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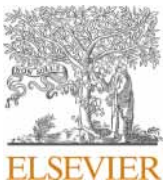

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Standardization of instantaneous fluoroprobe measurements of benthic algal biomass and composition in streams

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ABSTRACT

Fluoroprobes, such as BenthoTorch, offer a relatively rapid *in situ* method for monitoring benthic algae, provide new opportunities for aquatic ecological research, and enable early warning of algal proliferation in municipal water supplies. Currently, however, BenthoTorch is limited for the measurement of heterogeneous samples, and homogenizing samples is rather difficult. In this study, we compared chlorophyll-a (Chl-a) measurements between BenthoTorch and standard laboratory methods to quantify and describe the biomass and biovolume proportion of benthic algae growing on artificial and natural substrates. We used the difference in Chl-a measurements between these methods (n = 359) to evaluate the effects of environmental variables. Moreover, we used artificial and natural substrates to determine whether either of them reduced variation in BenthoTorch measurements. There was a general concordance in measurements between the two methods, but this agreement was stronger for artificial substrates. Artificial substrates also led to significantly less variation in BenthoTorch readings compared with natural substrates. This may be attributed to the formation of relatively thin algal mats on artificial substrates. To enable early warning of algal proliferation in an aquatic ecosystem, we recommend the use of BenthoTorch and artificial substrates combined with appropriate calibration using standard laboratory methods. The ability of BenthoTorch to produce instantaneous results facilitates replicate collection, which reduces sampling error and increases sampling area on the same day. Despite substantial variation in readings on natural substrates, BenthoTorch enables relatively rapid measurements, facilitating easy monitoring of large areas. Therefore, BenthoTorch may be worth incorporating into aquatic ecosystem studies, depending on the specific research questions.

1. Introduction

Benthic algal monitoring can provide key insights into the structure and function of stream ecosystems. Under specific conditions (e.g., stable-flowing water, high temperature, and labile nutrients), benthic algae proliferate, potentially reducing water quality and altering ecosystem functions (Scanlan et al., 2015; Ceola et al., 2013). Although quantifying benthic algal groups over wide temporal and spatial scales is economically and logistically challenging, it is essential for monitoring aquatic ecosystems under diverse conditions, both natural (e.g., climate) and artificial (e.g., nutrient loading) (Paerl et al., 2001; Echenique-Subiabre et al., 2016).

Until recently, quantification of algal biomass and pigment concentration as well as assessment of algal taxonomic groups relied upon time-consuming standard laboratory methods. In addition to the laborious sample preparation and high level of taxonomic expertise required, standard methods pose logistical constraints for replication while maintaining accuracy (Steinman et al., 2017). As an alternative, *in situ* techniques (e.g., fluoroprobes [BenthoTorch]) have emerged in the last decade for rapid estimation of phytoplankton and benthic algal characteristics (Catherine et al., 2012), thereby increasing the scope of temporal and spatial measurements. This, in turn, may broaden the research questions that aquatic ecologists can pursue. However, we must better understand the conditions under which BenthoTorch measurements are the most accurate and precise as well as quantify their reliability.

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Comparative studies have employed different approaches to assess the reliability of *in situ* versus standard laboratory methods and have shown a satisfactory agreement among measurements when the algal concentrations are relatively low (Table 1) (Steinman et al., 2017). However, all these studies (Table 1) used natural substrates and found it overall challenging to standardize chlorophyll-a (Chl-a) readings for benthic algal biomass and relative abundance based on mature mat formation. BenthoTorch and standard methods were comparable in terms of Chl-a measurements in benthic algae in oligotrophic streams (Kahlert and McKie, 2014). Similarly, Harris and Graham (2015) found a strong concordance in measurements between BenthoTorch and standard methods when Chl-a concentration was below 4 $\mu g \cdot cm^{-2}$ in an urban stream in Kansas (USA). In contrast, Echenique-Subiabre et al. (2016) found a weak agreement between these methods in eutrophic streams when benthic algal mats were relatively thick. Regarding the biovolume proportion of benthic algae and primary producers, Echenique-Subiabre et al. (2016), Kahlert and McKie (2014), and Harris and Graham (2015) have presented contradictory results for diatoms and cyanobacteria (Table 1). Recently, Kaylor et al. (2018) examined the sensitivity of BenthoTorch readings to light exposure and suggested that physical factors affecting benthic algal proliferation (i.e., light intensity, temperature, water velocity, and nutrients) must be considered when assessing the reliability of Chl-a readings using this fluoroprobe.

Despite the general agreement among previous studies on the concordance in readings between BenthoTorch and standard methods, variability in Chl-a concentrations and biovolume proportion of major algal groups must be reduced. In this context, we hypothesized that substrate variability would substantially contribute to differences in measurements and that standardization of substrates would decrease this variability. In addition. standardized substrates provide an improved foundation for monitoring temporal trajectories of algal accumulation or loss and produce responses similar to natural substrates (Cattaneo and Amireault, 1992; Tuchman and Stevenson, 1980). Standardized artificial substrates facilitate the monitoring of different benthic algal mat formation stages; moreover, their uniform texture may enable a more robust comparison of Chl-a readings between BenthoTorch and standard methods.

To this end, our study assessed the performance of BenthoTorch and compared it with that of standard laboratory methods using artificial and natural substrates to identify the conditions under which BenthoTorch is the most and least reliable. Specifically, we aimed to (1) compare total Chl-a concentration and biovolume proportion of algal groups grown on artificial and natural substrates between BenthoTorch and standard methods; (2) evaluate the effects of environmental factors, such as light intensity, temperature, algal mat thickness, water column depth, water velocity, and water discharge on Chl-a readings using BenthoTorch and laboratory methods; and (3) assess the variability of BenthoTorch readings using standardized artificial versus natural substrates. To the best of our knowledge, this study is the first to use standardized artificial substrates for a comparison between BenthoTorch and standard methods.

2. Materials and methods

2.1. Study area

The study was conducted in the headwaters of the Chalpi Grande River watershed, with a basin area of 95 km^2, located inside the Cayambe-Coca National Park in the northern Andes of Ecuador ($0^{o}16'45''S$, $78^{o}4'49'W$, 3,835 m altitude) (Fig. 1). Streams are predominantly fed by rainfall-draining volcanic geology, with substrates varying from boulders to silt, and tussock grasslands covering the stream banks. Quito's water supply system is located in the highest part of the Chalpi Grande watershed. Low-head dams are placed throughout the stream network to facilitate water withdrawal to the system of water supply pipes (water intake).

The Chalpi Norte stream is the primary headwater tributary, and small dams provide an ideal setting for studying benthic algae in natural (free flow) and artificial (regulated flow) stream reaches, because hydrological conditions (i.e., streamflow) vary over short spatial and temporal scales (Jacobsen and Dangles, 2017; Rosero-López et al., 2019). Thin mats of benthic algae are commonly found in free-flowing waters, while iron-oxidizing bacteria accumulate beneath benthic algae, which might influence the performance of BenthoTorch, under regulated flow conditions.

2.2. Sampling design

We conducted sampling during the high-flow season of 2017, from July to September (Supplementary Material FS1). Sampling methods are described in detail in section 2.3.2. To characterize the spatial and temporal dynamics of benthic algae, we measured Chl-a as a surrogate of algal accrual on artificial substrates and biomass on natural substrates (Fig. 2). We selected four sites each of free-flowing (upstream of water intake) and regulated flow (downstream of water intake) conditions. Except one site in an unaltered tributary (control site), all sites were located in the Chalpi Norte stream (Fig. 1). Artificial substrates were created by cementing unglazed ceramic tiles (4 cm × 4 cm × 0.5 cm) to clay bricks (15 cm × 25 cm). In the field, we placed 10 bricks with 5 tiles at each site, arranged in two parallel rows perpendicular to the current. We collected samples on days 0, 7, 14, 21, 28, 35, 42, 49, 56, and 63, with no replacement: five tiles per site (artificial substrates, total n =

Table 1
Previous comparisons of *in situ* (BenthoTorch) and standard methods (laboratory) for the measurement of chlorophyll-a concentration and biovolume proportion (%) of benthic algae and primary producers (e.g., diatoms, green algae, and cyanobacteria).

Reference	*n*	Chl-a concentration	Laboratory method	*n*	Proportion (%) biovolume
Kaylor et al., 2018	50	Benthotorch underestimated Chl-a when laboratory measures where > 4 $\mu g/cm^2$	95% acetone, Fluorometer	–	–
Echenique-Subiabre et al., 2016	288	High correlation of methods for thin biofilms < 4 $\mu g/cm^2$, ($R^2 = 0.60$, $p < 0.001$)	90% absolute methanol, Spectrophotometer	120	High correlation for cyanobacteria when proportion < 50% ($R^2 = 0.53$, $p < 0.001$)
		Low correlation of methods for biofilms > 4 $\mu g/cm^2$, ($R^2 = 0.23$, $p = 0.008$)		64	High correlation for diatoms when proportion > 40% ($R^2 = 0.54$, $p < 0.001$)
				117	Low correlation for green-algae when proportion < 20% ($R^2 = 0.10$, $p < 0.001$)
Harris and Graham, 2015	30	High correlation for concentrations < 4 $\mu g/cm^2$ ($R^2 = 0.47$, $p < 0.01$)	96% heated ethanol, Fluorometer	6	Benthotorch underestimated diatoms by 5.4X and green algae by 1.3X
				6	Benthotorch overestimated cyanobacteria
Kahlert and McKie, 2014	24	Benthotorch not significantly different from laboratory method	90% acetone, Spectrophotometer	24	Benthotorch estimation of diatoms ~ 85% and laboratory ~ 35%
				24	Benthotorch estimation of green algae ~ 11% and laboratory ~ 27%
				24	Benthotorch estimation of cyanobacteria ~ 4% and laboratory 32%

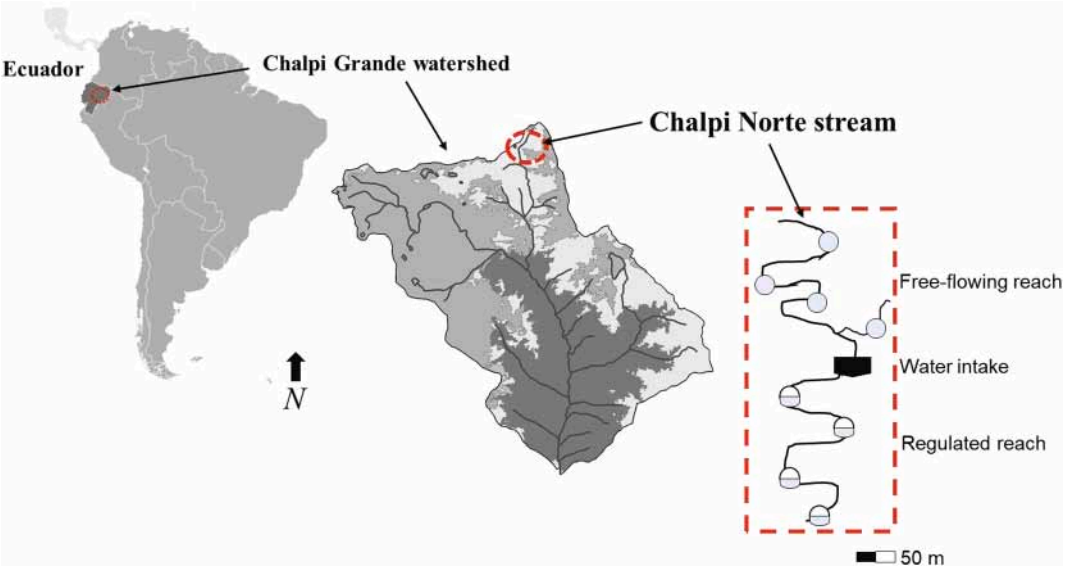

Fig. 1. Chalpi Norte stream study sites above (free-flowing reach) and below the water intake (regulated reach) in the Chalpi Grande watershed, Northeast Ecuador.

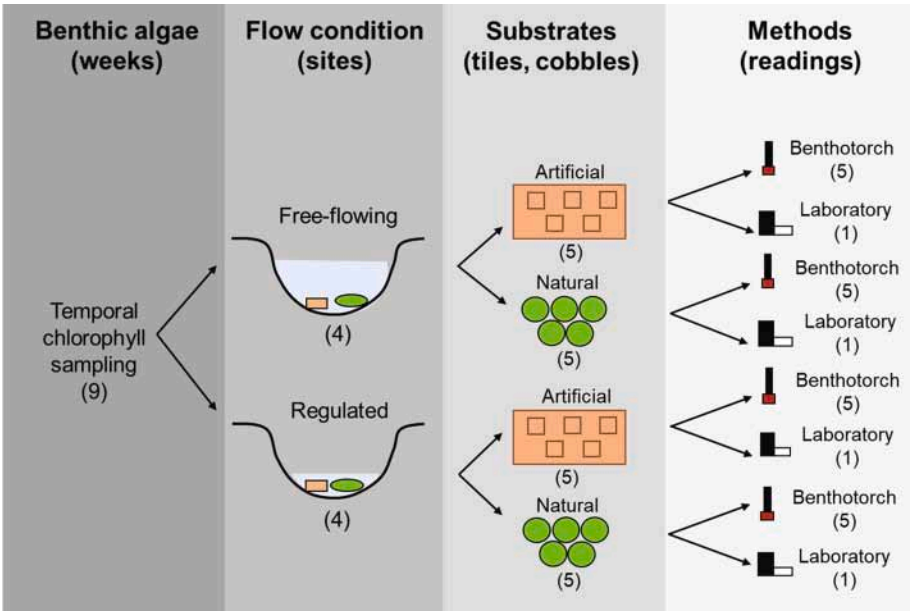

Fig. 2. Survey design for the comparisons of benthic algal chlorophyll-a concentration and biovolume proportion on artificial and natural substrates under different flow conditions: free flow and regulated flow; numbers in parentheses indicate replicates.

359) and five cobbles per site (natural substrates, total n = 355, diameter = 5–9 cm). For each set of samples, we applied two methods to determine benthic algal Chl-a concentration and biovolume proportion of algal taxonomic groups (see section 2.3). We also measured physical variables on artificial and natural substrates (see section 2.4). Based on previous findings (see Rosero-López et al., 2020), these headwater streams show low aquatic invertebrate density in the high-flow season; therefore, we assumed that the grazing effect was negligible.

2.3. Measurements of benthic algae on artificial and natural substrates

We used a fluoroprobe (BenthoTorch) for *in situ* measurement of Chl-a concentrations in each algal group. We also applied standard methods for laboratory Chl-a extraction and analysis as well as microscopy for taxonomic identification.

2.3.1. In situ method: BenthoTorch

The BenthoTorch fluorometer (BG36700-V, bbe Moldaenke GmbH Schwentinental, Germany) is a deployable pulse-amplitude-modulated spectrofluorimetric tool that uses predefined algorithms to instantaneously identify the Chl-a fluorescence signal of benthic algae and primary producers such as diatoms, cyanobacteria, and green algae (Carpentier et al., 2013). This instrument emits light pulses at 470, 525, 610, and 700 nm and records the Chl-a response at 690 nm (Kahlert and McKie, 2014). We placed BenthoTorch on artificial and natural substrates, avoiding light entering the algal surface area of excitation (Kaylor et al., 2018). The sampled area where the beam of light excited Chl-a measured 1 cm^2. Ten seconds of exposure provides Chl-a concentrations in diatoms, cyanobacteria, and green algae, and the fluorescence algorithm calculates the proportion of each group (Catherine et al., 2012). To reduce variation, we obtained three readings for each measurement and averaged them, as suggested by the manufacturer.

2.3.2. Standard methods: Laboratory readings and microscopic analysis

We measured Chl-a concentrations following DIN 38 412-L16 in the laboratory. We followed the manufacturer's laboratory method for Chl-a extraction with ethanol and quantification using a spectrophotometer to avoid variances caused by different solvents and equipment (i.e., acetone and methanol, see Steinman et al., 2017). Following the bbe Moldaenke protocol, samples (algae and substrate) were stored in foil bags with a moist sponge and placed in individual boxes with ice packs (at ~ 7 °C) for transportation to the laboratory. Under minimal light exposure, benthic algae were removed from the substrates using a nylon brush and collected in a tray with filtered water. The sample slurry of each substrate was homogenized and placed in a graduated cylinder to measure the total volume (V_P). We calculated the scraped area (A_{SCR}) on natural substrates using ImageJ2 (Rueden et al., 2017). We delineated the contour of the substrate surface colonized by benthic algae using a known reference area for scaling. We filtered 2.5 mL of the sampled slurry (V_E) through glass microfiber filters with funnel (Whatman GF/F 0.7 µm nominal pore size). Filters were introduced into dark film canisters filled with 10 mL of 90% ethanol, ensuring complete submersion. Canisters were closed and incubated for 12 h at 20℃. After incubation, 3 mL of the extract was poured into a cuvette, and a spectrophotometer (Agilent 8453, Thermo Scientific) equipped with UV–visible spectroscopy software running on a personal computer was used for measurements at a wavelength of 665 nm, followed by 750 nm; both readings were obtained against the offset of 90% ethanol solution as the reference (DIN 38 412-L16). Then, we used the 750 nm measurement for the compensation of sample turbidity. Finally, 3 M HCl (10 µL for 10 mL of extraction volume) was added to the filtered extract. After incubation for 10 min in the dark, we measured Chl-a converted to pheophytin at wavelengths of 665 and 750 nm. We calculated Chl-a concentrations using the following equation:

$$[\text{Chl} - \text{a}] = 29.6 \cdot \left[\left(A_{v_{665}} - A_{v_{750}}\right) - \left(A_{n_{665}} - A_{n_{750}}\right)\right] \cdot \frac{V_E}{A_{SCR} \cdot d} \quad (1)$$

where [Chl-a] is the chlorophyll-a concentration (µg·cm^{-2}), A_v and A_n are absorbances at 665 and 750 nm before (v) and after acidification (n), V_E is the extracted volume, A_{SCR} is the scraped area, and d is the cuvette width (cm).

We subsampled 2 mL of the scrubbed benthic algal slurry (see below) and placed it into a labeled round bottom cryogenic vial. Samples were preserved in Lugol's iodine solution and stored at room temperature. We left the samples to settle for at least 8 h and started the identification process no later than 24 h after collection. We identified and enumerated benthic algal groups in random fields using the Utermöhl sedimentation chamber under an inverted microscope (40–45 × objective, 400–450 total system magnification; Zeiss Axio Vert. 40, Germany). We enumerated 300 algal units (1 unit = 1 cell for all species) in a minimum of 10 and a maximum of 100 fields on a 12 mm × 12 mm grid. We counted algal species and grouped them into diatoms, cyanobacteria, and green algae in samples collected from artificial (n = 356) and natural (n = 144) substrates. All analyses were performed at the laboratory of the Quito water supply company Empresa Pública Metropolitana de Agua Potable y Saneamiento. We grouped taxa to calculate the biovolume proportion of benthic algal groups (i.e., diatoms, cyanobacteria, and green algae).

2.4. Physical variables (light intensity, temperature, algal mat thickness, water column depth, water velocity, and water discharge)

We performed our study during the high-flow season between July and August 2017 (Table 2). We registered environmental light intensity five times, every 10 s for 60 s each time, at a standard height of 1.6 m from the ground (LI-200R, Pyranometer sensor LI-COR®, USA). We measured instantaneous water temperature using a thermo-probe at five points around the artificial and natural substrates (VWR Thermometer probe, VWR Scientific™, USA). We measured the benthic algal mat thickness on substrates (Digital caliper, Mitutoyo 500–196-30 CAL, USA). We measured water velocity twice (Acoustic digital current meter OTT Hydromet, Germany) at a depth of 2 cm and water column depth with a current meter wading rod. We also measured integrated velocities at 20, 60, and 80% of the total depth, at a minimum of six locations across the stream, using a current meter to calculate water discharge at the reach scale (i.e., using the area-velocity method).

2.5. Statistical analysis

We applied the Tukey test (Bland–Altman test) to evaluate the interval of agreement between the two methods (Giavarina, 2015; Datta, 2017). Of note, strong agreement between the two datasets is insufficient for assessing method comparability (Giavarina, 2015). We plotted the averages (x-axis) and differences (i.e., difference = laboratory measurement – field measurement) (y-axis) of paired data (see Rosero-López, 2020 for further details). We assigned the laboratory method as the reference for comparison with BenthoTorch (field). The deviation of the mean of differences from the line of equality corresponds to the potential bias between the methods. In this study, this deviation

Table 2
Mean and ranges of physical variables measured at the study sites in the Chalpi Norte stream with different flow conditions between July and August 2017.

Site identification	Flow condition	Dominant substrate	Benthic algal mat thickness (mm)		Light (Watt/dm^2)	Water temperature (°C)	Water velocity at 2 cm from bottom (m/s)	Water depth (m)	Stream flow (m^3/s)
			Artifical subs.	Natural subs.					
Chalpi 01	Free-flowing	Gravel (Pebble - Cobble)	1.5 (0–3)	5.9 (1.67–9.5)	1.91 (0.55–4.02)	8.41 (7.02–8.99)	0.71 (0.31–1.08)	0.15 (0.14–0.19)	0.34 (0.17–0.54)
Chalpi 02	Free-flowing	Gravel (Pebble - Cobble)	1.4 (0–5)	6.5 (0.5–13.1)	2.39 (0.4–3.99)	8.86 (8.21–9.25)	0.68 (0.34–0.97)	0.15 (0.13–0.21)	0.35 (0.17–0.55)
Chalpi 03	Free-flowing	Gravel (Pebble - Cobble)	1.2 (0–4)	8.1 (1.4–14.3)	2.65 (1.55–4.22)	8.43 (8.01–8.99)	0.86 (0.21–1.42)	0.21 (0.18–0.27)	0.38 (0.18–0.56)
Chalpi 04*	Free-flowing	Gravel (Pebble - Cobble)	0.9 (0–2)	7.1 (1.8–12.7)	1.73 (0.55–3.72)	8.14 (7.44–9.93)	0.67 (0.33–0.99)	0.14 (0.11–0.21)	0.04 (0.03–0.21)
Chalpi 05	Regulated	Gravel (Cobble)	1.2 (0–4)	5.8 (0.4–13.9)	2.38 (0.33–4.55)	9.61 (8.54–10.09)	0.32 (0.21–0.59)	0.10 (0.09–0.13)	0.016 (0.012–0.034)
Chalpi 06	Regulated	Gravel (Cobble)	0.9 (0–2.5)	6.5 (1.3–13.1)	1.91 (0.56–3.99)	9.22 (9.05–9.84)	0.57 (0.32–0.88)	0.12 (0.07–0.15)	0.025 (0.015–0.038)
Chalpi 07	Regulated	Gravel (Cobble)	1.4 (0–3.5)	5.8 (0.4–11.4)	1.63 (0.45–4.81)	9.35 (8.09–9.93)	0.72 (0.29–0.99)	0.14 (0.07–0.16)	0.092 (0.024–0.101)
Chalpi 08	Regulated	Gravel (Cobble)	1.1 (0–3.5)	7.3 (2.2–14.0)	1.89 (0.45–4.21)	9.16 (8.64–9.77)	0.61 (0.25–0.99)	0.13 (0.07–0.16)	0.191 (0.088–0.391)

* this site corresponds to a tributary of the Chalpi Norte stream.

indicated whether BenthoTorch underestimated (+bias) or overestimated (−bias) measurements compared with the laboratory method. The mean of differences may be systematic and significant when the line of equality is not within the limits of the interval of agreement. To test the significance of differences, a two-tailed test of means was necessary. Using the "blandr" package (Datta, 2017; R Core Team, 2017), we performed Pearson correlation analysis to complement our method agreement analysis with untransformed data of Chl-a concentrations (μg·cm^{-2}) and biovolume proportion (%) of taxonomic groups (i.e., diatoms, cyanobacteria, and green algae) of benthic algae on both substrates.

To determine the effects of physical variables on the accuracy of Chl-a measurements, we modeled variables known to be associated with benthic algal accrual and biomass in streams (Grubicic et al., 2010). We evaluated benthic algal mat thickness, water temperature, light, water velocity, water column depth, and water discharge measured at sites with different flow conditions (free and regulated flow) (Table 2). We assessed differences between the two methods for each substrate using a linear mixed-effects model, which allows correlation patterns to be explicitly modeled (Bates et al., 2015). We used the difference in paired readings as the response variable; physical variables as the fixed effects; and flow conditions (free flow vs. regulated flow), sites, and sampling dates as the random effects as follows:

$$\Delta_{(laboratory-Benthotorch)} = \beta_0(\text{flow}) + \beta_1(\text{velocity}) + \beta_2(\text{temperature}) + \beta_3(\text{depth}) + \beta_4(\text{benthic algal mat thickness}) + \beta_5(\text{light}) + (1|\text{flowcondition}) + (1|\text{site}) + (1|\text{date}) \quad (2)$$

We assumed a Gaussian distribution of differences and visually checked the goodness-of-fit and homoscedasticity of the residuals. After testing for collinearity, we compared the models with all terms and a model with the interaction of significant variables. We selected models for each substrate using p-values obtained by the likelihood test and Akaike information criteria (AIC); a lower AIC value indicates a better model fit. We built models with the "lmer" function in the lme4 package v.3.2.3 (Bates et al., 2015; R Core Team, 2017). To visualize the distribution of differences, we plotted a heat map of physical variables with significant effects using R.

We assessed BenthoTorch measurements with substrate standardization first by evaluating biomass accrual over time for diatoms, cyanobacteria, and green algae found on artificial substrates at sites with

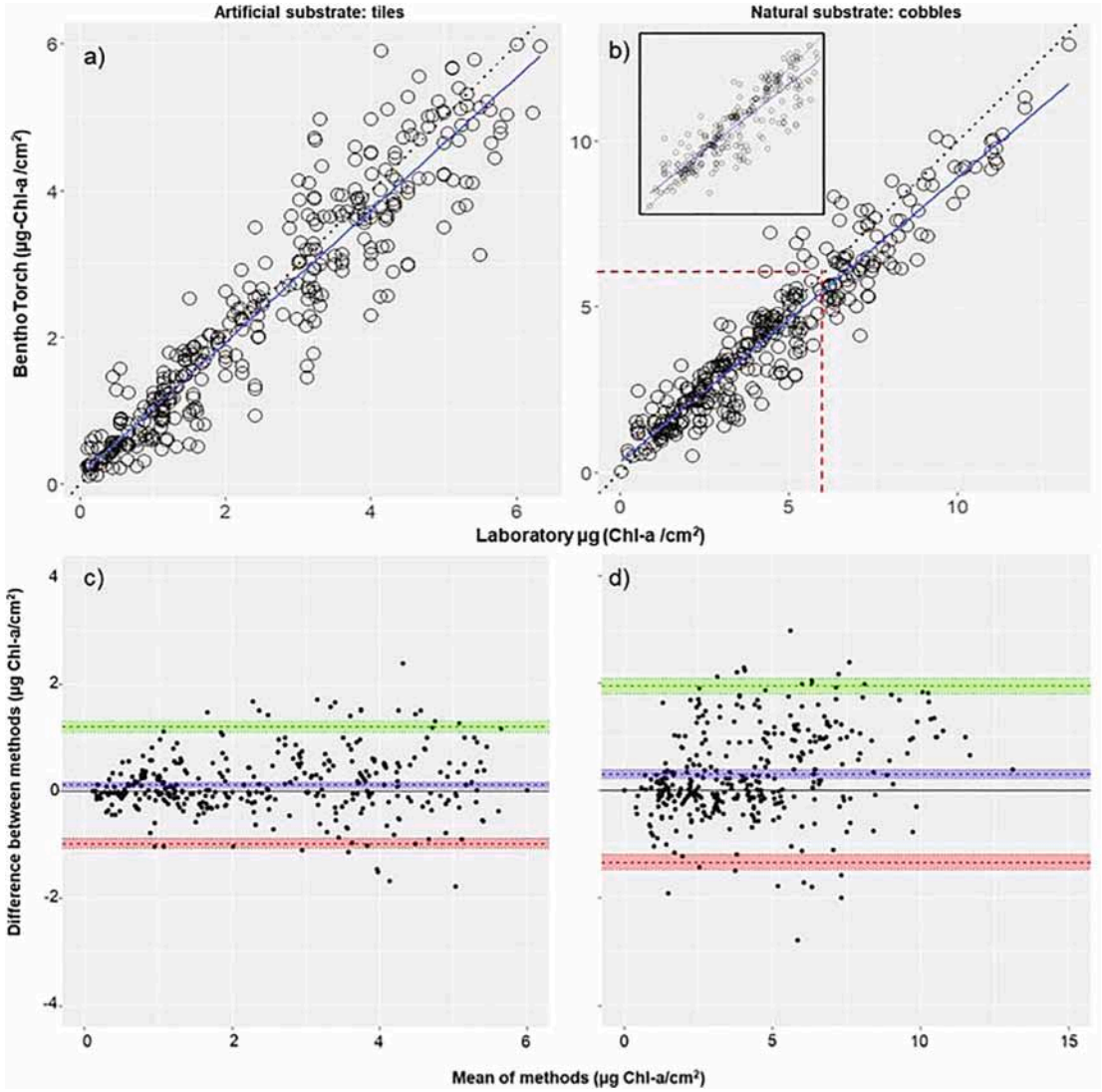

Fig. 3. Chlorophyll-a (Chl-a) concentrations on artificial and natural substrates measured with BenthoTorch (*in situ*) and laboratory (standard) methods. Pearson correlations between the paired measurements are shown; blue line indicates deviation from the line of equality (dotted line); small plot indicates the regression relationships between values on natural and artificial substrates (0–6 μg·cm^{-2}) (red dotted lines) (a, b). Bland–Altman intervals of agreement and systematic differences (c, d).

different flow conditions (free flow vs. regulated flow). Second, we used a separate dataset collected from the Chalpi Norte stream in 2018 to apply rarefaction to BenthoTorch readings on artificial and natural substrates. We calculated the coefficient of variation for five BenthoTorch readings obtained on five artificial and five natural substrates from five different sites of an experimental stream reach (n = 25). To calculate the coefficient of variation according to the number of samples, we randomized the data (n = 25) and then averaged the obtained values and calculated standard error. Finally, we plotted the coefficient of variation against the number of samples for artificial and natural substrates (R Core Team, 2017).

3. Results

3.1. Agreement between the methods

3.1.1. Chl-a concentrations

Chl-a concentration ranged from 0 to 6 μg·cm^{-2} (n = 359) on artificial substrates (Fig. 3a) and from 0 to 15 μg·cm^{-2} (n = 355) on natural substrates (Fig. 3b). Among all sampling events and under both free-flowing and regulated flow conditions, the agreement between BenthoTorch and the standard method was significant on both substrates (artificial: $R^2 = 0.84$, p = 0.0074; natural: $R^2 = 0.85$, p = 0.0032). Systematic difference analysis showed that compared with the standard method, BenthoTorch underestimated laboratory Chl-a concentration by 11.4% (>3 μg·cm^{-2}) on artificial substrates (Fig. 3c) as well as Chl-a concentration by 31% (>5 μg·cm^{-2}) on natural substrates (Fig. 3d).

3.1.2. Biovolume proportion of benthic algal taxonomic groups

On artificial substrates, BenthoTorch readings were significantly and positively correlated with microscopic biovolume proportions (n = 359) of diatoms ($R^2 = 0.76$; p = 0.018), cyanobacteria ($R^2 = 0.65$, p = 0.004), and green algae ($R^2 = 0.89$, p = 0.007, Fig. 4a, b, c). When the proportion of diatoms exceeded 40%, BenthoTorch underestimated it by ~ 2%. Similarly, when the proportion of cyanobacteria exceeded 40%, BenthoTorch underestimated it by ~ 2.8% (Fig. 4d, e). Finally, when the proportion of green algae exceeded 50%, BenthoTorch underestimated it by 1.5% (Fig. 4f). We found that 95.4%, 95.3%, and 95.1% of the diatom, cyanobacterial, and green algal data, respectively, fell within the 95% confidence limit (Fig. 4). These results indicate that the performance of BenthoTorch was consistent with that of the standard methods on artificial substrates.

Contrary to that on artificial substrates, the correlation between BenthoTorch and the standard method was not significant for diatoms (n = 144) ($R^2 = 0.49$, p = 0.132) and marginally significant for cyanobacteria ($R^2 = 0.39$, p = 0.07) and green algae ($R^2 = 0.65$, p = 0.05) on natural substrates (Fig. 5a, b, c). We found that above a threshold of 25%, BenthoTorch underestimated the proportions of cyanobacteria and green algae relative to the standard method on natural substrates (Fig. 5e, f). Systematic difference analysis showed that 95.8%, 93.7%, and 91.6% of the green algal, diatom, and cyanobacterial data, respectively, fell within the 95% confidence interval. These results indicate that the performance of BenthoTorch was generally consistent with that of the standard method for taxonomic identification on natural substrates but better on artificial substrates.

3.2. Physical variables and the differences in methods

Mat thickness and light intensity were the only significant (p < 0.05) predictors of the bias in Chl-a concentration measurements on artificial and natural substrates (Table 3). Linear mixed model comparisons between the two substrates were significant [χ^2 (1) = 61.25, p < 0.0001], and only mat thickness explained the bias on artificial substrates, while both mat thickness and light intensity explained the bias on natural

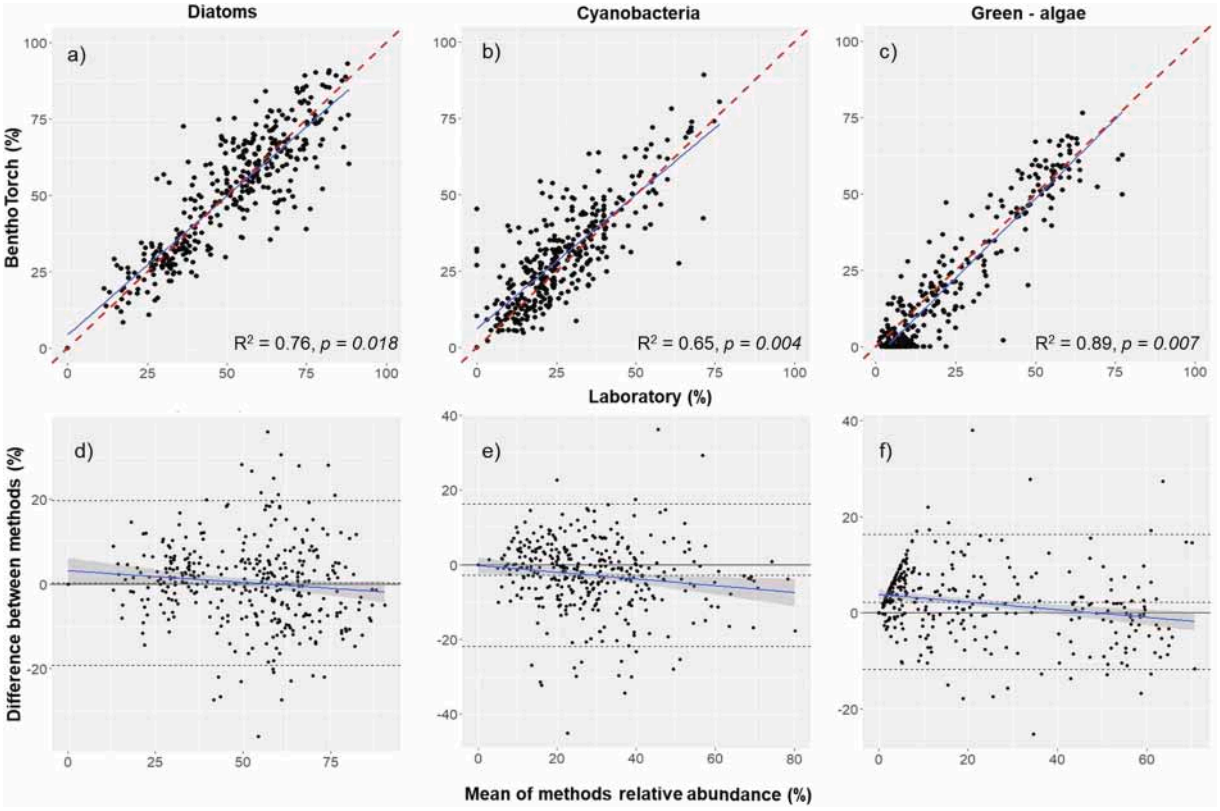

Fig. 4. Biovolume proportions (%) of diatoms (a, d), cyanobacteria (b, e), and green algae (c, f) measured with BenthoTorch (*in situ*) and laboratory (standard) methods on artificial substrates (n = 359). Top panels show regression lines (blue) with respect to the equality line (red). Bottom panels show the bias between method agreement (black) with 95% confidence intervals (dotted lines) and regression lines (blue).

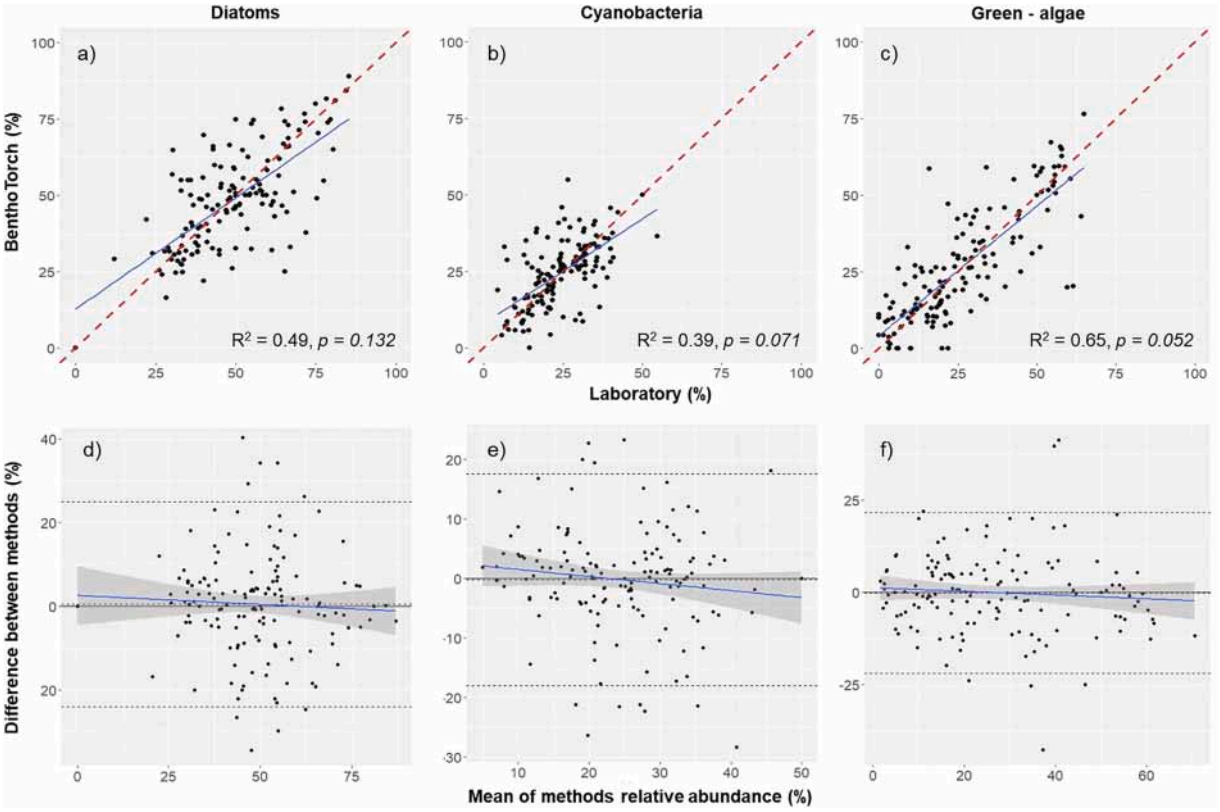

Fig. 5. Biovolume proportions (%) of diatoms (a, d), cyanobacteria (b, e), and green algae (c, f) measured with BenthoTorch (*in situ*) and laboratory (standard) methods on natural substrates (n = 144). Top panels show regression lines (blue) with respect to the equality line (red). Bottom panels show the bias between method agreement (black) with 95% confidence intervals (dotted lines) and regression lines (blue).

Table 3
Linear mixed model comparisons for the difference between chlorophyll-a concentrations (difference = laboratory–BenthoTorch) and the effects of light intensity and benthic algal mat thickness on artificial and natural substrates.

	Artificial substrates n = 359				Natural substrates n = 355			
Fixed effects	**Estimate**	**Std. Error**	**t value**	***p***	**Estimate**	**Std. Error**	**t value**	***p***
(Intercept)	-0.07	0.13	-0.55		0.92	0.20	4.65	
Light	0.04	0.05	0.83		-0.25	0.08	-2.99	0.001
Thickness	-0.09	0.04	-2.06	0.05	-0.31	0.03	-11.18	0.0001
Light*Thickness	0.01	0.02	0.62		0.09	0.01	8.18	0.0001
Random effects	**Variance**	**Std. Dev.**			**Variance**	**Std. Dev.**		
Date	0.000	0.000			0.000	0.000		
Site	0.054	0.233			0.000	0.000		
Flow condition	0.000	0.000			0.000	0.000		
Residual	0.312	0.559			0.619	0.780		

substrates. Moreover, there was no consistent bias between the measurement methods on artificial substrates for mat thicknesses between 1 and 5 mm and light intensity between 500 and 1,500 μmol·m^{-2} s^{-1}. On the contrary, on natural substrates, bias decreased when the mat thickness increased from 2 to 5 mm within this range of light intensity (Fig. 6). BenthoTorch underestimated (+bias) Chl-a concentrations on artificial substrates when the light intensity decreased below 460 μmol·m^{-2} s^{-1} but overestimated Chl-a concentrations on natural substrates when the light intensity increased above 1,400 μmol·m^{-2} s^{-1} at the benthic algal mat thicknesses of 3–5 mm (Fig. 6). Temperatures in the studied reaches were relatively low; therefore, temperature might be an important factor in warmer streams or streams that experience great temperature variations.

Table 3. Linear mixed model comparisons for the difference between chlorophyll-a concentrations (difference = laboratory − BenthoTorch) and the effects of light intensity and benthic algal mat thickness on artificial and natural substrates.

3.3. Standardization of substrates and BenthoTorch measurements

BenthoTorch measurements on artificial substrates provided information on biomass accrual of diatoms, cyanobacteria, and green algae

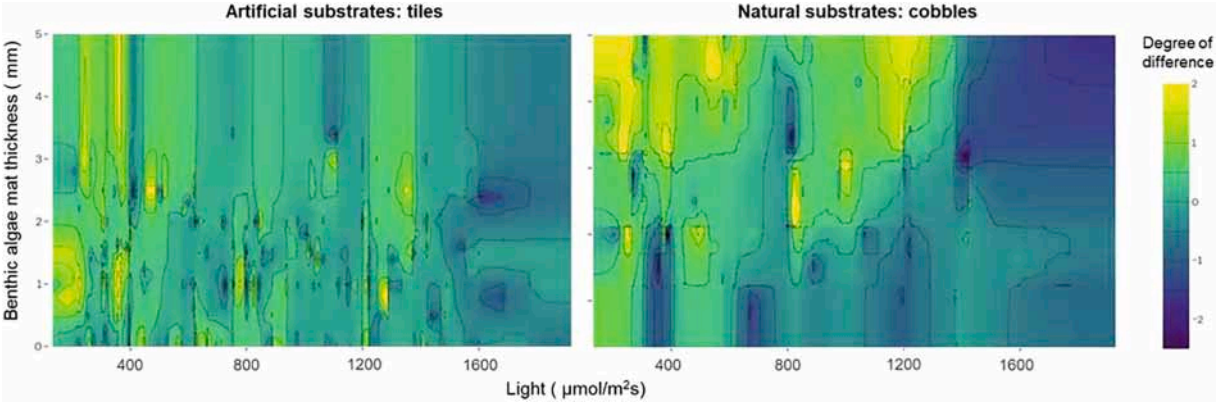

Fig. 6. Heat map of benthic algal biomass (µg Chl-a cm^{-2}) indicated by contour lines of the degree of difference (±2) (left, colored scale) in mat thickness and light intensity on artificial (n = 359) and natural (n = 355) substrates in the Chalpi Norte stream between July and August 2017.

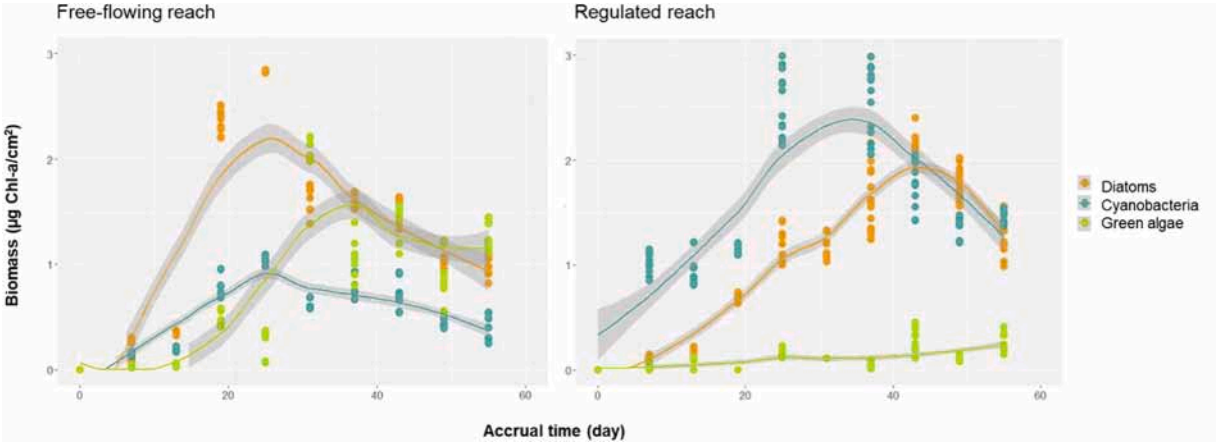

Fig. 7. Biomass of benthic algae and primary producers (i.e., diatoms, cyanobacteria, and green algae) measured weekly during 55 days as accrual on artificial substrates (n = 20) in free-flowing (a) and regulated (b) reaches of the Chalpi Norte stream, Ecuador.

under both free-flowing and regulated flow conditions (Fig. 7). Under free-flowing conditions, the biomasses of diatoms and cyanobacteria peaked simultaneously, but showed different values, on day 24. The biomasses of green algae peaked on day 36, albeit with lower biomass than that of diatoms but higher biomass than that of cyanobacteria. Cyanobacteria presented the lowest biomass among the three groups (Fig. 7a). Under regulated flow conditions, the biomasses of cyanobacteria peaked on day 36, with higher biomass than that of diatoms, which peaked on day 42 (Fig. 7b). The biomass of green algae was lower and presented no obvious peak of accrual (Fig. 7b).

Rarefaction analysis showed that the coefficient of variation for BenthoTorch measurements on artificial and natural substrates decreased when the number of samples increased (Fig. 8). The range of variation in BenthoTorch measurements on artificial substrates was lower than that on natural substrates. With the same number of samples (n = 13), we obtained a lower coefficient of variation for artificial substrates (5%) than that for natural substrates (21.3%). Of note, 1–2 artificial substrate samples showed the same coefficient of variation as the 13 natural substrate samples (Fig. 8). To obtain a coefficient of variation of 5%, we were required to sample 90 cobbles from natural substrates (Fig. 8).

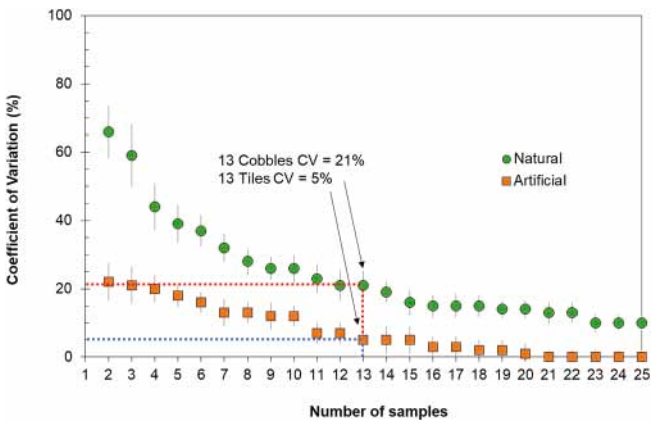

Fig. 8. Rarefaction analysis of the coefficient of variation of BenthoTorch measurements for an equal number of samples (n = 13) from artificial (blue dotted line; tiles) and natural (red dotted line; cobbles) substrates.

4. Discussion

The main goal of this study was to assess the performance of BenthoTorch compared with that of standard laboratory methods using artificial and natural substrates. We assessed: (1) the Chl-a concentration and biovolume proportion of algal groups; (2) the effects of physical variables on BenthoTorch and laboratory measurements; and (3) the potential use of artificial substrates to standardize BenthoTorch measurements.

Previous comparisons between BenthoTorch and laboratory methods were performed exclusively on natural substrates, with contradictory results of Chl-a concentration and biovolume proportion of algal groups

(Table 1). In our study, measurements obtained on artificial substrates under different flow conditions demonstrated that BenthoTorch provides comparable results to standard laboratory methods. Overall, there was a higher agreement between the two methods in the measurement of Chl-a concentration on artificial substrates than on natural substrates. Nonetheless, our study did not represent the full range of biodiversity in rivers across the planet.

On both substrates, BenthoTorch underestimated Chl-a concentration compared with the standard method. Our findings are consistent with those reported by Harris and Graham (2015) and Kaylor et al. (2018) that BenthoTorch underestimated Chl-a measurements when concentrations exceeded 4 $\mu g \cdot cm^{-2}$, although the threshold in our study was 3 $\mu g \cdot cm^{-2}$. The high agreement between the methods in the measurement of Chl-a concentration on artificial substrates noted in this study is consistent with observations reported in previous studies (Kaylor et al., 2018, Echenique-Subiabre et al., 2016, Harris and Graham, 2015), which found that BenthoTorch worked the best when the algal film were thin and Chl-a concentrations were low. However, this pattern is starkly contradictory to observations on natural substrates (Harris and Graham, 2015; Echenique-Subiabre et al., 2016).

Biovolume proportions of algal groups measured by the two methods were more comparable on artificial substrates than on natural substrates. This result could be explained by the physiological state of photopigments of diatoms in the upper layers of biofilms formed on artificial substrates as opposed to the distinct layers formed on natural substrates (Escoffier et al., 2015). Our results on artificial substrates are consistent with the findings reported by Harris and Graham (2015) and Echenique-Subiabre et al. (2016) on natural substrates at the early stages algal mat formation, which likely exhibited a morphology similar to the algal mats formed on artificial substrates in our study. The biomass of cyanobacteria was overestimated by BenthoTorch (~5%) when their proportion exceeded 40% of all communities on artificial substrates, corroborating the previously reported findings on natural substrates (Harris and Graham, 2015). However, this result contradicts the results reported by Echenique-Subiabre et al. (2016) and Kahlert and McKie (2014), who found that BenthoTorch underestimated the proportion of cyanobacteria on natural substrates. Echenique-Subiabre et al. (2016) attributed this underestimation of cyanobacteria to the greater abundance of phycoerythrin-containing cyanobacteria, which fluoresce at a different wavelength from phycoerythrin-lacking cyanobacteria. Further assessment of phycoerythrin-containing cyanobacteria on artificial substrates would help explain these discrepancies. However, despite the systematic overestimation (~2.8%) of cyanobacteria on artificial substrates, the results showed strong agreement between the two methods. Our results regarding green algae on artificial substrates are consistent with the reports in eutrophic and oligotrophic streams, where BenthoTorch systematically underestimated the proportion of green algae (Echenique-Subiabre et al., 2016; Harris and Graham, 2015; Kahlert and McKie, 2014).

Modeling of differences between the two methods revealed a weak association with mat thickness on artificial substrates but a strong association on natural substrates. According to Kaylor et al. (2018) and Echenique-Subiabre et al. (2016), light intensity and benthic algal mat thickness are the important factors influencing BenthoTorch performance. Kaylor et al. (2018) found that the light conditions before BenthoTorch measurements affect Chl-a estimates and, to reduce this error, they moved rocks into the shade. However, this increases the sampling time and logistic complexity. Evaluating the association between light conditions and Chl-a concentration using laboratory methods may reduce logistic constraints of using BenthoTorch.

We demonstrated that artificial substrates are a resourceful complement for standardizing BenthoTorch measurements, allowing the study of temporal dynamics of benthic algae while reducing the variations in Chl-a measurements even with a few samples. Owing to their rapid change rates, capturing such patterns of algal accrual and loss over relatively large areas is rather difficult using standard methods. However, although previous studies have shown a reasonably high degree of similarity between artificial and natural substrates (Cattaneo and Amireault, 1992; Tuchman and Stevenson, 1980), we observed less overall biomass the former. Therefore, to accurately reflect natural conditions, periodic sampling of natural substrates using standard methods is warranted to calibrate readings on artificial substrates.

Inefficiency in registering the incident and reflected signals from irregular substrates explains part of the bias in BenthoTorch measurements, although this has been addressed in recent BenthoTorch versions (Escoffier et al., 2015). Artificial substrates provided data on benthic algal colonization without considering potential detachment during high flows, which might have limited Chl-a yield on artificial substrates compared with that on natural substrates (Graba et al., 2014). Indeed, uniformity of the surface of artificial substrates reduces benthic algal self-shadow, favoring pigment fluorescence (Carpentier et al., 2013). In contrast, irregularities on natural substrates increase biomass accumulation in crevasses, which may affect the fluorescence of algae in the underlying layers (Hauer and Lamberti, 2011). Overcoming variability in BenthoTorch measurements seems plausible using artificial substrates and calibration with standard laboratory methods. Finally, substrate standardization enhanced BenthoTorch measurements, providing the opportunity for extensive sampling with in-field replication while reducing the variability in readings.

5. Comments and recommendations

Benthic algal and microbial communities are the key basal components of the aquatic food web, because they serve as the primary source of energy for higher trophic levels (Besemer et al., 2013; Battin et al., 2016). Prompt surveys of the presence, abundance, and population dynamics of certain algal groups is a strategy worth incorporating into aquatic and marine ecosystem monitoring. Factors triggering aquatic algal proliferation have limited the ability of the currently available standard methods to provide succinct responses to changing environmental conditions (Watson et al., 2008; Vörösmarty et al., 2010; Franks, 2018). In the present study, we highlighted the advantage of using BenthoTorch and artificial substrates relative to the standard methods for assessing the change rates of Chl-a concentration and biovolume proportion of benthic algal groups. However, this approach is particularly useful when coupled with readings on natural substrates to assess the simultaneous presence of very thick algal mats, which may be composed of numerous cyanobacteria. The ability of BenthoTorch to produce instantaneous results facilitates replicate collection, which can reduce sampling error and increase sampling area on the same day. Incorporating artificial substrates reduces the variability in BenthoTorch measurements, although deployment is laborious and substantially lower benthic algal biomass is observed on these substrates relative to that on natural substrates, indicating that they are not a perfect proxy for natural substrates. BenthoTorch, like most methods, should always be accompanied by routine comparison with standard methods. When BenthoTorch was compared with different standard laboratory methods reported in the literature, neither provided unequivocally correct measurements. For comparison, analysis of agreement may provide a better understanding of method performance than correlation analysis, specifically in the interest of deciphering conditions responsible for differences between these methods. Future comparisons of BenthoTorch with laboratory methods should follow the manufacturers' guidance to avoid intrinsic variations among standard methods. Incorporating fluoroprobes, such as BenthoTorch, into standard methods for monitoring benthic algal groups would amplify the opportunities for promptly responding to and developing an early warning system for algal proliferation in municipal water supplies.

CRediT authorship contribution statement

D. Rosero-López: Methodology, Data curation, Formal analysis,

Writing - original draft. **M.T. Walter:** Visualization, Supervision. **A.S. Flecker:** Writing - review & editing. **D.F. Ontaneda:** Methodology, Data curation. **O. Dangles:** Conceptualization, Methodology, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.107185.

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