterial rates were in Jungle Creek (≤51.7 µg N m^{-2} hr^{-1}), which are comparable to rates of 20–62 µg N m^{-2} hr^{-1} observed in similar conditions in Idaho mountain streams (Marcarelli & Wurtsbaugh, 2006, 2007), but lower than rates of ≤160 µg N m^{-2} hr^{-1} observed in other Idaho streams (Eberhard et al., 2018), all obtained using 3:1 conversion ratios. Our rates were much lower than those observed in warmer environments; in warm deserts, rates can be as high as 51,000 µg N m^{-2} hr^{-1} (3:1 conversion ratio; Grimm & Petrone, 1997). Our study focused only on *Nostoc* cyanobacteria rates as they are the predominant N-fixers, but it is possible that other cyanobacteria that we did not measure, such as diatom endosymbionts, also may contribute to the N budget of these streams. The cyanobacterial N fixation rates were surprisingly low, given the low N concentrations in our streams. Perhaps the low rates were a result of micronutrient limitation (see "Factors impacting cyanobacteria N fixation", below) or a combination of low temperatures at northern latitudes with micronutrient limitation. Although it is possible that we captured a chance low point for cyanobacteria N fixation, we did measure rates during the season when cyanobacteria were most abundant and stream temperatures were relatively high.

4.1.2 | Comparing cyano-only and cyano-midge rates

We found that cyano-midge rates were usually higher than cyano-only rates, but the difference was not large. Few studies have measured *Nostoc* N fixation in relation to the midge symbiosis. Dodds (1989) found that in still water (0 cm s^{-1}), cyano-only fixed more N and had higher photosynthesis than cyano-midge, whereas in currents of >10 cm s^{-1} differences between the two types were small, with cyano-midge rates slightly higher. The shape of the midge colony may allow it to stick out farther from the rock and take advantage of higher diffusion rates in higher flows. Our incubations took place inside sealed chambers, so our experimental design did not allow us to look more closely at how flow rates impacted N fixation. However, cyano-midge colonies were more prevalent than cyano-only colonies in all streams, possibly as a consequence of the extra stability provided by the midge. Of the four sites that developed cyanobacteria, Jungle and Taneum Creeks had velocities above 10 cm s^{-1} (10.5 and 17.3 cm s^{-1}, respectively), but this did not result in a higher ratio of cyano-midge to cyano-only colonies compared to other streams.

4.1.3 | Sediment microbial rates

We measured sediment N fixation rates in sediment that were higher than previous studies, probably because of our low ethylene:N_2 conversion ratio. The few reported rates of sediment N fixation are low, including from below detection in an Ontario stream (Tam et al., 1981), <10 µg N m^{-2} hr^{-1} for Idaho streams (Eberhard et al., 2018), and 1.4–42 µg N m^{-2} hr^{-1} for prairie streams in Kansas (Caton et al., 2018), whereas our rates ranged from 0 to 52.1 µg N m^{-2} hr^{-1}. All previous measurements used the acetylene reduction method and either reported rates in moles of ethylene (Tam et al., 1981) or used ratios from the literature (3:1, Eberhard et al., 2018; 3.8:1, Caton et al., 2018) to convert from ethylene to N fixed. Had we used a 3:1 ratio, our rates would have ranged from 0 to 2.7 µg N m^{-2} hr^{-1}, which is within the range for Idaho streams reported previously. In light of this, conversion ratios for sediment N fixation deserve further scrutiny and future studies should consider a site-specific $^{15}N_2$ calibration.

4.2 | Factors influencing N fixation rates

4.2.1 | Cyanobacterial distribution and density

Contrary to our expectations, *Nostoc* colonies did not develop at all sites, but only occurred at lower elevations (≤768 m above sea level)

where temperatures and conductivity were higher. Warmer temperatures often are better for *Nostoc* colony development (Carmiggelt & Horne, 1975; Dodds et al., 1995; Grimm & Petrone, 1997), although conductivity may be more important than temperature (Mollenhauer et al., 1999; Monteagudo & Moreno, 2016). For example, *Nostoc verrucosum*, a similar species to *N. paramelioides* that also hosts midge larvae (Sabater & Muñoz, 2000), was most frequently observed in conductivities between 0.145 and 0.430 mS cm^{-1}, a similar range to our observations of *N. paramelioides* (0.155 to 0.247 mS cm^{-1}). In addition to conductivity, TP also was positively related to cyanobacteria density, which is commonly associated with cyanobacterial growth (Marcarelli & Wurtsbaugh, 2006).

4.2.2 | Factors impacting cyanobacteria N fixation

Contrary to our hypothesis, light did not drive N fixation rates. Although we observed a large range of light values (64–988 µmol photons m^{-2} s^{-1}), it is possible that most were sufficiently high to not be limiting. In an Oregon Cascade stream, sites that had light fluxes >3.5 mol photons m^{-2} day^{-1} tended to be limited by nutrients rather than by light (Warren et al., 2017). Most transects had light fluxes of >3.5 mol m^{-2} during our incubations (4–8 hr). Likewise red alder did not drive N fixation rates, probably as a result of comparable alder coverage across sites.

It is possible that other nutrients such as iron (Fe) also may affect cyanobacteria N fixation rates. We did not measure Fe in our study, but we did observe abundant rust-coloured rock, often indicative of iron oxides, in Jungle Creek, which had the highest cyanobacteria N fixation rates in our dataset. Mesocosm experiments found that N-fixing cyanobacteria (including *Nostoc*) are most prevalent with higher P and Fe concentrations (Larson et al., 2015). Micronutrient limitation, such as Fe or molybdenum, both of which are components of nitrogenase, could help to explain our low observed N fixation rates (Rubio & Ludden, 2008).

4.2.3 | Factors affecting sediment fixation rates

Iron or other micronutrients may have influenced N fixation rates in cyanobacteria, yet TP was important for sediment N fixation rates. As a component of nitrogenase, P often drives N fixation rates in cyanobacteria (Kunza & Hall, 2013; Marcarelli & Wurtsbaugh, 2006, 2007) and in sediment in other ecosystems (e.g., mangroves; Romero et al., 2012). Given the P-rich geology of the area (Leland, 1995), it is possible that sediment microbes had better access to P than cyanobacteria colonies through direct proximity to the benthos. Additionally, heterotrophic activity often increases with P addition especially when labile C is available (Ardón & Pringle, 2007), which may indicate that there is sufficient C in these stream sediments to fuel heterotrophic N fixation. For example, in a parallel nutrient-diffusing substrate study, we found that water column TP, net ecosystem production and sediment N fixation rates were positively correlated in these same streams (E. A. H. Bakker and J. T. Lilly, unpublished data).

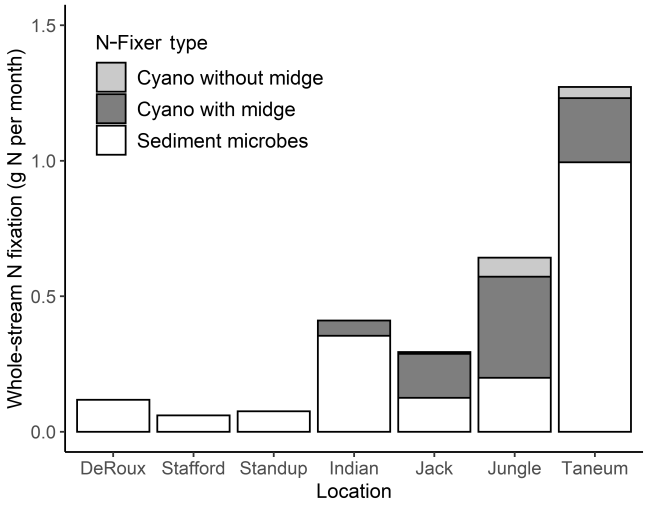

FIGURE 6 Estimates of N fixation contributions to whole stream reaches. Mean N fixation values for each type of N-fixer were scaled up to the whole reach using percentage substrate coverage and total area of each stream reach. Note that we measured cyanobacteria without the midge contributions only in Jungle, Taneum and Jack creeks as these streams had sufficient quantities to measure. The slightly thicker line at the top of the Jack Creek column depicts cyanobacteria without the midge contributions

4.3 | $^{15}N_2$ Calibration

4.3.1 | Sediment conversion ratios

Our conversion ratios (moles of ethylene produced to moles of N fixed) varied widely between the sediment and cyanobacteria, suggesting important differences in their N fixation pathways. Observed conversion ratios vary greatly even within N-fixer type, including 2.8:1 to 6.6:1 and 2.1:1 to 11.9:1 for cyanobacteria in a single ecosystem (Graham et al., 1980; Paerl, 1982) and 2.5:1 for sediments in a subarctic river (DeLuca et al., 2013). Why do conversion ratios vary? A ratio greater than 3:1 can result from hydrogen (H_2), produced during fixation, also reducing acetylene (Paerl, 1982) or from fixed N released into the water instead of maintained in the tissue (Graham et al., 1980). Ratios less than 3:1, as observed herein, could be a result of microbial community differences (Aoki & McGlathery, 2019) or exposure to acetylene altering the ambient community (Fulweiler et al., 2015).

Simultaneous ethylene production by nitrogenase and ethylene consumption by other microbial processes also could lower conversion ratios and affect uncalibrated data; however, little is known about ethylene consumption processes in stream sediments. In lakes, ethylene availability may motivate a shift in the microbial community toward ethylene consumption (Elsgaard, 2001); however, to the best of our knowledge, there has been no systematic quantification of ethylene consumption in streams alongside microbial

community composition data. Acetylene can inhibit ethylene decomposition in soils (Hendrickson, 1989; Zechmeister-Boltenstern & Smith, 1998), and most studies seem to assume that this also applies to aquatic settings. Of 18 papers that measured stream N fixation with the acetylene reduction method, none reported controls for the purpose of measuring ethylene consumption (Arango et al., 2009; Berrendero et al., 2016; Buckley & Triska, 1979; Carmiggelt & Horne, 1975; Caton et al., 2018; Eberhard et al., 2018; Francis et al., 1985; Grimm & Petrone, 1997; Hiatt et al., 2017; Kunza & Hall, 2013, 2014; Marcarelli & Wurtsbaugh, 2006, 2007, 2009; Scott et al., 2009; Tam et al., 1981, 1982; Welter et al., 2015), although most performed blanks to measure ethylene production by processes other than N fixation. Most of our ethylene controls (86% of sediment and 72% of cyanobacteria) consumed ethylene, emphasising the importance of calibration.

In oceans, N fixation rates were higher when measured with $^{15}N_2$ than when measured with acetylene reduction, implying that for marine sediments, ratios lower than 3:1 may be more common. Historically, marine sediment fixation was considered to be low and unimportant (Howarth et al., 1988); however, recent measurements with $^{15}N_2$ concluded that N fixation in sediments significantly contributed to N budgets (Aoki & McGlathery, 2019; Newell et al., 2016; Rao & Charette, 2012), with rates ranging from 476 to 10,757 $\mu g\,N\,m^{-2}\,hr^{-1}$. In one example, acetylene reduction with a 3:1 conversion ratio yielded rates from 28 to 266 $\mu g\,N\,m^{-2}\,hr^{-1}$, whereas an isotopic method yielded rates from 3,502 to 7,704 $\mu g\,N\,m^{-2}\,hr^{-1}$ (Aoki & McGlathery, 2019), which translates to conversion ratios ranging from 0.024:1 to 0.10:1. Perhaps sediment fixation has been similarly underestimated in freshwater sediments.

4.4 | Whole-stream N contributions

Cyanobacteria are typically assumed to contribute more N to streams than sediment microbes, yet whole-stream rates suggest that sediment microbes are more important to food webs in streams with higher coverage of fine sediment (Figure 6). Sediment microbes also have the potential to fix N year-round, whereas cyanobacteria colonies were well-established for just a two-month growing season because snow melt disturbance shortens the growing season. Thus, cyanobacteria may contribute more in streams with stable flow regimes. Cyano-midge were so much more abundant than cyano-only that their impact to streams over the whole summer outweighs the cyano-only. Thus, even though the two colony types fixed N at similar rates, cyano-midge dominated reach-scale N fixation.

Other sources of N to Cascade streams include leaf litter, atmospheric deposition and spawning salmon, all of which appear to contribute more N than fixation. For example, assuming two months of cyanobacteria fixation and three months of sediment fixation for the summer (June to August), our habitat-weighted estimate of N fixation contributions from all fixer types ranged from 0.00026 to 0.0062 $g\,N\,m^{-2}$ per summer. By contrast, atmospheric N deposition contributed 0.010 $g\,N\,m^{-2}$ over the same period and 0.083 $g\,N\,m^{-2}$ for the year (National Atmospheric Deposition Program, 2018 data for NTN Site WA99, http://nadp.slh.wisc.edu/data/sites/siteDetails.aspx?net=NTN&id=WA99). Litter fall estimated from the nearby Oregon Cascades would contribute ~0.30 $g\,N\,m^{-2}$ during the growing season and ~1.35 $g\,N\,m^{-2}$ annually (Triska et al., 1984). A single pink salmon excretes 13.6 g N while migrating upstream to spawn (Tiegs et al., 2011), which is nearly four times more than the total input from summer fixation in our highest-rate stream (Taneum Creek, 3.5 g N per summer).

Nonetheless, the timing of N fixation contributions may still make them important. For these snow-pack fed streams, N inputs from runoff peak in spring, inputs from spawning salmon and leaf litter peak in the autumn, and inputs from atmospheric deposition are lowest in summer when precipitation is low. Therefore, N fixation may supplement stream food webs in the summer, when other contributions decline and in-stream demand surges.

4.5 | Future studies

Our study highlighted some potential problems with the acetylene reduction technique and the possibility of micronutrient limitation for cyanobacteria N-fixers. Our understanding of stream N fixation would benefit from an examination of the relationship between N fixation and Fe and other micronutrients. Furthermore, measuring N fixation and denitrification simultaneously may help to clarify the fate of fixed N (Eberhard et al., 2018). Given that many herbivores avoid cyanobacteria as unpalatable (Arango et al., 2009), a study comparing the fate and transfer rate of fixed N in the food web from both cyanobacteria and sediment microbes could further solidify the importance of each N-fixer type to overall stream function. Moreover, a direct method using $^{15}N_2$ to measure N fixation, or at the very least, a calibration of the acetylene reduction method with $^{15}N_2$, will better assess the importance of sediment microbes for stream N cycling when attempting to budget sources and fates of N in streams.

5 | CONCLUSION

Both cyanobacteria and sediment microbes were important in creating new N inputs to Cascade Mountains streams. Colonies with a midge symbiont were more abundant and therefore collectively fixed 11.9-fold more N at the reach-scale than colonies without a symbiont. Although sediment microbes often have been viewed as unimportant to N fixation in streams, our study suggests their N fixation contributions are comparable to those of cyanobacteria colonies on an areal basis. However, sediment heterotrophs are probably present for a longer portion of the growing season than cyanobacteria, which could result in more N fixed in sediments annually than N fixed via cyanobacteria. Though observed rates were low, N fixation by all three groups actively contributes N to these streams, helping to support stream food webs especially during summer.

AUTHOR CONTRIBUTIONS

EAB, CPA and SSR developed the idea and designed the experiment; EAB, SSR and CV developed methods; EAB completed field and laboratory measurements; EAB and CV analysed the data; EAB wrote the manuscript; and all authors edited the manuscript.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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