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# Nitrogen fixation varies spatially and seasonally in linked stream-lake ecosystems

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**Abstract** We performed surveys of nitrogen ($N_2$)-fixation in three oligotrophic lake-stream systems in the Sawtooth Mountains of central Idaho to address two questions: (1) Which habitat types within linked lake-stream systems (lake pelagic, lake benthic, and stream) exhibit the highest rates of $N_2$ fixation?, and (2) How does $N_2$ fixation compare to the hydrologic flux of nitrogen? A seasonal survey showed that $N_2$ fixation in a single lake and its outlet stream peaked in late summer, when hydrologic N fluxes were lowest. Benthic lake $N_2$-fixation rates by epiphytes were highest at mid-lake depths, where their percent cover was highest, while rates by epipelon were greatest at shallow lake depths. Pelagic $N_2$ fixation was below detection. Stream $N_2$-fixation rates were greatest on rock substrates and in the lake outlet stream. These patterns were supported by a baseflow survey (late July) in three lake-stream ecosystems which confirmed that $N_2$-fixation rates peaked in the lake benthos at shallow depths and on rock substrates in outlet streams. Scaling $N_2$-fixation rates to whole lake and stream areas revealed that $N_2$ fixation could exceed the nitrate, and sometimes the total dissolved nitrogen flux during baseflow in lakes and outlet streams. Despite low rates, total $N_2$-fixation contributions (kg/day) from lakes were greater because they had far larger surface areas than the stream environments. Fixed nitrogen contributions from stream outlets were also relatively high because of high $N_2$-fixation rates and despite low surface areas. This study suggests that $N_2$ fixation could be a seasonally important nitrogen source to nutrient deficient subalpine lake-stream ecosystems. In addition, the frequency and location of lakes could control $N_2$-fixation contributions to watersheds by providing a large area for within-lake $N_2$ fixation, and creating conditions favorable for $N_2$ fixation in outlet streams.

**Keywords** Hydrologic N flux · Linked lake-stream ecosystems · Nitrogen fixation · Oligotrophic · Subalpine watersheds · Cyanobacteria

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## Introduction

It is unclear how natural lakes are ecologically linked with their inlet and outlet streams. In relation to streams, lakes have long hydraulic residence times and large surface areas for collecting solar radiation (Wetzel 2001), and act as important nutrient and carbon processors in watersheds (Brown et al. 2008).

Therefore, lakes can have profound effects on their outlet streams, including flow regimes (Arp et al. 2006; Milner et al. 2007), geomorphology (Arp et al. 2007; Myers et al. 2007), temperature patterns (Wotton 1995), carbon, nitrogen (N), and phosphorus (P) forms and supply rates (Kling et al. 2000; Wurtsbaugh et al. 2005; Fairchild and Velinsky 2006; Larson et al. 2007; Brown et al. 2008), biotic community structure (Robinson and Minshall 1990; Marcarelli and Wurtsbaugh 2006), and biotically-mediated nutrient processes (Marcarelli and Wurtsbaugh 2006; Arp and Baker 2007). Moreover, inlet stream characteristics such as nutrient concentration and water temperature can control within-lake physical and chemical properties including mixing regimes and depth of nutrient delivery (Carmack et al. 1986). Despite these important lake-stream linkages, nutrient cycling studies typically consider lakes and streams as independent entities rather than as holistic, interacting ecosystems.

Few studies have attempted to quantify the importance of nitrogen ($N_2$) fixation in N-deficient lake-stream ecosystems. In lakes, pelagic $N_2$ fixation is often associated with eutrophic lakes and cyanobacterial blooms, and in oligotrophic lakes pelagic $N_2$ fixation typically contributes <0.1% of the N budget annually (Howarth et al. 1988a). Nutrient processing in the benthos of lakes has received much less attention than the more easily and frequently studied pelagic zones (Vadeboncoeur et al. 2002). However, in N-deficient subalpine lakes $N_2$ fixation is frequently important to benthic periphyton dynamics (Reuter et al. 1985; Reuter and Axler 1992), but not a quantitatively important contributor to whole-lake N cycling (Reuter et al. 1983). In streams, $N_2$ fixation has seldom been measured, despite the common presence of $N_2$-fixing taxa in benthic stream communities (Marcarelli et al. 2008). Estimates in a desert stream indicated that annual $N_2$ fixation was comparable to annual rates measured in eutrophic lakes and rice fields (Grimm and Petrone 1997), and natural and experimental N enrichments have demonstrated that increased inorganic N concentrations decrease cyanobacterial abundance and $N_2$-fixation rates in N-limited streams (Henry and Fisher 2003; Marcarelli and Wurtsbaugh 2007). However, a comprehensive analysis of the importance of $N_2$ fixation to N-limited streams including seasonal trends and comparison to other N fluxes is lacking (but see Grimm and Petrone 1997).

The objective of our study was to determine where $N_2$ fixation occurs in linked lakes and streams, and how important it could be to these aquatic ecosystems. We asked the following two questions: (1) Which "habitat types" within linked lake-stream systems (lake pelagic, lake benthic, and stream) will exhibit the highest rates of $N_2$ fixation?, and (2) How does $N_2$ fixation compare to the hydrologic flux of total and dissolved N? To answer these questions, we conducted two surveys of $N_2$ fixation in oligotrophic, N-deficient watersheds in the Rocky Mountains. In the first, we focused on one inlet-lake-outlet system for two years to examine temporal changes in $N_2$ fixation and N flux. Second, we broadened our spatial focus to include three inlet-lake-outlet systems during a single time period (summer baseflow) to determine whether similar patterns were observed among systems in the study area. These surveys, combined with measurements of physical and chemical factors in the streams and lakes, suggest what factors could potentially control the observed patterns of $N_2$ fixation in these linked lake-stream systems.

## Methods

### Study area

This study was conducted in three watersheds in the Sawtooth Mountains of central Idaho (44°10′ N, 114°56′ W). These watersheds have very low atmospheric (ca. 130 kg N $km^{-2}$ $year^{-1}$; National Atmospheric Deposition Program 2006) and geologic nutrient input. The waters in this area have low conductivity (20–100 μS $cm^{-1}$), low alkalinity, and are relatively pristine (Emmett 1975). The annual hydrograph in these high elevation (ca. 2,000 m) watersheds is dominated by a spring runoff pulse peaking in early June, followed by a prolonged base flow period beginning in mid to late July (Arp et al. 2006).

Our work focused on three terminal moraine lakes and their inlet and outlet streams (Bull Trout, Stanley, and Yellow Belly; Fig. 1). There is a 0.050 $km^2$ pond ca. 0.7 km upstream of Yellow Belly Lake, and a 0.014 $km^2$ pond below Bull Trout Lake. For the purpose of this study, the lakes and ponds were considered a single unit, which was a reasonable assumption based on orientation and location of the terminal moraines (C. Arp, personal communication).

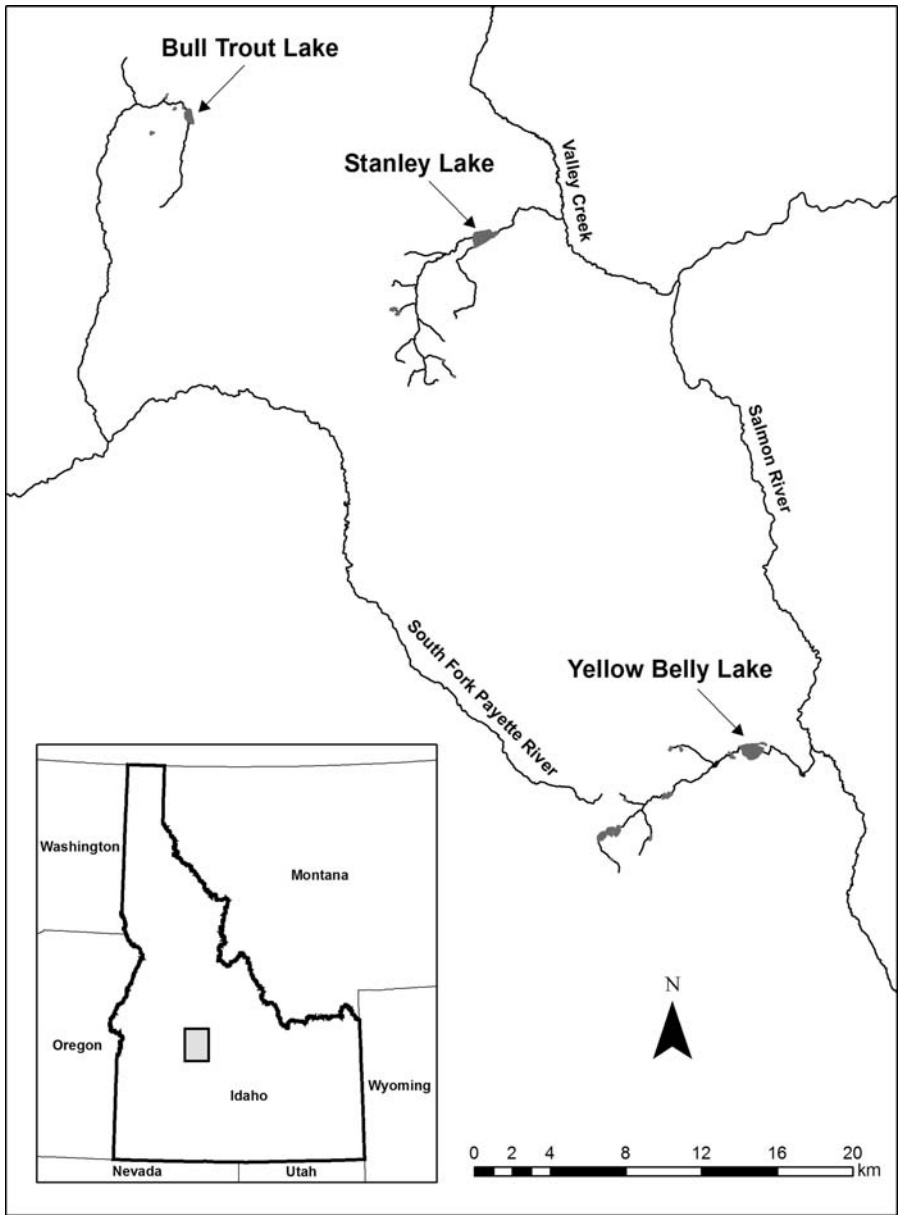


**Fig. 1** Map showing the location of the three study watersheds: Stanley, Yellow Belly, and Bull Trout. Inset shows study area within Idaho, USA

Therefore, for our analysis the inlet of Yellow Belly Lake was considered to be the inlet of the upstream pond, and the outlet of Bull Trout Lake is actually the outlet of the downstream pond.

The study lakes are all oligotrophic, with Secchi depths ranging from 6 to18 m (Budy et al. 1995; Wurtsbaugh, unpublished; Table 1). Lakes are typically covered by ice up to 1-m thick from mid-November through late May or early June, and epilimnetic temperatures usually peak near 19°C in late July. Epilimnia typically extend to a depth of 5–6 m during the summer. Previous laboratory and in situ bioassays have shown that N limits lake phytoplankton growth more often than P, but the two nutrients are always co-limiting (Wurtsbaugh et al. 1997; Sawatzky et al. 2006). Pelagic heterocystous cyanobacteria have never been observed in these lakes, although some do have large numbers of unicellular *Synococcus* sp. (Budy et al. 1995).

For this study we focused on the inlet and outlet streams within 300 m above and below the study lakes. These 3rd and 4th order streams had widths

**Table 1** Characteristics of the study lakes

| Characteristic | Bull Trout | Stanley[a] | Yellow Belly[a] |
|---|---|---|---|
| Surface area ($km^2$) | 0.28 | 0.81 | 0.73 |
| Watershed:surface area | 41.8 | 48.6 | 41.6 |
| Mean depth (m) | 4.6 | 13 | 14 |
| Maximum depth (m) | 15 | 26 | 26 |
| Extinction coefficient | 0.59 | 0.36 | 0.28 |
| Nitrate-N ($\mu g\ L^{-1}$) | 2.5 | 6.0 | 7.5 |
| Total N ($\mu g\ L^{-1}$) | 106 | 116 | 84 |
| Total P ($\mu g\ L^{-1}$) | 4 | 10 | 7.5 |

Chemical concentrations and extinction coefficients for all lakes are from August epilimnetic measurements

[a]Nutrient concentrations are means from 1992–1993 (Steinhart et al. 1994)

ranging from 3–12 m and summer baseflow discharge from 230–860 L $s^{-1}$ (Table 2). Mean daily temperatures in inlet streams ranged 6–13°C from June to August, while temperatures were usually 16–18°C in outlet streams (J. Garrett and W. Wurtsbaugh, unpublished). Riparian vegetation consisted largely of willow (*Salix* sp.) and lodgepole pine (*Pinus contorta*) forests and the canopy was primarily open. Stream periphyton are co-limited by N and P (Marcarelli and Wurtsbaugh 2007), and the most common $N_2$-fixing taxa are the cyanobacteria *Calothrix* and *Anabaena*, and diatoms with cyanobacterial endosymbionts, including *Epithemia* sp. and *Rhopalodia gibba* (Marcarelli and Wurtsbaugh 2006).

## Sample collection

To measure ambient rates of $N_2$ fixation in both lakes and streams, we performed two $N_2$-fixation surveys. In the first, seasonal sampling was conducted in Bull Trout Lake, inlet, and outlet during the ice-free season in 2002 and 2003 (henceforth referred to as the seasonal survey). In the second survey, we collected samples from all three study lakes and their inlet and outlet streams during baseflow for two weeks in late July 2003 (henceforth referred to as the baseflow survey).

For the seasonal survey in Bull Trout Lake, $N_2$ fixation was measured on both pelagic and benthic samples. Pelagic samples were collected at 0.5, 3, 5.5, and 10 m from one centrally-located station using a 10-L Kemmerer bottle. Benthic samples were collected using SCUBA to separate the different benthic substrates in the lake into macrophytes (classified to genus) and periphyton communities on sediments (hereafter called "epipelon"). Four sampling transects were established from the shoreline to the deepest part of the lake, with sampling depths of 0.5, 3, 5.5, and 10.5 m. At each depth on each date, SCUBA divers visually estimated the percent areal coverage of the dominant benthic substrates within a 0.5 $m^2$ area that had not been previously sampled. Divers collected cores of epipelon using a 3.5-cm diameter polyethylene tube plunged into the sediment. The top 1 cm of the core was collected and suspended in 50 mL of water for $N_2$-fixation analysis. Epiphytes attached to macrophytes were collected with a 2-L polycarbonate jar with 500-μm mesh Nitex netting on the top, which allowed water to escape and thereby minimized disturbance of macrophytes and associated epiphytes when divers placed the jar over the plant. The entire plant was cut above the roots with scissors and enclosed in the jar with a solid bottom cover. The jar was kept upright as it was brought to the surface to prevent loss of material through the mesh. At the surface, the mesh was removed and replaced with a solid cover, and the macrophyte was shaken vigorously for 1 min to separate epiphytes from the plant surface. The volume of the epiphyte slurry was measured and sub-sampled for $N_2$-fixation measurement (see below).

For the baseflow survey in the three study lakes, pelagic samples were collected as described above. Benthic samples were collected with an Eckman dredge from a boat along 4–5 transects in each lake, which was less time intensive than the SCUBA method described above. Both benthic and pelagic samples were collected at 4–5 depths, with intervals varying depending on the maximum lake depth (Table 1). The dredge effectively sampled bare sediments, but had some difficulty severing macrophytes and collecting gravel substrates. Therefore, sometimes the dredge was deployed several times before a successful sample was collected (where material was retained in dredge until it could be retrieved to the boat). Dredge material was a mixture of sediment and macrophyte material when the latter was very abundant, but likely overrepresented the contribution of epipelon attached to sediment because of the collection limitations. After a successful dredge collection, the material in the dredge was

**Table 2** Characteristics of the study streams during baseflow

| Factor | Bull Trout | | Stanley | | Yellow Belly | |
|---|---|---|---|---|---|---|
| | Inlet | Outlet | Inlet | Outlet | Inlet | Outlet |
| Width (m)[a] | 3.1 | 4.9 | 8.0 | 11.8 | 9.3 | 11.9 |
| Width:depth ratio | 4 | 13 | 12 | 27 | 13 | 37 |
| Stream gradient (%)[a] | 0.25 | 0.49 | 0.10 | 0.14 | 0.13 | 0.41 |
| Median sediment size ($D_{50}$, mm) | 11 | 19 | 5 | 29 | 9 | 45 |
| Mean daily temperature (°C)[b] | 6.2 | 16.4 | 10.1 | 17.7 | 13.8 | 18.6 |
| Discharge (L $s^{-1}$)[a] | 230 | 240 | 680 | 830 | 700 | 760 |
| Nitrate-N (μg $L^{-1}$)[a] | 5.1 | 1.2 | 13.3 | 1.5 | 24.8 | 7.1 |
| Total dissolved N (μg $L^{-1}$)[a] | 27.1 | 78.4 | 36.4 | 24.0 | 60.7 | 48.0 |
| Total N (μg $L^{-1}$)[a] | 74.8 | 110.3 | 70.4 | 129.0 | 82.5 | 86.2 |
| Total dissolved P (μg $L^{-1}$)[a] | 4.8 | 2.6 | 2.4 | 2.3 | 2.2 | 2.6 |
| Total P (μg $L^{-1}$)[a] | 10.3 | 14.0 | 2.6 | 2.6 | 2.7 | 3.1 |

[a] Data collected as part of a collaborative study (Arp and Baker 2007)

[b] Mean daily temperature for July–August, calculated from 15-min interval measurements (Garrett and Wurtsbaugh, unpublished)

cored with a 3.5 cm diameter polyethylene tube, and cores were sectioned and suspended as described above.

$N_2$-fixation rates of stream benthos were measured on four different benthic substrates: wood, rocks, coarse benthic organic matter (CBOM) and fine benthic organic matter (FBOM). Benthic samples in streams were collected using the same methods for both the seasonal and baseflow surveys: at 3 random locations in the study reach, either a 2,660 $cm^2$ plastic or a 490 $cm^2$ steel cylinder was forced into the stream bottom, and the substrate from the stream surface (top 0.5 cm) in that known area was collected and separated into FBOM and CBOM, which were suspended in water for $N_2$-fixation measurement. In addition, three samples of wood were collected randomly from within the reach, and the periphyton community was scraped from a known area and suspended in water for $N_2$-fixation measurement. Finally, five rock samples were collected randomly from the thalweg of each stream reach. When the dominant rock size in the stream was cobble, samples were comprised of three randomly selected rocks that were pooled into a single sample. When the dominant rock size was gravel or smaller, five samples were collected from a known area using the same cylinder used to collect CBOM and FBOM. Periphyton was collected by gentle scrubbing with a soft brush and suspended in water. Total volume of the slurry was measured, then it was sub-sampled for $N_2$-fixation measurement. Rock planar area was measured by tracing the rocks and weighing tracings to determine the approximate surface area (Bergey and Getty 2006). Proportional benthic cover of stream substrates was determined by walking five transects along a 100 m stream reach. For each transect, substrate at 20 evenly spaced points across the stream width were classified as CBOM, FBOM, wood, or rock.

### Acetylene reduction and chlorophyll *a* analysis

$N_2$ fixation was measured using an acetylene reduction assay (Flett et al. 1976; Capone 1993) with 50-mL aliquots in 62-mL glass serum vials. Samples were injected with 4 mL of acetylene generated from calcium carbide to achieve a headspace of approximately 20% acetylene gas and shaken to ensure equal partitioning of gas between the liquid and vapor phases (Flett et al. 1976). All samples were incubated for 2 h in situ between 1,100 and 1,600 h. Stream samples were suspended in the center of the stream thalweg to minimize shading by the stream bank. Lake samples were suspended on an incubation line at the depth of collection. Standards with known concentrations of ethylene and 4 mL of acetylene in deionized water were also incubated with the samples. After the incubation, vials were shaken again to repartition the gas, and final gas samples were collected into cleaned, re-evacuated 3 mL Vacutainers®. Ethylene and acetylene in each sample and standard were measured

using a SRI 8610 gas chromatograph equipped with a Poropak T column, He carrier gas, and a flame ionization detector. Concentrations of ethylene in the samples were compared to the known concentrations in the standards and then converted to the amount of $N_2$ fixed using an assumed 3:1 ethylene:$N_2$ conversion ratio (Capone 1993).

After the acetylene reduction assay was completed, the solution in each vial was filtered through a 0.8-μm GF/F filter and frozen for chlorophyll (chl) *a* measurements as an index of periphyton biomass. After several weeks, filters were extracted in 95% ethanol overnight and chl *a* in the extract was measured using a non-acidification technique and a Turner 10-AU fluorometer (Welschmeyer 1994).

### $N_2$ fixation and hydrologic N flux calculations

Contributions of fixed N from lakes and streams were calculated as mass of N fixed per $m^2$ of the various substrate types (sediment and macrophytes in lakes; CBOM, FBOM, rock and wood in streams). Rates were then scaled up to the total benthic surface area of lakes and streams by accounting for the relative cover of the different substrate types. These rates will henceforth be referred to as the "substrate-scaled" (per $m^2$ of a particular substrate) and the "cover-scaled" (per $m^2$ of lake or stream) rates, respectively. For the baseflow survey, the dredge samples were assumed to be representative of the benthic lake cover and rates were not scaled. Overall benthic areas at different lake depth strata (e.g., 0–1 m, 1–4 m, etc.) were determined using hypsographic curves, with the depth of rate measurement set as the median depth in each stratum. In streams, total benthic area was calculated using measured stream widths and an assumed reach length of 1.4 km. This length was based on a topographic analysis of streams in the Sawtooth mountains, which showed that the average stream distance between lakes was 2.8 km (Wurtsbaugh, unpublished). For this purpose, we allocated half of the inter-lake distance to the inlet, and half to the outlet.

For both the lake and stream habitats, cover-scaled rates were extrapolated to daily rates to compare to N fluxes. For this purpose, we assumed that nighttime rates in our system were zero, which is supported by studies showing that $N_2$-fixation rates decrease significantly (but do not always cease) in the dark when cyanobacteria are the primary $N_2$ fixers (Livingstone et al. 1985; Grimm and Petrone 1997). We are likely underestimating the daily contribution of $N_2$ fixation by assuming that nighttime rates are zero and that light/dark $N_2$-fixation ratios do not vary by cover type (Enrich-Prast and Esteves 1998), so our estimates should be considered conservative. Hourly rates measured in our study were scaled to a day assuming 15 h of light during June and July, 14 h during August, and 12 h during September and October.

Fluxes of dissolved and total N were calculated to compare to our $N_2$-fixation contributions in each habitat. Nitrate-N, total dissolved N (TDN; dissolved inorganic + dissolved organic N) and total N (TN; TDN + particulate N) fluxes were calculated either from water samples collected with an ISCO automated water sampler and preserved with sulfuric acid (seasonal survey; Baker, unpublished) or from grab samples (baseflow survey; Arp and Baker 2007). Ammonium was typically below detection so fluxes were not calculated. Concentrations of total P (TP) and total dissolved P (TDP) were also measured; phosphate-P was always $<2$ μg $L^{-1}$ and is not reported. Samples for dissolved nutrients were filtered through pre-combusted 0.8-μm GF/F filters and frozen, while samples for total nutrients were simply frozen. Nitrate-N concentrations were determined using ion chromatography on a Dionex DX500 (detection limit = 0.2 μg $L^{-1}$). Total and total dissolved nutrient concentrations were analyzed using a persulfate digestion followed by second derivative analysis of N (Crumpton et al. 1992, detection limit = 35 μg $L^{-1}$) and malachite-green analysis of TDP and TP (Motomizu et al. 1983, detection limit = 2 μg $L^{-1}$). Daily loads were calculated using concentrations and mean daily discharge measured with water stage loggers (Arp et al. 2006).

### Statistical methods

To determine the effects of habitat, substrate, and season on cover-scaled $N_2$ fixation and chl *a*, the seasonal survey data were analyzed separately for the lake and the streams using two or three-way analysis of variance (ANOVA). For Bull Trout Lake, epiphytes and epipelon were analyzed separately because epipelon was not measured at 3.0 m, and epiphytes were rarely present at 10.5 m, leading to an unbalanced data set. Therefore, cover-scaled epiphyte and epipelon chl *a* and $N_2$ fixation was analyzed

using two-way ANOVA (factors = date, depth). For Bull Trout inlet and outlet, cover-scaled $N_2$ fixation and chl *a* were analyzed with a three-way ANOVA (factors = date, stream type, substrate); stream type refers to inlet versus outlet stream. For the baseflow survey, the lake data were not analyzed statistically because the sampling depths in the three lakes were different. For the streams, cover-scaled $N_2$-fixation rates were compared among watersheds using a two-way ANOVA (factors = stream type, substrate). All analyses were conducted using SAS V. 9 (SAS Institute, Cary, NC, USA) and significance was considered at the $\alpha = 0.05$ level.

## Results

### Lakes

#### *Seasonal survey*

During the seasonal survey in Bull Trout Lake, pelagic $N_2$ fixation was never detected (7 sampling dates, 67 total samples assayed). The general pattern of benthic substrate cover, summarized as the annual average during 2003, varied strongly with lake depth (Fig. 2). Epiphyte cover was <20% at 0.5 m and they were nearly absent at 10.5 m. At 3 and 5 m, epiphyte coverage was 65–70%, and consisted of *Potamogeton* sp., *Elodea* sp., and *Chara* sp., with sediment and associated epipelon comprising the additional 30–35% of coverage. Sediments with epipelon ranged from 95% of total cover at 10.5 m to ca. 30% at 3 m (Fig. 2), and when corrected for hypsographic shape, epipelon covered 60% of the entire lake bottom. Mean substrate-scaled $N_2$-fixation rates were lowest on *Ceratophyllum* at 3 μg N $m^{-2}$ $h^{-1}$ and greatest on *Potamogeton* sp. at 275 μg N $m^{-2}$ $h^{-1}$, but were similar between the rest of the lake benthic substrates when averaged across the entire seasonal survey (10–45 μg N $m^{-2}$ $h^{-1}$; Fig. 3).

When the substrate-scaled $N_2$-fixation rates were corrected for benthic cover, $N_2$-fixation rates by epiphytes were higher than by epipelon in both 2002 and 2003. The highest cover-scaled $N_2$-fixation rates for epiphytes were measured in August and September at 3.0 and 5.5 m (Fig. 4a) where the epiphyte coverage (Fig. 2) and substrate-scaled $N_2$-fixation rates were both greatest. $N_2$-fixation rates of epiphytes varied

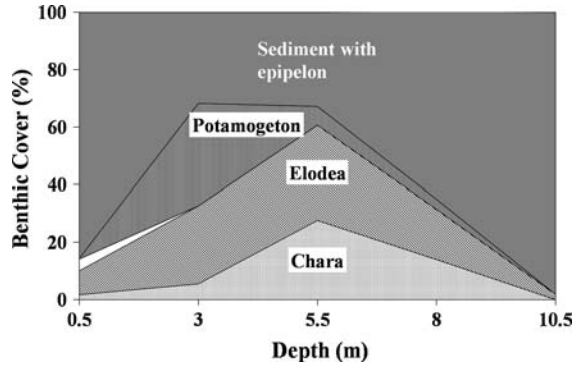


**Fig. 2** Mean benthic cover in Bull Trout Lake in 2003. Cover type labels are shown, except for grass/sedge cover, which is shown in *white* (only found between 0 and 3 m depths)

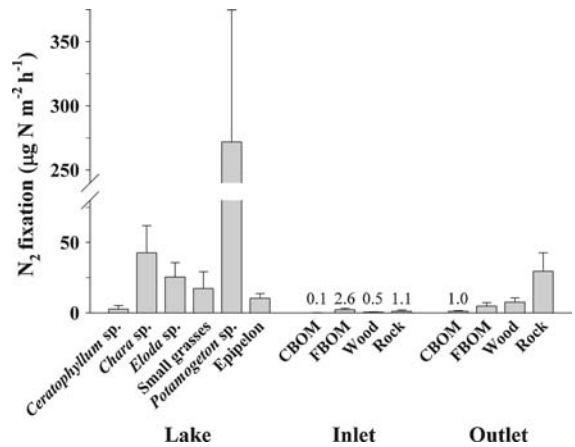


**Fig. 3** Mean $N_2$-fixation rates measured on various substrates in Bull Trout Lake and its inlet and outlet streams during the seasonal survey. Rates are scaled per $m^2$ of substrate and averaged across all measurements on all dates to compare the relative amount of $N_2$ fixation on different substrates and in different habitats. Error bars are ±1 S.E., *n* ranges from 2 to 25 for each data point

significantly with date and depth (two-way ANOVA, date $F_{7,\ 45} = 4.1$, $p = 0.002$, depth $F_{2,\ 45} = 11.2$, $p < 0.001$, no significant interaction). Cover-scaled $N_2$ fixation by epipelon was greater at 0.5 m than at either 5.5 or 10.5 m on all study dates but one (Fig. 4b), due to a combination of low epipelon cover at 5.5 m (Fig. 2), and low $N_2$-fixation rates at 10.5 m. Cover-scaled epipelon $N_2$-fixation rates peaked in July and August, particularly at 0.5 m depth, although the two-way ANOVA did not reveal a significant interaction between date and depth (Fig. 4b; date $F_{7,\ 54} = 5.7$, $p < 0.001$, depth $F_{3,\ 54} = 30.6$, $p < 0.001$).

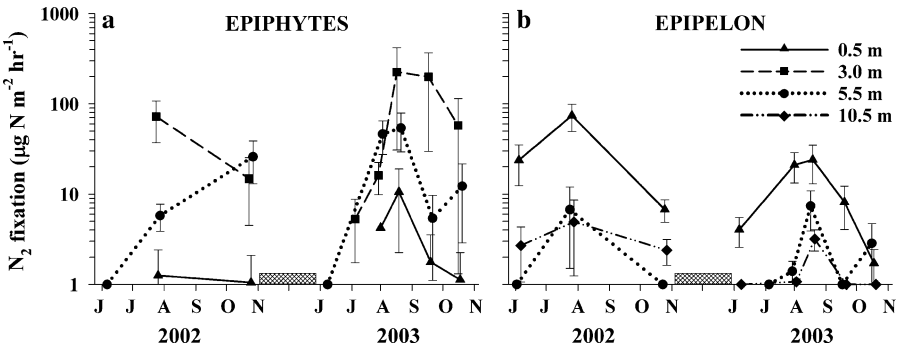


**Fig. 4** $N_2$-fixation rates for (**a**) epiphytes and (**b**) epipelon in Bull Trout Lake during the seasonal survey. The *shaded boxes* represent winter when the lake surface was frozen. Rates are corrected for the proportional area of different benthic cover and scaled per $m^2$ of lake bottom. Epiphyte cover was negligible at 10.5 m and therefore isn't included, while $N_2$ fixation by epipelon was not measured at 3.0 m. Note log scale on the *y*-axes. Error bars ± 1 S.E., *n* ranges from 3 to 8 for each data point

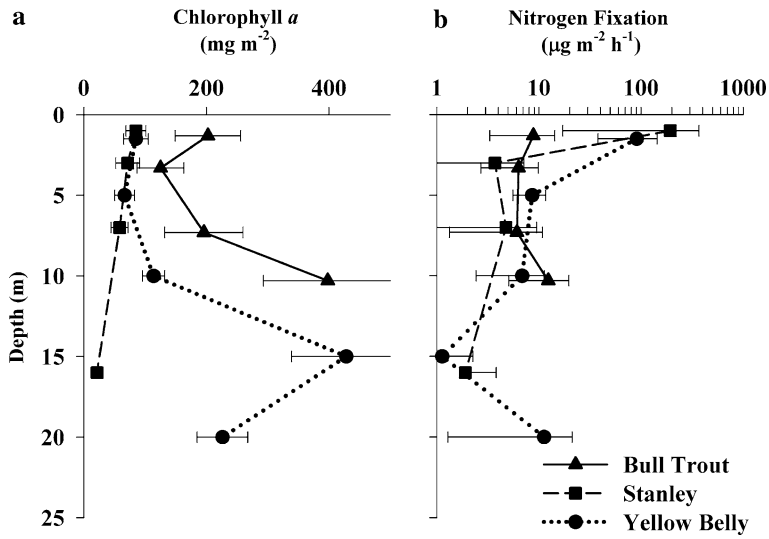


**Fig. 5** Benthic profiles of (**a**) chlorophyll *a* concentration and (**b**) $N_2$ fixation in all three study lakes during the baseflow survey. Note the log scale on the *x*-axes of (**b**). Error bars ±1 S.E., *n* ranges from 3 to 8 for each data point

*Baseflow survey*

During the baseflow survey, $N_2$-fixation rates were low and both chl *a* and $N_2$ fixation varied with depth in all three study lakes. Chlorophyll *a* concentrations in Yellow Belly and Bull Trout Lakes increased 2–7 fold with increasing depth, but declined slightly with increasing depth in Stanley (Fig. 5a). Pelagic $N_2$ fixation was undetectable in all lakes on all study dates (3 sampling dates, 36 total samples assayed). Benthic $N_2$-fixation rates were very variable, but were highest at 0.5 m in Stanley and Yellow Belly Lakes (Fig. 5b). $N_2$ fixation was an order of magnitude lower at 0.5 m in Bull Trout Lake than in the other two lakes. Mean $N_2$ fixation at depths below 2 m in all three lakes ranged from 1–10 µg N $m^{-2}$ $h^{-1}$, with the highest rates observed at 20 m in Yellow Belly Lake (Fig. 5b). When expressed relative to the amount of chl *a*, $N_2$ fixation showed a similar pattern to area scaled $N_2$-fixation rates, with greatest rates observed at 0.5 m in Stanley (2.5 µg N (mg chl *a*)$^{-1}$ $h^{-1}$) and Yellow Belly Lakes (2.9 µg N (mg chl *a*)$^{-1}$ $h^{-1}$), whereas all other rates were around 1 µg N (mg chl *a*)$^{-1}$ $h^{-1}$.

Streams

*Seasonal survey*

$N_2$-fixation rates varied strongly with season, substrate and stream type in Bull Trout inlet and outlet during the seasonal survey. Substrate cover area was

similar in the inlet and outlet streams and dominated by rock (Inlet: rock = 75%, FBOM = 11%, wood = 9%, CBOM = 4%; Outlet: rock = 78%, FBOM = 10%, wood = 6%, CBOM = 6%). Substrate-scaled $N_2$-fixation rates were highest on rock substrates in the outlet stream. Otherwise, $N_2$-fixation rates were low on all other substrates in both the outlet and particularly in the inlet stream (Fig. 3).

When the Bull Trout inlet and outlet $N_2$-fixation rates were corrected for substrate cover, the pattern of high $N_2$-fixation rates by periphyton on rock substrates became even more pronounced. Cover-scaled $N_2$ fixation on rocks in the outlet peaked in late July or mid-August, and were low on all other substrates in both streams on all sampling dates (Fig. 6a, b; 3-way ANOVA date × substrate interaction $F_{24,\ 113} = 2.3$, $p = 0.002$). This was due to a combination of low substrate-scaled $N_2$-fixation rates on CBOM, FBOM and wood (Fig. 3) and low benthic cover of all substrates besides rock. Cover-scaled $N_2$-fixation rates on rocks were always 1–2 orders of magnitude greater in the outlet compared to the inlet stream (Fig. 6); the interaction between substrate type and stream type was highly significant (three-way ANOVA, substrate x stream type interaction $F_{3,\ 86} = 27.0$, $p < 0.001$).

*Baseflow survey*

In all three inlet–outlet stream pairs during the baseflow survey, $N_2$ fixation on substrates in lake outlets were greater than or equal to those in lake inlets. Substrate cover varied more widely between the inlet and outlet streams in Stanley and Yellow Belly than in Bull Trout. In Stanley and Yellow Belly, rock covered ca. 60% of the inlet stream area, while it covered more than 80% in the outlet stream. In contrast, in Bull Trout, rock made up 75–78% of the cover in both the inlet and outlet (Fig. 7a). The greatest substrate-scaled $N_2$-fixation rates were measured on rock substrates in the lake outlet streams, but rates were much higher in Yellow Belly and Stanley outlets (460 and 730 μg N $m^{-2}$ $h^{-1}$) than in Bull Trout outlet (Fig. 3). High substrate-scaled $N_2$-fixation rates were also measured on wood substrates in Yellow Belly and Stanley outlets (215 and 120 μg N $m^{-2}$ $h^{-1}$), and they were 10–20 times higher than those on wood in the inlet streams of these lakes, as well as in Bull Trout outlet (Fig. 3). $N_2$-fixation rates were also moderately high on FBOM substrates in Yellow Belly outlet (90 μg N $m^{-2}$ $h^{-1}$), and similar on CBOM substrates in all streams.

When corrected for the relative cover of the substrates, $N_2$-fixation rates were significantly higher in the outlet (10–610 μg N $m^{-2}$ $h^{-1}$) than the inlet streams (0.2–8 μg N $m^{-2}$ $h^{-1}$; Fig. 7b). In particular, cover-scaled $N_2$-fixation rates were significantly greatest on rock substrates in outlet streams (two-way ANOVA stream-type x substrate interaction $F_{7,\ 15} = 7.7$, $p < 0.001$). This pattern was likely driven by higher $N_2$-fixation rates on rocks, rather than differences in substrate coverage between outlet and inlet streams (Fig. 7a). When corrected for chl *a* biomass, the patterns of $N_2$ fixation were mostly maintained, with higher rates in lake outlets and on rock substrates compared to inlet streams and other stream substrates (Fig. 7c).

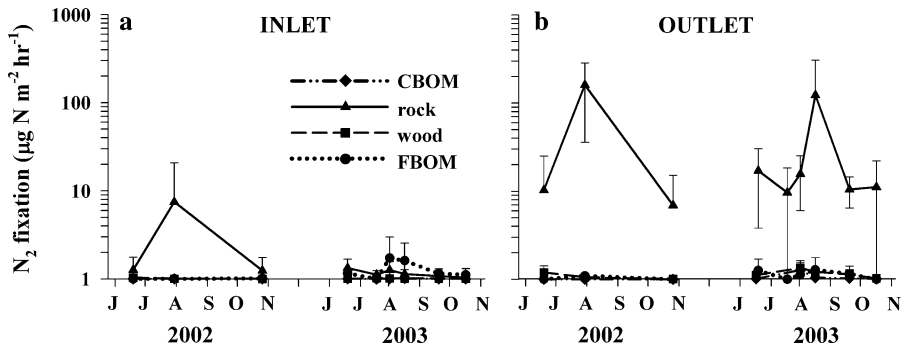


**Fig. 6** $N_2$ fixation for the different substrates in (**a**) Bull Trout inlet and (**b**) outlet during the seasonal survey. Rates are corrected for the proportional area of different benthic cover and scaled per $m^2$ of stream bottom. Note log scale on the *y*-axes. Error bars ±1 S.E., $n = 3$ for wood, CBOM and FBOM, $n = 5$ for rock

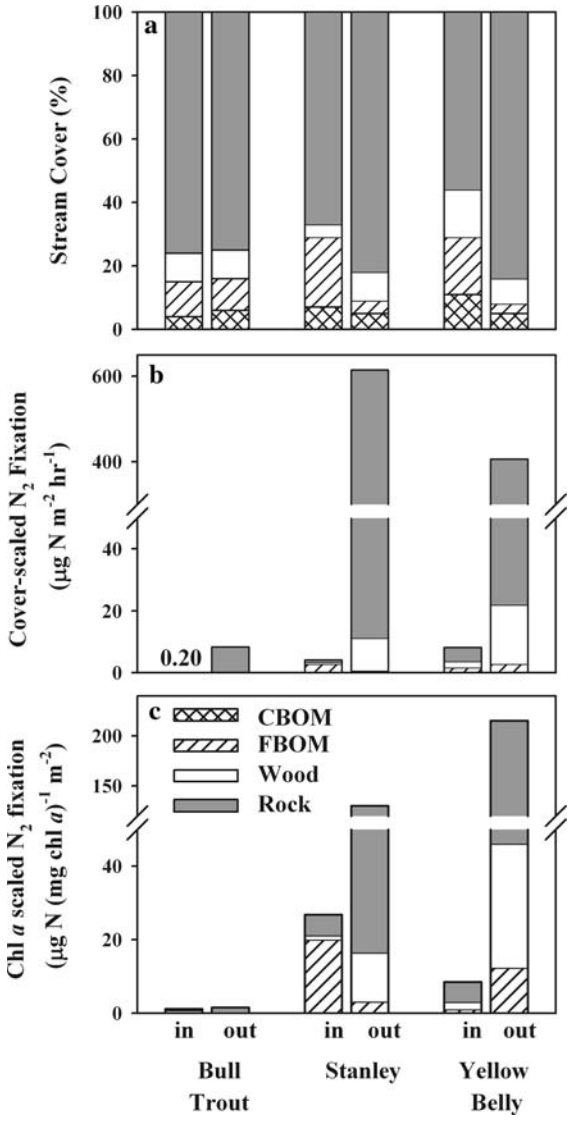


**Fig. 7** (**a**) Benthic stream cover, (**b**) cover-scaled and (**c**) chl *a* scaled $N_2$-fixation rates on stream substrates during the baseflow survey in the three inlet-outlet streams. The different shading patterns show the proportion of different substrate types. Note different *y*-axis scales on the different panes. For (**a**) and (**b**), $n = 3$ for wood, CBOM and FBOM, $n = 5$ for rock

## Comparing $N_2$-fixation rates in lakes and streams to N flux

As expected, nutrient concentrations in all of the study lakes and streams were exceedingly low and fluxes of N were closely linked to the annual hydrograph. Baseflow N and P concentrations were similar between the study lakes and streams (Tables 1 and 2). Total annual TN load into Bull Trout Lake averaged 1,240 kg year$^{-1}$; TDN composed 65% whereas nitrate-N composed only 8% of the TN flux. Loads of TN, TDN and nitrate-N into Bull Trout Lake peaked in late May or June (Fig. 8a), which is coincident with the spring-snowmelt water pulse.

When $N_2$-fixation contributions were scaled up to the entire lake or stream reach, inlet streams had much lower $N_2$-fixation contributions than either outlet streams or lakes. For the seasonal survey in the

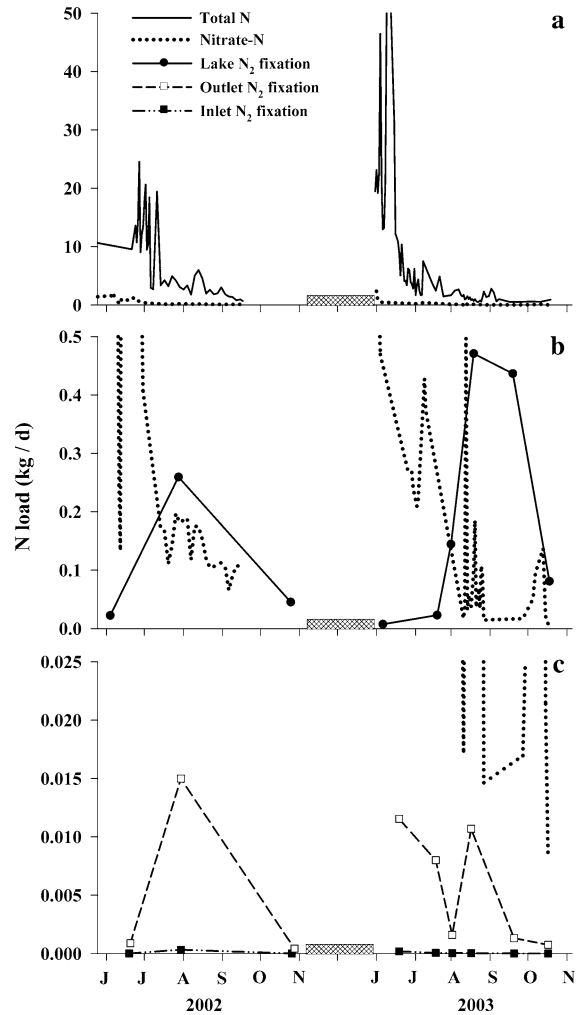


**Fig. 8** (**a**) Hydrologic N (TN and nitrate-N) flux in Bull Trout inlet, (**b**) whole-lake and (**c**) stream-reach scaled estimates of daily N inputs from $N_2$ fixation for the ice-free season in 2002–2003. Note the different *y*-axis scales on the panes, and that the dotted line represents nitrate-N flux on all panes. The *shaded boxes* represent winter when the lake surface was frozen. See methods section for details on how rates were scaled to whole lake and stream areas

Bull Trout lake-stream system, annual $N_2$-fixation contributions averaged 26.9 kg N $year^{-1}$ in the lake, 0.7 kg N $year^{-1}$ in the outlet stream and only 0.01 kg N $year^{-1}$ in the inlet stream. The large difference between stream and lake contributions can almost entirely be attributed to the much larger surface area of the lake compared to the streams; the benthic area of Bull Trout Lake was $2.6 \times 10^5$ $m^2$, while the benthic areas of the inlet and outlet stream were only $3.4 \times 10^3$ $m^2$ and $8.0 \times 10^3$ $m^2$, respectively. Similar patterns were observed in the baseflow survey, where lake contributions were 1–2 orders of magnitude larger than inlet $N_2$-fixation contributions. However, in the Stanley and Yellow Belly systems, $N_2$-fixation contributions of the outlet streams were similar to those of the lake (Table 3). In this survey, the very large surface area of the lake habitat lead to large $N_2$-fixation contributions despite low $N_2$-fixation rates, while in outlet streams very high $N_2$-fixation rates lead to large contributions despite small habitat surface areas.

For the study lakes, $N_2$-fixation contributions were generally low but asynchronous with the fluxes in dissolved and particulate N. On an annual basis, $N_2$-fixation contributions into Bull Trout Lake were equal to 22–31% of the nitrate-N load, 3–4% of the TDN load, and only 2% of the TN load. During the spring, nitrate-N, TDN, and TN fluxes into the lake were high and $N_2$-fixation contributions were low (Fig. 8b). In contrast, in August and September of both years $N_2$-fixation contributions peaked while dissolved and total N fluxes were lowest (Fig. 8b). At this time, the in-lake $N_2$-fixation contributions exceeded the nitrate-N flux into the lake by 25–1,700%. For all three lakes during the late July baseflow survey, in-lake $N_2$-fixation contribution was equal to 12–32% of the nitrate-N flux from the inlet stream, 5–7% of the TDN flux, and 2–4% of the TN flux (Table 3).

During the seasonal and baseflow surveys for all inlet streams, hydrologic fluxes always greatly exceeded $N_2$-fixation contributions (Fig. 8c, Table 3). In the outlet streams, $N_2$-fixation contributions were higher relative to hydrologic N fluxes. During the seasonal survey in Bull Trout outlet during mid-summer (July–August), the $N_2$-fixation contribution was greater than or similar to the nitrate-N flux (Fig. 8c, outlet fluxes not shown). However, in the same stream in June and October, nitrate-N flux was 2–3 orders of magnitude greater than $N_2$-fixation contribution, TDN was 3–4 orders of magnitude greater, and TN was 3–5 orders of magnitude greater. During the baseflow survey in the lake outlet streams, $N_2$-fixation contributions were similar to the lakes for two of the three systems studied (Table 3), and equaled 4–136% of the nitrate-N, 0.06–9% of the TDN and 0.03–2% of the TN fluxes.

## Discussion

### The magnitude and importance of $N_2$ fixation in the study lakes and streams

The results of our $N_2$-fixation surveys showed that $N_2$-fixation rates in oligotrophic lake-stream systems are typically low, but peak in late summer, when the flux of TN and nitrate-N are low. The seasonal pattern of $N_2$ fixation we observed was similar to that observed in a eutrophic reservoir where pelagic $N_2$ fixation was seasonally asynchronous with stream N loading (Scott et al. 2008) and with $N_2$-fixation rates measured by Reuter et al. (1983) on benthic substrates in Lake Tahoe. The highest substrate-scaled $N_2$-fixation rate observed in any of our lakes or streams was approximately 1,000 μg N $m^{-2}$ $h^{-1}$; peak rates for epiphytes in the lake and on rocks in streams were more frequently around 200–300 μg N $m^{-2}$ $h^{-1}$. These rates were quite similar to benthic rates observed on rock substrates in tropical and subalpine oligotrophic lakes (Loeb and Reuter 1981; Higgins et al. 2001) and by epiphytes in a tropical lake (Doyle and Fisher 1994). The lack of pelagic $N_2$ fixation was expected and has been observed in other oligotrophic lakes (Mague and Burris 1973; Flett et al. 1980; Reuter and Axler 1992). We did not, however, measure $N_2$ fixation at night and consequently cannot rule out $N_2$ fixation by unicellular cyanobacteria, which are abundant in our study lakes (Budy et al. 1995). The $N_2$-fixation rates in our streams were low compared to those reported in desert streams (Grimm and Petrone 1997) or California coastal streams with large populations of *Nostoc* sp. (Horne and Carmiggelt 1975), but similar to those observed in two Antarctic streams (Howard-Williams et al. 1989).

Although our study suggests that $N_2$-fixation contributions may be most important during times

**Table 3** Daily contributions of $N_2$ fixation, nitrate-N, total dissolved N, and total N to lakes and streams in the baseflow survey

| Watershed | | Total N[a] (g N day$^{-1}$) | Total dissolved N[a] (g N day$^{-1}$) | Nitrate-N[a] (g N day$^{-1}$) | $N_2$ fixation (g N day$^{-1}$) |
|---|---|---|---|---|---|
| Bull Trout | Inlet | 1,500 | 540 | 100 | 0 |
| | Lake[b] | – | – | – | 33 |
| | Outlet | 2,290 | 1,630 | 25 | 1 |
| Stanley | Inlet | 4,140 | 2,140 | 780 | 1 |
| | Lake[b] | – | – | – | 143 |
| | Outlet | 9,250 | 1,720 | 107 | 146 |
| Yellow Belly | Inlet | 5,000 | 3,670 | 1,500 | 1 |
| | Lake[b] | – | – | – | 186 |
| | Outlet | 5,660 | 3,150 | 470 | 70 |

[a] Nitrate N, TDN and TN loads were calculated from Arp and Baker (2007)

[b] Contributions to the lakes are the same as the contributions to the inlet stream, since most of the N input into the lakes is from stream flow

when hydrologic N fluxes are low, determining the magnitude of how these compare is problematic. First, $N_2$-fixation contributions are dependent on the total surface area of the habitat studied, because as surface area increases the amount of N introduced via $N_2$ fixation increases. In contrast, hydrologic N fluxes are not spatially dependent (Cummins et al. 1983, Grimm and Petrone 1997). The very large surface areas of the lakes certainly contributed to the more important role for $N_2$ fixation in lakes versus streams in our analysis: lakes are simply much larger areas where $N_2$ fixation can occur, while streams comprise a relatively small overall portion of our study watersheds. While the surface area of the habitat was relatively easy to determine for the study lakes, the stream reach length of 1.4 km used was relatively arbitrary based on typical geomorphic characteristics of Sawtooth Mountain watersheds. Increasing or decreasing this reach length would alter our assessment of the importance of $N_2$ fixation versus hydrologic N fluxes in the study streams. However, regardless of how long of a stream reach we selected over which to apply our $N_2$-fixation rates, or how large the study lake area was, on a whole watershed scale it is likely that $N_2$ fixation in aquatic habitats would continue to appear inconsequential compared to atmospheric N inputs and N processing in the much larger terrestrial environment.

Directly equating $N_2$ fixation to hydrologic fluxes also ignores the potential importance of fixed N for stream organisms. Studies with *Trichodesmium* sp., a marine $N_2$-fixing cyanobacteria, suggest that about 50% of the N obtained via $N_2$ fixation is assimilated into biomass, but this can range from 5–95% (Mulholland et al. 2006). The fixed N lost might then be an important N source for other algae or microbes living in close proximity to $N_2$ fixers (Mulholland et al. 2006, Marcarelli et al. 2008). Because N obtained via $N_2$ fixation may be quickly incorporated into algal or bacterial biomass, it may play a crucial role for algal assemblages (Baker et al. 2009, Scott et al. 2009) and stream food webs (Marcarelli et al. 2008). In contrast, hydrologic N flux does not consider the fate of that N once it enters a stream reach, where it can pass through unaltered, be retained temporarily via cycling by the biota, be transformed and exported in a different N form, or be lost permanently via denitrification. A more useful comparison may be between $N_2$ fixation and the amount of N retained as periphyton biomass (e.g., Grimm and Petrone 1997) or uptake rates of inorganic N (Newbold 1992). By comparing $N_2$-fixation rates and cell N content, Scott et al. (2007) found that $N_2$ fixation contributed about 30% of the N content of metaphyton (floating algal and microbial mats) in a wetland. $N_2$-fixation rates during July and August in our study streams are typically equal to or greatly exceed nitrate-N uptake rates in lake outlet streams, but not lake inlets (Arp and Baker 2007; Marcarelli et al. 2008). However, $N_2$-fixation rates were always 1–2 orders of magnitude lower than ammonium-N uptake rates in Bull Trout inlet and outlet (Koch 2005; Marcarelli et al. 2008). There is a need for more comprehensive seasonal studies to

determine the usefulness of comparing $N_2$-fixation rates, biofilm N content, and N uptake rates in a range of stream ecosystems (Marcarelli et al. 2008).

Our $N_2$-fixation rates are likely underestimates of the true $N_2$-fixation potential in our systems. First, collecting periphyton for the slurry technique disturbs the biofilm structure, and likely aerates the anoxic microzones that may promote $N_2$ fixation (Paerl 1985). Calibrations of our slurry technique against simultaneous measurements on intact rock substrates from streams in recirculating chambers indicate that the slurry $N_2$-fixation rates are about 8 times lower than those by undisturbed wood and rock communities (log [undisturbed rate + 1] = 0.9*log[slurry rate + 1] + 0.76, $r^2 = 0.73$; Marcarelli 2006). Second, we assumed that nighttime $N_2$ fixation was zero. This assumption is reasonable based on stream studies that have shown that $N_2$-fixation rates by cyanobacteria decrease significantly during the night (Livingstone et al. 1985, Grimm and Petrone 1997), but at odds with studies of marine cyanobacterial mats where $N_2$-fixation rates peak at night, temporally separated from daytime peaks in photosynthesis (Villbrandt et al. 1991). Together, these indicate that $N_2$-fixation rates in our study system are likely even higher than reported here, and hence our estimates concerning the importance of $N_2$ fixation for these systems may be conservative. This could have important implications for our estimations of the importance of $N_2$ fixation; for example, if rates for the seasonal survey in Bull Trout Lake were eight times greater, the $N_2$-fixation contribution would be 2 times greater than the nitrate-N load and would equal approx. 20% of the annual TN load; these numbers are much higher than our previous estimates of 22–31% of nitrate-N load and 2% of the TN load.

Methodological differences may also explain the different conclusions reached regarding the importance of benthic lake $N_2$ fixation in the seasonal versus baseflow survey. Large differences in primary production have been observed on different benthic lake substrates (Vadeboncoeur et al. 2006), and we observed similar differences in $N_2$ fixation on benthic substrates during the seasonal survey in Bull Trout Lake. However, during the baseflow survey benthic substrates were integrated into dredge samples. This technique efficiently collected sediment and associated epipelon, but was much less efficient at collecting rocks and macrophytes, where we observed the highest rates of $N_2$ fixation. It is likely that we would have estimated higher $N_2$-fixation contributions during the baseflow survey had we partitioned benthic $N_2$-fixation rates by substrate type.

## Factors potentially controlling $N_2$-fixation rates in lake-stream systems

The seasonal peaks we observed in $N_2$ fixation were likely driven by changes in temperature, light intensity, and nutrient supply to our linked lake-stream systems. When $N_2$ fixation peaked, the intensity and duration of light and water temperature were at their annual high, and the dissolved nutrient concentrations had returned to the low levels characteristic of baseflow. $N_2$ fixation is strongly temperature dependent, because increased temperatures favor the inclusion of cyanobacteria in the periphyton community (e.g., Cairns 1956), and increase enzymatic activity, thereby increasing $N_2$-fixation rates (Reuter et al. 1983; Staal et al. 2003; Marcarelli and Wurtsbaugh 2006). $N_2$-fixation rates are strongly related to light intensity and duration because cyanobacteria need to maintain photosynthesis as an energy source for the energetically expensive $N_2$-fixation reaction (Lewis and Levine 1984). Finally, $N_2$-fixation rates are strongly related to ambient nutrient concentrations, and it has been demonstrated that N additions or high N concentrations suppress $N_2$-fixation rates, because $N_2$ fixation will not occur when cells have access to a less energetically expensive source of N (Howarth et al. 1988b). Clearly, these three factors are all interrelated, and the interactions among them are most likely responsible for the seasonal patterns we observed.

Temperature and nutrient supply also likely controlled spatial patterns of $N_2$ fixation in our study areas. In Bull Trout Lake, the highest rates of $N_2$ fixation were by epiphytes attached to macrophytes, which has also been observed in other lakes (Finke and Seeley 1978; Moeller and Roskoski 1978). Although macrophytes can obtain nutrients from the sediment pore water, studies have shown that during the growing season they can be a net sink of DIN within a lake (Carpenter and Lodge 1986). Additionally, macrophytes may be a net source of dissolved P to the water column, although release rates represent less than 5% of the P taken up from the sediment

(Carpenter and Lodge 1986). If macrophytes are a sink of N and a source of P, the aquatic environment surrounding the macrophytes may have a low N:P ratio, leading to an environment where $N_2$ fixers are favored over other taxa. Phosphorus limits $N_2$ fixation in epilithic stream communities in the Sawtooth Mountains (Marcarelli and Wurtsbaugh 2007), but its importance for $N_2$ fixers in the lakes has not been measured. In the streams, much higher rates of $N_2$ fixation were measured in lake outlet than lake inlet streams. In our study area, lakes alter nutrient concentrations (Brown et al. 2008), nutrient uptake rates (Arp and Baker 2007), and water temperature (Marcarelli and Wurtsbaugh 2007). We have experimentally demonstrated that high water temperature, and low nitrogen supply interact to stimulate $N_2$-fixation rates in our study streams (Marcarelli and Wurtsbaugh 2006). Therefore, the presence of upstream lakes may create conditions favorable for $N_2$ fixation in outlet streams.

Substrate stability could be another important factor favoring high $N_2$-fixation rates on rocks in streams and on epiphytes in lakes. Macrophytes provide more stable sites for periphyton colonization than shifting benthic sediments in the littoral zone, while simultaneously attenuating light below their canopies and shading underlying sediment (Carpenter and Lodge 1986). In some places, macrophyte beds in Bull Trout Lake were 1-2 m deep, and growing high in the macrophyte canopy could have provided a more favorable light habitat for epiphytes than the lake bottom. In our snowmelt dominated study streams, rocks should provide more stable substrates for slow-growing cyanobacteria (Douglas 1958) than more mobile CBOM, FBOM or small wood substrates. In our study streams, rock sizes are larger in outlet streams, and stream beds are less mobile in lake outlets than in lake inlets due to larger particle size and attenuated peak flows by upstream lakes (Arp et al. 2007; Myers et al. 2007), leading to more stable habitat for periphyton communities, and perhaps greater $N_2$ fixation, in lake outlets.

## Conclusions

Considering the location and timing of $N_2$ fixation in linked lake-stream ecosystems provides new insight into the role of $N_2$ fixation in freshwater, oligotrophic watersheds. Our study indicates that $N_2$-fixation rates are very patchy, strongly dependent on the location within lake-stream systems, and seasonally asynchronous with peaks in hydrologic N flux. By considering lakes and streams together, our study illustrates how they interact to create conditions where biotic processes, such as $N_2$ fixation, are favored. Lakes are important sites of new N production in a watershed due to a combination of high $N_2$-fixation rates when conditions are favorable and the large benthic area. Additionally, by increasing water temperature, increasing benthic substrate stability and decreasing dissolved nutrient concentrations, lakes may increase $N_2$-fixation rates in outlet streams. Altering lake location and frequency within a watershed will certainly alter the amount of N entering watersheds via $N_2$ fixation, both by increasing within-lake and downstream rates of $N_2$ fixation, and could be an important watershed characteristic controlling N supply and processing rates in subalpine aquatic ecosystems.

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