Towards a simple global-standard bioassay for a key ecosystem process: organic-matter decomposition using cotton strips

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ABSTRACT

Cotton-strip bioassays are increasingly used to assess ecosystem integrity because they provide a standardized measure of organic-matter decomposition – a fundamental ecosystem process. However, several different cotton-strip assays are routinely used, complicating the interpretation of results across studies, and hindering broader synthesis. Here, we compare the decay rates and assemblages of bacteria and fungi colonizing the three most commonly used cotton materials: Artist's canvas, Calico cloth, and Empa fabric. Cotton strips from each material type were incubated in 10 streams that span a wide range of physicochemical properties across five ecoregions. Additionally, to evaluate responses to environmental stress without potentially confounding biogeographical effects, we deployed identical bioassays in five streams across an acidification gradient within a single ecoregion.

Across all streams decomposition rates (as tensile strength loss [TSL]) differed among the three cotton materials; Calico cloth decomposed fastest (time to 50% TSL [T_{50}] = 16.7 d), followed by the Empa fabric (T_{50} = 18.3 d) and then Artist's canvas (T_{50} = 21.4 d). Despite these differences, rates of TSL of the three cotton materials responded consistently to variation in environmental conditions; TSL of each fabric increased with stream temperature, dissolved-nutrient concentrations and acid-neutralizing capacity, although Artist's canvas and Calico cloth were more sensitive than Empa fabric. Microbial communities were similar among the materials, and values of community structure (e.g., phylotype richness and diversity) were comparable to those reported for decaying leaves in streams from the same region, the major natural basal carbon resource in forested-stream ecosystems. We present linear calibrations among pairs of assays so that past and future studies can be expressed in a "common currency" (e.g., Artist's-fabric equivalents) 'past and future studies' repeated two times in the sentence. Lastly, given its relatively low within-site variability, and the large number of streams where it has been used (> 700 across the globe), we recommend Artist's fabric for future work. These results show that cotton provides an effective and realistic standardized substrate for studying heterotrophic microbial assemblages, and acts as a reasonable proxy for more chemically complex forms of detritus. These findings add to growing evidence that cotton-strip bioassays are simple, effective and easily standardized indicators of heterotrophic microbial activity and the ecosystem processes that result.

1. Introduction

Organic-matter decomposition is a fundamental ecosystem process that contributes to the global carbon cycle, governs concentrations of atmospheric CO_2 (Battin et al., 2008, 2009), and influences local food-web dynamics (e.g., Moore et al., 2004). In streams and rivers this process plays a central role in ecosystem functioning (see review by Tank et al., 2010) and, because it responds sensitively to critical anthropogenic stressors, decomposition has been proposed as an indicator of ecosystem condition that complements commonly used structural

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indicators (e.g., invertebrate community composition) (Boulton and Quinn, 2000; Gessner and Chauvet, 2002). Organic-matter decomposition is most commonly assessed using litter-bag assays, an approach that typically involves incubating a known mass of locally collected leaves in mesh bags in the field and retrieving them to determine leaf-mass loss over time (Boulton and Boon, 1991). Leaf-litter breakdown has been extensively studied during the past two decades in a variety of aquatic ecosystems to evaluate the impacts of anthropogenic stressors including organic pollution, acidification, hydromorphological alterations and land-use change (Young et al., 2008; Tank et al., 2010; Elosegi and Sabater, 2012; Woodward et al., 2012; Chauvet et al., 2016; Colas et al., 2017; Ferreira and Guérold, 2017). While understanding of the factors that influence this process has grown considerably, key knowledge gaps persist due in part to limitations of litter-bag assays.

Despite their widespread use, litter-bag assays have shortcomings that undermine reliable comparisons among studies (Boulton and Boon, 1991) and hinder applications at large temporal and spatial scales (Jackson et al., 2016; Tiegs et al., 2019). Notable among these is the difficulty in consistently obtaining leaf litter of uniform quality. Litter characteristics that influence decomposition, such as nutrient and lignin content, are highly variable both among and within tree species (Webster and Benfield, 1986; Gessner and Chauvet, 2002; Hladyz et al., 2008; Lecerf and Chauvet, 2008), and even among leaves of individual trees of the same species (e.g., Sariyildiz and Anderson, 2003). These and other sources of variability in litter quality (e.g., interannual variation) mean that decomposition rates among studies are contingent on not only the environmental conditions in which the litter was incubated, but also the particular organic matter used. Consequently, basic understanding of how the inherent capacity of streams to process organic matter – i.e., their decomposition potential (sensu Imberger et al., 2010) – varies through time and space, and in response to anthropogenic stressors, is impeded by a widespread lack of standardization and comparability.

Given the numerous advantages that they offer over litter-bag approaches, cotton-strip assays are a logical choice for use as a universal-standard bioassay because they enable researchers to evaluate the capacity of ecosystems to decompose organic matter. Moreover, given their ease of assembly, deployment, and portability, cotton-strip assays have potential to enable research at large spatial and temporal scales, addressing the pressing need for global monitoring and assessment (Jackson et al., 2016; Tiegs et al., 2019). Consisting of ≈95% cellulose, cotton is an ecologically relevant compound given that this key carbohydrate is the main constituent of plant litter, the most abundant organic polymer on Earth, and a major basal resource in most of the Earth's food webs (Egglishaw, 1972). Cotton contains very low concentrations of nutrients such as N and P, which are highly variable among and within the litter of other plant species, and are main intrinsic determinants of decomposition rates (e.g., Latter and Howson, 1977; Claret et al., 2001; Clapcott et al., 2010; Hladyz et al., 2008; Tiegs et al., 2007, 2013). Additionally, cotton fabric is less prone to fragmentation than leaves (Egglishaw, 1972), thereby reducing the variability due to hydraulic conditions. Cellulose is colonized by a wide range of microorganisms, including leaf-colonizing fungi and bacteria (Singh, 1982; Harrison et al., 1988). They provide practical and logistical advantages over litter-bag assays, including often-shorter incubation times in the field, relative ease of use, and low cost of the measurement (Tiegs et al., 2013). Importantly, cotton-strip decomposition, as with leaf-litter decomposition, is sensitive to a range of anthropogenic stressors including: acidification (e.g., Hildrew et al., 1984; Jenkins et al., 2013), temperature (Griffiths and Tiegs, 2016; Tiegs et al. 2019), metal contamination (e.g., Chew et al., 2001; Costello and Burton, 2014; Gardham et al., 2015), and nutrient loading (e.g., Boulton and Quinn, 2000; Piggott et al., 2015). Cotton-strip assays therefore offer promising opportunities for standardized measurements of microbial activity and community structure in streams, like those already used in terrestrial ecosystems (e.g., Williamson, 1994; Pankhurst et al., 1995).

The first cotton-strip assays were conducted in terrestrial ecosystems using a fabric produced by the Shirley Company (Manchester, UK), which became a benchmark industry standard in soil studies (Latter and Howson, 1977; Latter and Walton, 1988; Boulton and Quinn, 2000). The 'Shirley Soil Burial Test Fabric' was subsequently used in aquatic ecosystems where it was found to be sensitive to variation in environmental conditions (Hildrew et al., 1984; Boulton and Quinn, 2000; Claret et al., 2001; Tiegs et al., 2007). Unfortunately, the manufacture of Shirley material was discontinued in 2002, creating need for a new standard (Fritz et al., 2011). Several materials have since been used including 'Calico' cloth (Imberger et al., 2010), 'Artist's canvas' (Slocum et al., 2009), also known as 'Artist's fabric' (Tiegs et al., 2013) and 'Empa material' (Clapcott et al., 2010). Importantly, this lack of a single standard complicates the comparison of results across studies because the physical properties of the three cotton materials differ markedly (e.g., length and fineness of cotton fiber, strength, and elastic properties). Intercalibrations are therefore needed to compare studies involving different fabric types, past and future.

Here, we compared the performance of these three materials in streams along gradients of major environmental factors that influence organic-matter decomposition rates (e.g., ecoregions, acidification, temperature, nutrient concentrations). With this approach we derived intercalibrations that allow the conversion of decomposition rates into a 'common currency' for cellulose-decomposition potential among studies using different cotton types. We also characterized bacterial and fungal communities associated with each cotton material and evaluate attributes of community structure in the context of those published for leaf litter. Our aim is to provide a standardized methodology for quantifying cellulose-decomposition potential and heterotrophic communities in streams and other habitats across the globe, so that organic-matter decomposition and the factors that influence it can be better understood.

2. Materials and methods

2.1. Study site description

Cotton strips were deployed in headwater streams from five ecoregions that differ in their geology and climate (Olson et al., 2001; Omernik and Griffith, 2014): northern temperate deciduous forests of the United States (US); English lowland deciduous broadleaf woodlands (UK); Vosges Mountains in northeast of France (FRNE); western-European broadleaf forests of Switzerland (CH); and Mediterranean mountains of Pyrenees in southwestern France (FRSW). These five ecoregions encompass broad latitudinal, longitudinal and altitudinal gradients, with a wide range of environmental and climatic conditions including oceanic, humid continental and subalpine climates. A pair of streams was selected in each ecoregion. In FRNE, five additional streams were included to span an acidification gradient within a single ecoregion. Water samples were taken on the first and last day of the cotton-strip-incubation period and assessed for concentrations of ammonium, nitrate, nitrite, and phosphorus, conductivity and pH using established protocols (e.g., NF EN ISO 13395). The study streams in the five ecoregions represented a range of nitrate concentrations from 0.11 to 1.85 mg NO_3^--N/L, phosphate concentrations from 1.4 to 28.3 µg/L and ammonium concentrations from 1 to 111 µg NH_4^+-N/L. The five sites in FRNE represented a gradient of pH ranging from 4.9 to 6.9, from −910.1 to 134.4 µeq/L for acid-neutralizing capacity values (ANC) and from 52.3 to 343.3 µg/L for aluminum concentrations. Detailed information on characteristics of individual streams across the five ecoregions are available in Table 1.

Table 1
Ranges of values of main physicochemical characteristics of water among streams across the five ecoregions examined: western European broadleaf forests in Switzerland (CH); the Mediterranean mountains of Pyrenees in southwestern of France (FRSW); the Vosges mountains in northeastern of France (FRNE); the English lowlands deciduous forests (UK); the temperate deciduous forests of the northern United States (US). Concentrations of Al and acid neutralizing capacity (ANC) were determined following the methods detailed in Ferreira and Guérold (2017). Nutrient gradient (n = 10). ANC, pH and Al were determined in 5 streams in FRNE that span an acidification gradient.

	FRNE		CH		UK		FRSW		US	
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Degree days	135.2	172.0	135.2	186.8	n.a.	n.a	205.1	231.1	52.9	60.4
NO_3^--N (mg/L)	0.11	0.73	0.93	1.66	0.26	1.34	0.74	1.85	n.a	n.a
NO_2^--N (µg/L)	< 10	< 10	2.0	45.0	0.60	2.00	< 10	37.0	n.a	n.a
NH_4^+-N (µg/L)	< 10	< 10	6.0	10.0	1.00	7.00	45.0	111.0	n.a	n.a
PO_4^{3-}-P (µg/L)	2.0	5.0	14.0	17.0	1.40	1.60	14.0	28.3	n.a	n.a

Acidification gradient (n = 5)

	TH	MR	BR	RR	CE
ANC (µEq/L)	134.4	38.9	10.1	9.4	−10.0
pH	6.9	6.3	5.6	5.7	4.9
Al (µg/L)	52.3	174.3	207.1	176.0	343.3

2.2. Cotton-strip bioassays

Cotton strips were prepared from bolts of 'Calico' cloth (Lincraft Pty. Ltd., Miranda, New South Wales, Australia), 'Artist's' canvas (Fredrix-brand unprimed 12-oz. heavyweight cotton fabric, Style #548, Lawrenceville, GA, USA), and 'Empa' fabric (Swissatest Product no. 222; Empa, St. Gallen, Switzerland). Detailed information on each substrate is available in Table 2. Cotton strips were prepared and deployed according to methods previously reported in the literature (e.g., Tiegs et al., 2013, 2019).

In each stream, one 'block' with one strip of each material type was located in six riffle-type habitats separated by approximately 7 times the bankfull-channel width. Each block consisted of cotton strips attached to a stake hammered into the stream substrate. Strips were incubated in streams for 20–27 days beginning in November 2014, a duration predicted to yield an approximate average of 50% tensile-strength loss, an amount of decay that is believed to maximize sensitivity of the assay (Tiegs et al., 2013). In the five streams that spanned the acidification gradient, one additional strip of each material type located in three riffle-type habitats was incubated for 6 weeks to evaluate fungal and bacterial abundance, and to compare microbial assemblages that colonized the three types of cotton materials. Additionally, a time-series experiment was conducted in the UK and French reference streams (i.e. FRNE and FRSW) to compare the time to yield 50% tensile loss of each material. To this end, six strips for each cotton type were anchored to three additional stakes and removed weekly for 6 weeks. Temperature was recorded hourly in each stream for the duration of the experiments with a logger, except for streams in southeast UK. After incubation in the field, cotton strips were removed and placed individually into labeled plastic bags for transport to the laboratory.

2.3. Tensile loss determination

In contrast to litter-bag assays that quantify decomposition rates by measuring the loss of mass of organic matter through time, cotton-strip assays quantify the loss of tensile strength, a process that equates to the catabolism of cellulose. In the laboratory, following the protocol of Tiegs et al. (2013), each strip was placed in a shallow tray containing 70% ethanol and cleaned gently for 30 s with a small paint brush to remove adhering sediment and debris. The strips were then transferred to small aluminum pans, dried at 40 °C for several days, and stored in desiccators. The strips were shipped to the Aquatic Ecology Lab at Oakland University for tensile-strength determination.

The tensile strength of each strip was measured on a tensiometer (Mark-10 brand, Model #MG100, Copiague, NY, USA) mounted to a motorized test stand, and pulled at a rate of 2 cm/min. The initial tensile strength of the strips was determined using a set of control strips, i.e., for each ecoregion, seven additional strips of each cotton material that were not incubated in the streams were wetted in tap water, dried in an oven, and stored in a desiccator before being processed identically to the treatment strips (Table 2). Tensile-strength loss (TSL) was expressed as percent of the initial tensile-strength lost per day of incubation according Equation (1) (after Tiegs et al., 2013):

$$TSL = \left[1 - \left(\frac{Tensile\ Strength_{treatment\ strips}}{Tensile\ Strength_{reference\ strips}}\right)\right] \times 100/Incubation\ Time \quad (1)$$

where Tensile $Strength_{treatment\ strips}$ is the maximum tensile strength

Table 2
General characteristics of the cotton materials before they were incubated in the field.

	Calico	Empa	Artist's
Weight (g/m^2)	115	205	407
Average (± SD) initial tensile strength (lbs)	42.7 ± 6.7	44.6 ± 9.0	57.7 ± 7.3
Cotton strip dimensions (mm)	30 × 60	24 × 50	25 × 80
References	Imberger et al., (2010)	Clapcott et al., 2010	Tiegs et al., (2013)

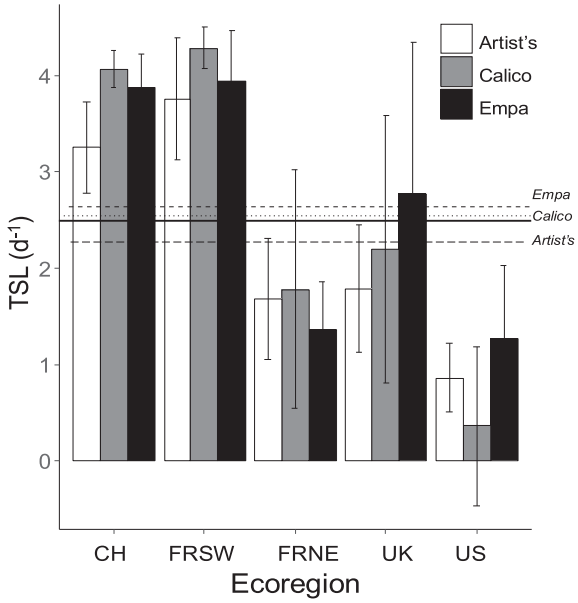

Fig.1. Mean TSL per day (± SD) across fabric types and ecoregions. CH: western European broadleaf forests in Switzerland; FRSW: the Mediterranean mountains of Pyrenees in southwestern France; FRNE: the Vosges mountain in northeastern France; UK: English lowland beech forests; US: temperate deciduous forests of the northern United States. Continuous black line indicates the mean percent tensile strength loss per day for all cotton fabrics; the dashed line indicates the mean value for each of the three fabric types across all streams.

recorded for each strip incubated in the field, Tensile $Strength_{reference\ strips}$ is the mean tensile strength of control strips that were not incubated in the field, and incubation time is the number of days the strips were incubated in the field. To account for differences in decomposition rate due to temperature differences across the acidification gradient, tensile strength was also expressed as a percentage of the initial TSL per degree-day by substituting degree-days for time in Eq. (1). Degree days were estimated by summing the mean daily temperatures at each stream, based on hourly readings greater than 0 ^{0}C. Additionally, the time to yield 50% TSL (T50) was estimated by fitting the tensile loss to a function of incubation time according to a linear model using the time series experiments performed in three reference streams. The linear model was used because it fits the data better than the exponential model according to AICc.

2.4. Microbial abundance and community assemblages

Before DNA extraction, strips were placed in a shallow tray filled with distilled water (not ethanol as with the other strips) and cleaned gently with a small paint brush to remove any adhering sediment. Pieces of cotton (1.5 × 1.5 cm) were crushed in Eppendorf tubes containing 750 µl of the PowerBead Tube solution from the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Microbial suspensions were transferred in the PowerBead Tubes and total DNA was extracted using the PowerSoil DNA Isolation Kit according to the manufacturer's instructions.

The abundance of fungi and bacteria was estimated by qPCR using the primer sets Fung5F/FF390R (Lueders et al., 2003), 968F/1401R (Felske et al., 1998), which target fungal 18S rRNA genes and the bacterial 16S rRNA genes, respectively. The SYBR green qPCR assays were performed as previously described (Thion et al., 2012; Cébron et al., 2015) using a CFX96 Real-Time PCR detection system (Bio-Rad). Data were expressed as 18S to 16S rDNA copy number ratio. PCR primers ITS3GC and ITS4 were used for PCR amplification of the ITS2 region of fungal ribosomal DNA (Nikolcheva and Bärlocher, 2005). PCR primers 341F-GC2/907R were used for partial amplification of the bacterial 16S rRNA genes (Muyzer et al., 1998, 1993).

The amplification products were separated with Denaturing Gel Electrophoresis (DGGE) on the DCODE Mutation Detection System (Bio-Rad, Hercules, CA). Electrophoresis was performed on 8% poly-acrylamide gels with a denaturing gradient from 30 to 70% for the fungal PCR products and from 40% to 60% for the bacterial PCR products [100% denaturant corresponds to 40% (v/v) formamide and 7 M urea]. DGGE was run 16 h at 55 V and 56 °C for fungal PCR products and 16 h at 100 V and 60 °C for bacterial PCR products. The gels were stained with SYBR Green I and imaged with a STARION FLA-9000 scanner (Fujifilm Life Sciences FSVT, Courbevoie, France) before being analyzed using GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium). DGGE profiles were aligned using control samples as migration markers. The molecular richness was calculated as the total number of bands/phylotypes (PR = phylotype richness) for each DGGE profile. Microbial diversity was calculated using the Shannon-Weaver index (H') and relative abundance of phylotypes (based on relative band intensity).

2.5. Statistical analyses

Nested ANOVA was used to evaluate differences in mean percent TSL per day among ecoregions and cotton materials (as a fixed effect) using streams nested within ecoregions as a random effect. A linear mixed model was used to test the variation of percent TSL per degree-day along the acidification gradient using the fabric type and the acid neutralizing capacity, or the aluminum concentrations, as fixed effects and the streams as random effect. Similarly, the relationships between percent TSL per day and nutrient concentrations were examined using linear mixed models. Nutrients concentrations and fabric type were used as fixed effects and ecoregions and streams nested within ecoregions as random effects. Variation in microbial abundance, phylotype richness and diversity among cotton fabric incubated along the acidification gradient was examined using mixed ANOVA with cotton type as fixed effect and streams as random effect. All mixed models were performed using restricted maximum likelihood (REML) and the package lmerTest (Kuznetsova et al., 2015). Significance of fixed effects was derived using Satterthwaite approximation for degrees of freedom, as it produces acceptable Type 1 error rates for smaller samples (Luke, 2017). Post-hoc comparisons of means were performed using 'glht' function of the 'multicomp' package and a Bonferroni-Holm correction (Hothorn et al., 2008). Significance of random effects was determined using 'rand' function of 'lmerTest' package. Model checking included homogeneity of variance and normal distribution of model residuals and did not reveal any obvious deviations from homoscedasticity or normality.

Non-metric multidimensional scaling (NMDS) analyses of fungal and bacterial community profiles were used to assess differences among sites and among cotton fabric using the function 'metaMDS' in vegan. The Bray-Curtis coefficient was used to quantify dissimilarity among sites and among cotton fabrics based on community profiles. Goodness-of-fit was estimated with a stress function, which ranges from 0 to 1, with values close to zero indicating a good fit. Stress < 0.15 corresponds to good ordination (Clarke, 1993). Axes values from the NMDS analyses were correlated using Spearman rank correlation with ANC and aluminum concentration values to identify variables that corresponded to among-site differences in microbial community profiles using the function 'cor.test' of the 'stats' package. Linear regression models were performed to link percent TSL per day of each cotton fabrics using the caret (Kuhn et al., 2017) and mass (Venables and Ripley, 2002) packages. To run the models, data were divided into two parts, a training data set (i.e. 80% of the original data set) used to build the model and a validation data set (i.e. 20% of the original data set) used to gauge the model's performance. Model performance and accuracy were estimated using root mean square error and the Pearson correlation coefficient between the observed and predicted values

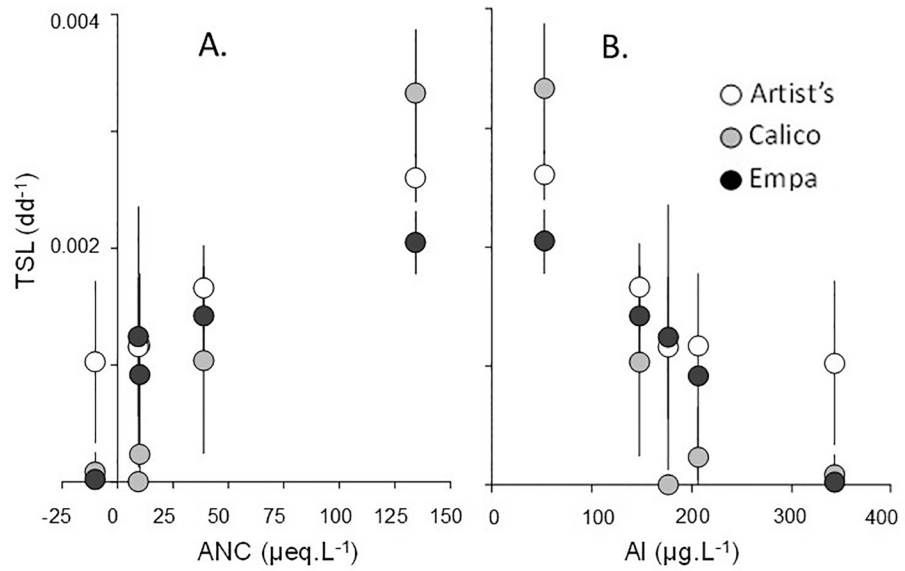

Fig 2. Mean TSL per degree day (± SD) along acid-neutralizing capacity (A) and aluminum concentrations (B) gradients. N = 5 streams in each panel.

coming from the validation data set. All statistical analyses were performed using R version 3.4.3 (R Development Core Team, 2008).

3. Results

3.1. Tensile-strength loss across ecoregions and environmental gradients

Patterns of TSL across ecoregions were similar among the three cotton materials (Fig. 1). Across all streams, TSL ranged from 0.4 to 4.4% d^{-1}, with an overall mean ± SD of 2.3 ± 1.2, 2.6 ± 1.4 and 2.5 ± 1.7% for Artist's fabric, Empa and Calico, respectively (Fig. 1). Differences were observed among fabric types in terms of their overall rates of tensile-strength loss ($F_{2,108} = 5.6$, $p < 0.005$) with Artist's fabric being slower to decompose than either Empa or Calico. Differences in mean rates of TSL were also observed among streams within ecoregions but not among ecoregions, although the differences were close to being statistically significant ($F_{4,5} = 4.8$, $p = 0.058$).

For each of the three fabric types, TSL per degree day increased with increasing ANC ($F_{1,11} = 59.2$, $P < 0.001$; Fig. 2A), and decreased with increasing aluminum concentrations ($F_{1,11} = 43.4$, $P < 0.001$;

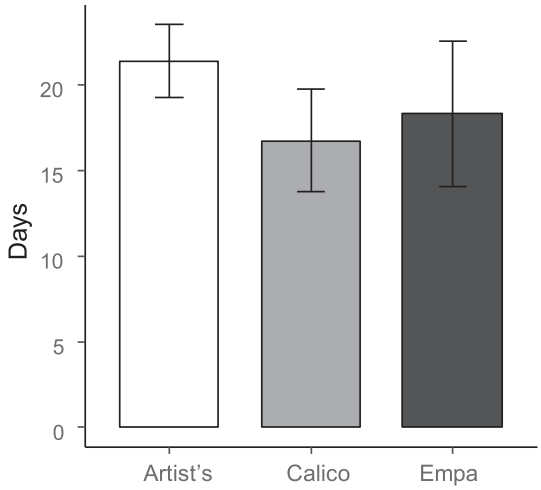

Fig.4. Barplot of T50 (± SD) calculated on three reference sites using linear models for Artist's fabric, Calico cloth and Empa's fabric. N = 3 streams.

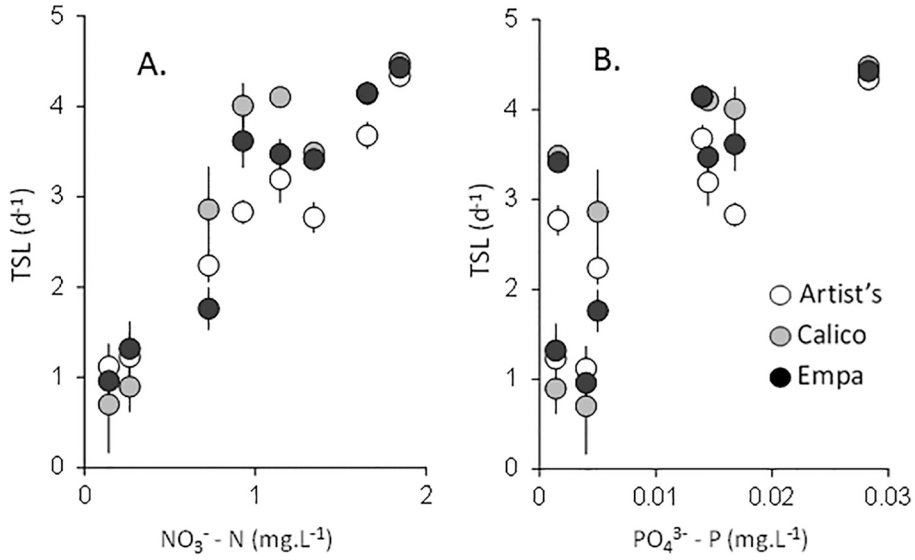

Fig 3. Mean TSL per day (± SD) across concentrations in NO_3^--N (A) and in PO_4^{3-}-P (B). N = 8 streams in each panel.

Table.3
Parameters of calibrations performed between the TSL per day of each material. ρ indicates the Spearman's correlation between the observed (i.e. data from training data sample) and predicted values (i.e. data from validation data sample). RMSE indicates the values of Root Mean Square Error values.

x	y		
	Artist's	Calico	Empa
Artist's		Intercept = -0.764 Slope = 1.388 R^2 = 85.3% *F*-value = 222.2 *P*-value < 0.001 AIC = 84.1 ρ = 0.95 RMSE = 0.59	Intercept = 0.238 Slope = 1.041 R^2 = 76.1% *F*-value = 121.8 *P*-value < 0.001 AIC = 85.1 ρ = 0.88 RMSE = 0.77
Calico	Intercept = -0.7426 Slope = 1.387 R^2 = 87.1% *F*-value = 373.5 *P*-value < 0.001 AIC = 52.5 ρ = 0.95 RMSE = 0.40		Intercept = 0.882 Slope = 0.717 R^2 = 81.2% *F*-value = 165.6 *P*-value < 0.001 AIC = 75.5 ρ = 0.95 RMSE = 0.58
Empa	Intercept = 0.315 Slope = 0.737 R^2 = 76.1% *F*-value = 121.8 *P*-value < 0.001 AIC = 71.6 ρ = 0.88 RMSE = 0.57	Intercept = -0.612 Slope = 1.141 R^2 = 81.2% *F*-value = 165.6 *P*-value < 0.001 AIC = 93.7 ρ = 0.95 RMSE = 0.61	

Fig. 2B). No significant differences were observed among fabric types, indicating that regardless of fabric type the decomposition of cellulose is strongly affected by acidification. Similarly, fabric type did not affect TSL along gradients of NO_3^--N (Fig. 3A) and PO_4^{3-}-P (Fig. 3B); TSL increased with concentrations of NO_3^--N ($F_{1,5.2}$ = 41.4, P < 0.005) and PO_4^{3-}-P ($F_{1,6}$ = 9.3, P < 0.05).

3.2. Time-series experiment

Time-series experiments and linear models revealed that the time to 50% TSL (T50) differed among the fabrics type (Fig. 4). Artist's fabric decomposed more slowly than Calico and Empa with 50% tensile-strength loss achieved after 21.2 ± 2.2, 16.7 ± 3.0 and 18.3 ± 4.2, respectively. Empa fabric was the most variable at T50; Artist's fabric was the least variable (CV: 10.4% for Artist's fabric, 17.9% for Calico and 23.0% for Empa).

The TSL of the three cotton materials was strongly related (Table 3). Each linear regression model performed between TSL of each cotton type was highly significant. On average, the models accounted for 81.2 ± 4.6% of the variation with correlations between the observed and predicted values ranging from 0.88 to 0.95. The information required to convert the tensile-loss rates of each strip type to another is shown in Table 3, for example, to express measured rates of Empa TSL in terms of Artist's-fabric TSL equivalents.

3.3. Comparison of microbial communities

Heterotrophic microbial assemblages were broadly similar across cotton types, but varied significantly among streams. Across all streams and cotton materials, fungi-to-bacteria ratios ranged between 0.2 and 4.1. Mean phylotype richness (PR) ranged from 13.7 to 22 phylotypes for bacteria and from 13.7 to 23.3 for fungi. Shannon-Weaver's diversity index (H') ranged from 2.2 to 2.9 for bacteria and from 1.9 to 2.7 for fungi. NMDS ordinations performed on fungal (Fig. 5A) and bacterial communities (Fig. 5B) exhibited stress < 0.15, with Axes 1 of the NMDS being strongly correlated with ANC and total aluminum

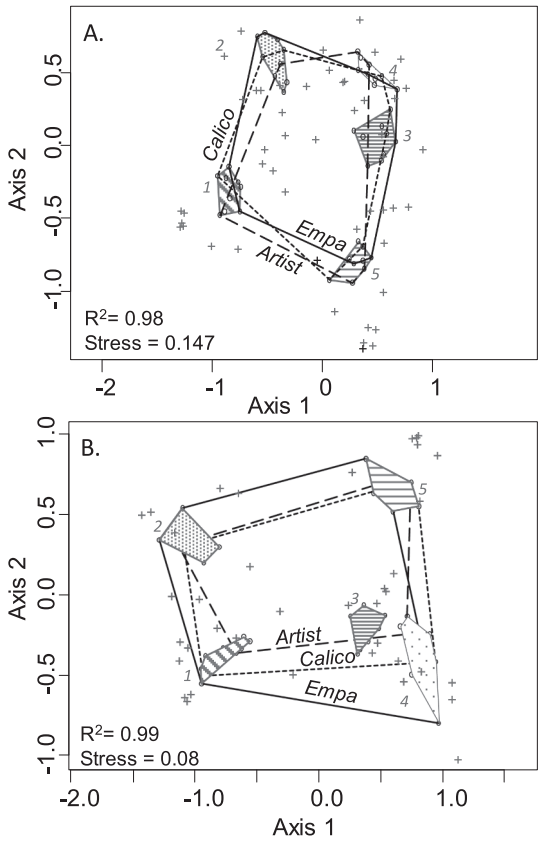

Fig.5. NMDS plots of DGGE fungal (A) and bacterial (B) community profiles from the five streams ordered according to the acidification gradient (i.e., from 1, the circumneutral stream, to 5, the most acidified stream) for each of the three materials. The plot shows both the communities (3 replicates × 3 blocks per stream; open circles) and phylotypes (crosses). Polygons connect the assemblages belonging to the same stream.

concentration for fungal (r = −0.70, p < 0.001; r = 0.66, p < 0.001, respectively) and bacterial community profiles (r = −0.61, p < 0.001; r = 0.80, p < 0.001, respectively). Detailed information on microbial abundances and assemblages across the five streams is available in Table 4.

4. Discussion

Ecosystem processes are rarely included as part of river-health-assessment programs, despite compelling advocacy for considering their role when gauging human impacts; part of the reason is a lack of easy-to-use standardized indicators. The results presented here further support a body of evidence indicating that cotton-strip assays are sensitive to a suite of environmental factors, including those that are influenced by human activities. Adding to the utility of cotton-strip assays are close relationships among the three fabrics we examined – relationships that enable direct comparisons of past, ongoing and future studies, thereby greatly expanding the number of streams for which there are comparable data. Given that the number of fungal and bacterial phylotypes that were found colonizing the cotton strips was similar to that found on leaves, the assay holds promise as a standardized means of sampling microbes in stream and other habitats. And given that Artist's fabric had the least variability among analytical replicates, and the large number of studies and field sites where Artist's fabric has been used (e.g., including this study, over 700 different streams across the globe), we recommend Artist's fabric as the standard material for researchers who require a reliable and sensitive process-based indicator. These and other findings are key steps towards understanding large-scale variation in

Table 4
Phylotype richness (PR) and diversity index (H') of bacteria and fungi for three cotton fabrics based on qPCR and DGGE performed on 16S and 18S rRNA. PR-Bac: Phylotype richness of bacteria, H-Bac: Shannon-Weaver diversity index of bacteria, PR-fungi: Phylotype richness of fungi, H-fungi: Shannon and Weaver diversity index of fungi, F/B ratio: fungi-to-bacteria ratio from qPCR. Streams are ordered according to the acidification gradient, from 1 (circumneutral stream) to 5 (acidified stream).

		PR-Bac		H'-Bac		PR-Fungi		H'-Fungi		F/B ratio	
Fabric	Stream	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Artist's	1	22.0	0.00	2.76	0.09	17.7	2.08	1.98	0.05	3.94	1.85
	2	22.0	0.00	2.62	0.08	17.3	0.60	1.94	0.51	2.66	1.20
	3	15.0	0.00	2.38	0.04	20.7	0.60	2.34	0.19	0.90	0.48
	4	14.0	0.00	2.19	0.20	15.7	0.58	2.34	0.04	2.61	1.29
	5	15.0	0.00	2.59	0.03	23.3	2.88	2.55	0.30	2.23	1.25
Calico	1	22.0	0.00	2.92	0.03	21.0	2.00	2.26	0.25	1.63	0.50
	2	21.0	0.00	2.48	0.04	17.3	0.60	2.00	0.29	4.08	2.78
	3	15.7	0.60	2.49	0.19	19.7	0.60	2.41	0.07	0.28	0.05
	4	15.0	0.00	2.28	0.07	17.0	1.00	2.41	0.08	0.91	0.12
	5	13.7	1.15	2.33	0.21	23.3	1.16	2.20	0.03	1.29	0.91
Empa	1	21.7	0.58	2.92	0.08	22.3	1.53	2.19	0.20	2.56	0.79
	2	20.7	0.60	2.40	0.17	13.7	2.08	2.04	0.18	3.38	2.24
	3	15.0	1.00	2.34	0.13	22.0	2.00	2.71	0.32	0.19	0.06
	4	15.7	0.58	2.37	0.24	19.3	0.58	2.61	0.14	0.84	0.35
	5	14.0	0.00	2.30	0.04	21.3	1.53	2.43	0.05	0.93	0.29

ecosystem functioning, and inclusion of process-based indicators in bioassessment programs across the globe.

We found consistent results across the three fabric types, and with studies using cotton-strips and litter-bags in terms of the responses to environmental gradients and stressors (e.g., Ferreira et al., 2006; Gulis et al., 2006; Ferreira and Chauvet, 2011; Fernandes et al., 2012; Tiegs et al., 2013, 2019; Wagenhoff et al., 2011). Organic-matter decomposition in aquatic ecosystems has typically been investigated without explicit consideration for the communities of microorganisms involved. Our study characterized bacterial and fungal communities and their role in cellulose decomposition, and paves the way for future work that will elucidate the roles of these communities in cellulose decay using next-generation molecular tools (e.g., Clivot et al., 2014).

Cotton differs from leaf litter (the most commonly used substrate in organic-matter decomposition studies and a key source of carbon in streams) in that it does not contain appreciable quantities of nutrients such as nitrogen and phosphorus that can promote microbial activity, nor does it contain lignin or secondary compounds that can inhibit it (French, 1988). Because of these and other differences in quality, different heterotrophic microbial communities between leaves and cotton strips might be expected. However, given the extremely wide ranging quality of litter and other organic matter that has been documented, including that from riparian trees (e.g., Boyero et al., 2017), microbes can also be expected to harbor adaptations that allow them to exploit diverse types of resources. Despite the differences between cellulose fabric and more-complex organic matter, the microbial communities from cotton strips were similar to those reported for leaf-litter in terms of phylotype richness and diversity (Manerkar et al., 2008; Clivot et al., 2014). Here we found between 14 and 23 OTUs of fungi along the gradient of acidification; in the same geographic same area, Clivot et al. (2014) reported between 17 and 22 OTUs of fungi on decaying alder (*Alnus glutinosa*) leaves in five streams across an acidification gradient. Regarding bacterial community profiles, Clivot et al. (2014) found a range of 15 to 28 OTUs on maple leaves in streams spanning an acidification gradient, compared to the range of 14 and 22 OTUs in our study. In a first-order stream in Nova Scotia (Canada), Manerkar et al. (2008) found a total of 23 OTUs of fungi on leaf disks of *Tilia cordata*. Our results, in the context of previously reported findings, suggest that in addition to providing a useful method for quantifying decomposition rates, cotton-strip assays offer a standardized means of sampling heterotrophic microbial communitie.

The community structure of microorganisms found on cotton strips, and any organic matter in streams, stems from several processes, including inoculum transport from the upstream environment, colonization on the substrate and subsequent microbial activity and growth. Of these, inoculum pressure and colonization would seem to be the least sensitive to substrate quality; this might explain the similar levels of phylotype diversity found between cotton strips and leaves. Regardless of the mechanisms behind the similarity in phylotype diversity between cotton strips and leaves, cotton strips seem to function as a standardized substrate for obtaining a representative sample of microbial heterotroph communities.

While the three cotton materials displayed similar responses to variable environmental conditions and stressors, such as acidification and nutrient loading, TSL differed among them; this demonstrates the need for intercalibrations across studies that use different assays. Since the microbial communities that colonize cotton strips were similar, simple physical differences in the properties of the cotton types may be driving the differences in TSL (e.g., thread density, thread strength). Artist's canvas is far denser than the Empa and Calico cotton (i.e., 407 g/m^2 for Artist's fabric and 205 and 115 g/m^2 for Empa and Calico, respectively), an observation that may explain why the Artist's fabric had the least within-site variability of the three fabric types examined. A notable finding was that the Empa material was less sensitive to environmental conditions than the other fabric types given its greater coefficient of variation. Despite the differences among them we nonetheless found very strong positive linear relationships between the TSL values. This enables direct comparisons of studies by providing researchers with information to convert data to a common currency. For example, with the calibration parameters provided in Table 3, TSL of Calico and Empa materials can easily and accurately be converted to Artist's-fabric equivalents.

Tensile-strength loss is the most commonly applied method for quantifying cotton-strip decomposition, an approach that requires a tensiometer. Tensiometers are common in materials-testing facilities across the globe, and in engineering departments at universities. Additionally, some laboratories perform tensile-strength determination on a contract basis for researchers who do not have ready access to a tensiometer. These instruments range widely in their degree of sophistication and price. For researchers who opt not to determine tensile strength, the cotton-strip assay can still be applied, and *in lieu* of tensile strength, closed chamber measurements of respiration can be performed (see Tiegs et al., 2013 for details). And as mentioned above, the assay can be used as a standard means of sampling microbial community structure. Lastly, mass loss can be measured on the strips, which relates to tensile-strength loss (Tiegs et al., 2007). However, we urge

caution with this approach since significantly longer incubations may be required, and the strips become prone to mass loss through unraveling, rather than carbon mineralization. Whether through determination of tensile strength, respiration, or heterotrophic-community structure, the cotton-strip assay offers utility and value to ecologists interested in organic-matter decomposition.

Here we helped develop the cotton-strip assay by deploying it in streams, however, it is increasingly used to evaluate microbial activity in other ecosystems, terrestrial and aquatic. For example, cotton strips have been deployed in shallow hyporheic sediments (Boulton and Quinn, 2000; Burrows et al., 2017) and deep groundwater (Lategan et al., 2010). Artist's fabric has been deployed in remote high-latitude wetlands (Vizza et al., 2017), in the flocculant sediments of lakes, wetlands and streams (Kincaid et al., in revision), and in spruce peatlands as part of a large scale warming and carbon-dioxide-enrichment experiment (SPRUCE). Additionally, the assay has tremendous potential to improve understanding of how decomposition rates vary in marine habitats, and to date, strips have been deployed in estuaries (Bierschenk et al., 2012), kelp forests (Filbee-Dexter unpublished data), and intertidal mangrove creek systems (Tiegs unpublished data). Lastly, cotton strips made of Artist's fabric have been deployed in hundreds of riparian zones across the globe to better understand the drivers of carbon processing in these ecosystems (Tiegs et al., 2019). Through time, and with the continued used of standardized decomposition assays, a finer resolution picture will emerge of how carbon is processed in Earth's ecosystems, such as identifying its hot spots and hot moments (sensu McClain et al., 2003).

The cotton-strip assay is believed to be most sensitive to variation in environmental conditions when values of tensile-strength loss are near 50% (Harrison et al., 1988) and the research presented here is useful for optimizing incubation durations in order to hit this target. These durations are 21d, 16d, 18d for Artist's fabric, calico and Empa material, respecively. Temperature and nutrient status are additional factors to consider when estimating an appropriate incubation duration, with moderate levels of enrichment stimulating decay rates. However, very high nutrient concentrations, such as those associated with agriculture and urbanization, can be associated with slowed decay (Woodward et al., 2012), and incubation duration may need to be adjusted accordingly. Notably, most of the streams studied here have somewhat high nutrient levels, and longer incubations with more degree day accumulations will be required to hit the target decay level of 50% in other systems. Moisture is believed to be limiting to microbial activity in terrestrial habitats (Tiegs et al., 2019), and incubation durations may need to be lengthened considerably (e.g., up to many months) when aridity is a factor. While 50% TSL should be the aim in most instances, appreciable deviations from this target will not invalidate studies, but may limit the sensitivity of the assay.

Our results validate the utility of cotton strips as effective indicators of both ecosystem functioning and the community structure of the microbes that drive it. These are needed steps towards a more universal, global bioassay. Hundreds of streams have now had their decomposition potential quantified with the cotton-strip assay and this data constitutes a substantial foundation for establishing baselines to track environmental change. And if developed within the framework of new global biomonitoring initiatives (e.g., Future Earth, Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services), the cotton-strip assay holds promise as a means to track the impacts of global change on vital aspects of carbon cycling, such as decomposition rates and the structure of heterotrophic microbial communities.

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