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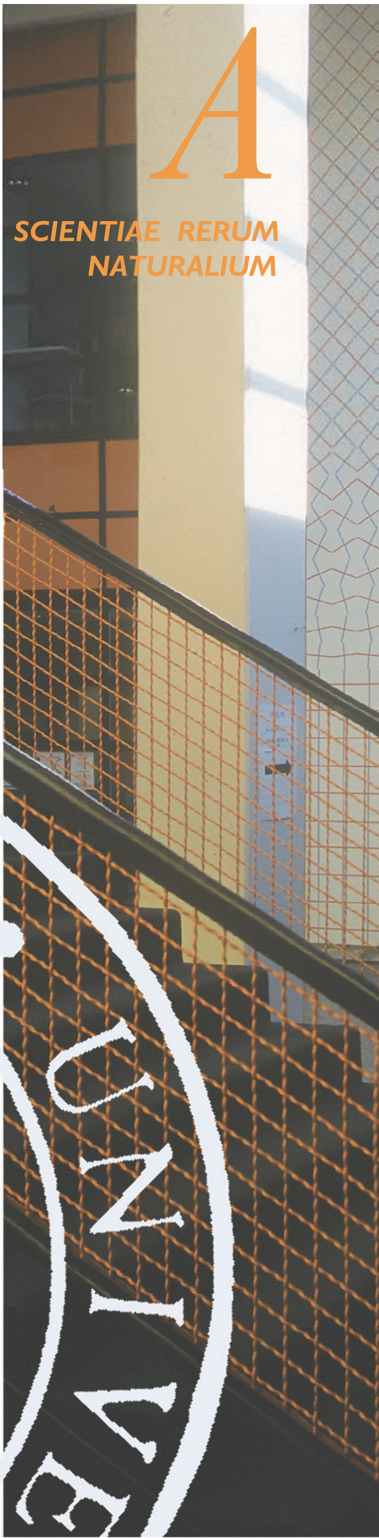

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Joanna Brüsecke

STREAM BROWNING AS A DRIVER OF BENTHIC COMMUNITY CHANGE AND DEGRADATION OF THE PERIPHYTIC NUTRITIONAL BASE

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FACULTY OF SCIENCE

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JOANNA BRÜSECKE

STREAM BROWNING AS A DRIVER OF BENTHIC COMMUNITY CHANGE AND DEGRADATION OF THE PERIPHYTIC NUTRITIONAL BASE

Academic dissertation to be presented with the assent of the Doctoral Programme Committee of Technology and Natural Sciences of the University of Oulu for public defence in the OP-Pohjola auditorium (L6), Linnanmaa, on 6 September 2024, at 12 noon

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Abstract

The browning of freshwaters, caused by increasing inputs of terrestrial organic matter, is an ongoing phenomenon in boreal regions. This terrestrially derived organic matter enters freshwaters mainly in the form of dissolved organic carbon (DOC). In Finland, the main driver of these increased inputs is peatland drainage for forestry purposes. My aims for this thesis were to elucidate the effects of browning in boreal streams on: 1) the nutritional quality of stream periphyton, 2) benthic macroinvertebrate communities and 3) bacterial biofilm communities. To achieve this, I collected samples from a set of streams representing a browning gradient in terms of DOC concentration. For the investigation of the periphyton nutritional quality, there was also an experimental component where DOC was manipulated at three different levels. I found a marked decrease in periphyton quality in terms of fatty acid composition with increasing DOC concentrations. Profound changes also occurred in the macroinvertebrate and bacterial communities along the DOC gradient. I detected a community shift at around 12 mg DOC L^{-1} for the macroinvertebrates, and two separate shifts for the bacterial community: one at 4–7 mg DOC L^{-1} and a second at very high concentrations >20 mg L^{-1}. Of all the macroinvertebrate functional feeding groups, only algal scrapers, who primarily feed on the stream periphyton, showed a strong negative response to browning. The bacterial communities showed a trend of increasing phylogenetic clustering with increasing DOC concentrations. The results I present here have important implications for management, bioassessment and conservation, as well as policies for land use practices concerning the future of boreal forest streams and connected waterbodies. An important aspect for further investigation is how browning interacts with other stressors, for example, droughts, which are expected to increase in boreal streams with climate change. Another interesting direction would be to trace if the change in fatty acid composition detected here in the periphyton cascades up the food web and through the stream-forest ecotone.

Keywords: bacteria, biofilm, boreal streams, browning, community shift, fatty acids, macroinvertebrates, periphyton

Brüsecke, Joanna, Virtavesien tummuminen muuttaa eliöyhteisöjen lajikoostumusta ja heikentää ravintoresurssien laatua
Oulun yliopiston tutkijakoulu; Oulun yliopisto, luonnontieteellinen tiedekunta
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Tiivistelmä

Valuma-alueelta tulevan eloperäisen, pääasiassa liukoisen, orgaanisen aineksen määrän lisääntyminen on johtanut sisävesien tummumiseen kaikkialla pohjoisen havumetsävyöhykkeen alueella. Suomessa liukoisen orgaanisen aineksen määrän kasvu johtuu ensisijaisesti soiden ojituksesta puun kasvun tehostamiseksi. Tässä väitöstutkimuksessa pyrin selvittämään tummumisen vaikutuksia 1) virtavesien tärkeimpien perustuottajien, päällyslevien, laatuun korkeampien eliöiden ravintona sekä 2) pohjaeläinten ja 3) biofilmin bakteerien yhteisöjen monimuotoisuuteen ja lajistokoostumukseen. Tutkimus perustui biologisiin näytteisiin, jotka kerättiin tummumisgradientilta kirkasvetisistä (3 mg C L^{-1}) erittäin tummavetisiin (lähes 30 mg C L^{-1}) puroihin. Biofilmin laadun muutoksia tutkittiin keinouomissa suoritetussa kokeessa, jossa liukoisen hiilen pitoisuutta manipuloitiin kolmella eri tasolla. Biofilmin rasvahappokoostumus kokeessa heikkeni liukoisen hiilen määrän kasvaessa. Myös sekä pohjaeläinten että bakteerien yhteisökoostumus muuttui voimakkaasti tummumisgradientilla, mutta eri kohdissa gradienttia. Pohjaeläinyhteisöjen koostumus muuttui nopeasti liukoisen hiilen pitoisuuden yltäessä tasolle 12 mg L^{-1}, kun taas bakteeriyhteisöt reagoivat jo selvästi alhaisemmissa pitoisuuksissa, n. 4–7 mg L^{-1}. Toinen nopean muutoksen kohta bakteeriyhteisöissä oli jo pitkälle tummuneissa vesissä, joissa liukoisen hiilen pitoisuus oli yli 20 mg L^{-1}. Pohjaeläinten ravinnonhankintaryhmistä vain kaapijat, jotka käyttävät puron pohjassa kasvavia päällysleviä ensisijaisena ravintokohteenaan, vastasivat selkeän negatiivisesti lisääntyneeseen tummumiseen. Bakteeriyhteisöjen fylogeneettinen rakenne muuttui tummumisen myötä siten, että taksonit olivat keskenään läheisempää sukua tummemmissa vesissä. Tässä väitöskirjassa esitettyjä tuloksia voidaan hyödyntää pohjoisten virtavesien hoidossa, tilanarvioinnissa ja suojelussa. Tulevissa tutkimuksissa pitäisi edelleen selvittää eliöyhteisöjen vasteita useisiin samanaikaisiin ympäristöpaineisiin, ottaen huomioon esimerkiksi tummumisen ja hydrologisten ääri-ilmiöiden (tulvat, kuivuusjaksot) mahdolliset yhteisvaikutukset. Erityisen mielenkiintoista olisi myös selvittää, voisiko tässä tutkimuksessa havaittu päällyslevästön laadullinen heikkeneminen heijastua purojen ravintoverkon korkeammille tasoille ja edelleen maa-vesi rajapinnan yli aina rantametsän selkärangattomiin petoihin saakka.

Asiasanat: biofilmin bakteerit, boreaaliset virtavedet, pohjaeläimet, päällyslevästö, rasvahapot, sisävesien luontokato, tummeneminen

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Although doing a PhD is often described as a lonely endeavour, it is not something you achieve by yourself. I have many people to thank for helping me get this far.

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resources and interesting news or helping each other with something often kept me grounded and reminded me that we are all just trying our best to get things done, despite all the challenges. So thanks for that and your cheerful attitude. Thank you Wille-Pekka Lepo for your collaboration as co-author of paper II. And thank you to the other members, Joel Nyberg, Aino Erkinaro and Aino Juutinen for all the shared experiences in the lab, field, courses or other work-related activities and many nice conversations. I thank also former master's students Sanni Litjo, for her collaboration as co-author of paper II, and Sanna Räsänen for her assistance in the field.

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The whole experience helped me grow, both on an academic and a personal level, and I want to thank you all for accompanying me and helping me on this journey.

June 2024 Joanna Brüsecke

List of abbreviations

ALA	Alpha-linoleic acid
ASV	Amplicon sequence variant
BFA	Bacterial fatty acid
BIC	Bayesian information criterion
C	Carbon
dbRDA	Distance-based redundancy analysis
DHA	Docosahexaenoic acid
DIN	Dissolved inorganic nitrogen
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EEM	Excitation-emission matrix
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
GF	Gradient forest
IndVal	Indicator value
LSAFA	Long-chain saturated fatty acid
MNTD	Mean nearest taxon distance
N	Nitrogen
NO_3	Nitrate-nitrogen
NTI	Nearest taxon index
P	Phosphorous
PCA	Principal component analysis
PERMANOVA	Permutational multivariate analysis of variance
PES	Polyethersulfone
Pt	Platinum
PUFA	Polyunsaturated fatty acid
RF	Random forest
SUVA	Specific ultraviolet absorbance
TITAN	Threshold indicator taxa analysis
TN	Total nitrogen
TP	Total phosphorus
UV	Ultraviolet
VIF	Variance inflation factor

List of original publications

This thesis is based on the following articles, which are referred to throughout the text by their Roman numerals:

I Jyväsjärvi, J., Rajakallio, M., Brüsecke, J., Huttunen, K.-L., Huusko, A., Muotka, T., & Taipale, S. J. (2022). Dark matters: Contrasting responses of stream biofilm to browning and loss of riparian shading. *Global Change Biology, 28*, 5159–5171.

II Brüsecke, J., Muotka, T., Huttunen, K.-L., Litjo, S., Lepo, W.-P., & Jyväsjärvi, J. (2023). Drainage-induced browning causes both loss and change of benthic biodiversity in headwater streams. *Limnology & Oceanography Letters, 8*, 620-627.

III Brüsecke, J., Muotka, T., Huttunen, K.-L., Lehosmaa, K., & Jyväsjärvi, J. (manuscript). Biofilm bacterial communities along a gradient of browning in boreal headwater streams.

Own contribution

The main conceptualisation of all three papers was done by my supervisors, Jussi Jyväsjärvi, Timo Muotka and Kaisa-Leena Huttunen. I contributed to the finalisation of the topics for the three articles. For all three papers, I was involved in study design, field and laboratory work, and editing of the manuscript. For paper II, I contributed also to the statistical analyses and manuscript writing. For paper III, I was responsible for sample processing in the laboratory, analysing the data and was involved in manuscript preparation. All tasks were completed under the guidance of my supervisors.

Contents

1 Introduction

Freshwater ecosystems are particularly vulnerable to environmental change due to their low location in the landscape, as was already emphasised by the pioneers of freshwater ecology (Lindeman 1942; Hynes 1975). This subjects them to both local catchment effects and regional atmospheric processes (Jackson et al., 2016). It also renders them particularly vulnerable to climate change (Piggott et al., 2015b). The hierarchical organisation of stream networks also means that stressors operating at a large scale, e.g. at the catchment level, will most likely also act as stressors or initiate other stressors at lower hierarchical orders, right down to the microhabitat scale (Meißner et al., 2019). Freshwater ecosystems cover only about 1% of the Earth's total surface area but are home to almost 10% of all known plant and animal species (Meißner et al., 2019). At the same time, they are among the most degraded ecosystems globally, with freshwater biodiversity in more severe decline than in marine or terrestrial ecosystems (Dudgeon et al., 2006; Meißner et al., 2019; Sala et al., 2000).

A stressor can be defined as any abiotic or biotic factor that exceeds its normal range of variation and significantly affects individual physiology, population performance or community stability in any ecosystem (Nõges et al., 2016). More specifically, anthropogenic stressors are those "biotic or environmental factors that exceed their natural ranges of variation due to human activities" (Crain et al., 2008). The impacts of these stressors can be measured at the level of individuals as changes in an organism's fitness, such as growth or reproduction (Nõges et al., 2016). At the community or ecosystem level, stressors cause a "decline in the number of organisms affecting biotic interactions and integrity" (Nõges et al., 2016). Broadly, the most impactful anthropogenic influences that each bring with them various stressors are habitat loss, over-exploitation of species, pollution, invasive species, and climate change (Crain et al., 2008). Examples of stressors that have been studied in various ecosystems include UV radiation, toxins, salinization, increased temperatures, nutrient enrichment, and sedimentation (Crain et al., 2008; Wagenhoff et al., 2012). Some of these (e.g. nutrients) can be referred to as "usable inputs" because they are utilised by the community and are required for ecosystem functioning at natural levels, but can cause stress when they are augmented by anthropogenic activities (Wagenhoff et al., 2011). That is, they only become a stressor at levels elevated above natural background levels, as first suggested by Odum et al. (1979). A distinction can be made between these and toxic substances, which can be expected to produce negative impacts at any level and have no subsidy effect associated with them when present at low levels (Wagenhoff et al., 2011).

The browning of freshwaters is an ongoing phenomenon in boreal regions (Evans et al., 2005; Monteith et al., 2007). This is caused by increasing inputs of terrestrial organic matter into freshwater systems, which originates from humic substances derived from the partial decomposition of vascular plants (Lennon et al., 2013). Most of this material enters freshwaters in the form of dissolved organic carbon (DOC) (Jones & Lennon, 2015; Lennon et al., 2013); for example, in a survey by Bergström & Karlsson (2019), 97% of the total organic carbon content in lakes was in the form of DOC. This dissolved fraction of organic material is most commonly defined by its passing through a filter with a 0.45 µm pore size (Creed et al., 2018).

There are several anthropogenic factors driving browning. Among these are land use changes, decreased acid deposition and climate-change induced increases in terrestrial vegetation productivity, precipitation, runoff and temperature (Evans et al., 2005; Monteith et al., 2007; Roulet & Moore, 2006). Although DOC concentrations can be naturally high in freshwater systems whose watersheds are dominated by peatlands, these factors can further amplify it and lead to a continuing increase in water colour. In Finland, more than half of all peatlands have been drained for forestry purposes, making peatland drainage the leading cause of stream browning in the country (Asmala et al., 2019; Nieminen et al., 2021). The DOC in the rivers and streams eventually reaches the Baltic Sea, where the total organic carbon load from rivers has increased by around 47% between the 1990s and 2017 (Asmala et al., 2019).

From a human perspective, freshwater browning jeopardises ecosystem services like drinking water quality, fishing opportunities, recreational use, and aesthetic values (Kritzberg et al., 2020; Härkönen et al., 2023). In lentic ecosystems, it also alters greenhouse gas emissions and carbon stocks (Ferland et al., 2014; Chmiel et al., 2015).

DOC is considered a poor-quality resource for aquatic systems, as it consists mainly of high molecular weight material that most aquatic organisms are unable to utilise, and lacks essential fatty acids that algae produce (Jones & Lennon, 2015; Tanentzap et al., 2017). Primary production in lakes is decreased by DOC concentrations above a certain threshold level due to its light attenuating effects (Bergström & Karlsson, 2019; Jones & Lennon, 2015; Karlsson et al., 2009; Fork et al., 2020). At low concentrations, primary productivity is nutrient-limited, then reaches a plateau at around 10 mg DOC L^{-1}, before becoming light-limited

(Bergström & Karlsson, 2019). This leads to decreased availability of autochthonous resources for consumers, which means there is a lack of essential fatty acids not found in terrestrially derived organic matter (Creed et al., 2018; Karlsson et al., 2015). This insight reveals that, above certain levels, increased DOC input no longer acts as a subsidy to lake communities by increasing macronutrient (C, N, P) levels but rather acts as a stressor, causing decreased productivity, also at higher trophic levels (Karlsson et al., 2009). Terrestrial DOC chelates micronutrients and may inhibit enzymatic activity, which can contribute to the reduction of primary productivity (Jones & Lennon, 2015). Although increased bacterial respiration (caused by an assemblage of heterotrophic bacteria capable of growth using only terrestrial DOC) was observed at increased DOC levels in experimental ponds, bacterial productivity remained the same at higher DOC treatments (Jones & Lennon, 2015).

While the ecological consequences of browning have been studied quite intensively in lakes for the last two decades, far less is known about its effects on stream food webs and biodiversity, particularly in benthic stream habitats. Moreover, organisms at different trophic levels may respond to different stressors and factors associated with a phenomenon like browning, and responses to the same stressor can be highly variable at different trophic levels (Mustonen et al., 2016). This highlights the importance of studying multiple taxonomic groups to measure community responses to stressors.

It is often recommended to compare the results from mesocosm experiments to observations from the field to gain a more complete picture of the real situation. While it is possible to disentangle the underlying mechanisms using mesocosms, these experiments are restricted in their temporal and spatial scales. Browning is associated with several concurrently changing factors, and the individual effects of, for example, increased DOC and nutrients (e.g., Bergström & Karlson, 2019) cannot be distinguished without experimentation. Nevertheless, broad-scale field surveys can help us understand and predict biotic responses to browning (see Solomon et al., 2015). Therefore, results from field surveys can be used as a “reality check” to confirm if the effects observed in experimental setups also occur in nature (Elbrecht et al., 2016; Piggott et al., 2015a; Tolkkinen et al., 2015).

2 Aims

The main aim of this study was to elucidate the effects of browning on different stream communities and the nutritional base of stream food webs. Rather than disentangle the causal effects of individual stressors associated with browning, the field survey component of this project is meant to provide a benchmark of real-world consequences of browning on natural streams, encompassing the responses of benthic periphyton, benthic macroinvertebrates and benthic biofilm bacteria. To do this, we collected samples of different taxonomic groups from a set of streams representing a browning gradient and examined community responses and diversity metrics along the gradient. To investigate changes in the nutritional base of stream food webs, we analysed the fatty acids of benthic periphyton in both natural streams and a mesocosm experiment where dissolved organic carbon concentrations were manipulated to simulate browning.

The task was divided into three sub-projects:

1. First, we set out to analyse the fatty acids of the stream biofilm in our browning gradient, as well as in a stream mesocosm experiment with three different levels of DOC. We hypothesised that algal growth would be impacted by increased DOC concentrations and that, in response, the composition of fatty acids in the stream periphyton would change. Diatoms generally dominate the periphyton in near-pristine streams. They contain eicosapentaenoic acid (EPA), which is essential for aquatic invertebrate consumers. Since browning is associated with an increase in nutrient availability, we expected that cyanobacteria would become more dominant. Cyanobacteria contain abundant alpha-linoleic acid (ALA) but no other polyunsaturated fatty acids (PUFAs), like the important EPA and docosahexaenoic acid (DHA). Similarly, green algae may be present in high DOC streams, but they also contain only low amounts of EPA. Therefore, due to changes in dominant algal groups, we expected to find a decrease in PUFAs (particularly EPA and DHA) and an increase in long-chain saturated fatty acids (LSAFAs) and ALAs along the DOC gradient.
2. Secondly, we wanted to elucidate the effects of browning on the benthic macroinvertebrate community. We expected to see either a gradual change in the community composition along the DOC gradient or an abrupt shift when DOC content exceeds a certain concentration. We hypothesised that sensitive

taxa like Ephemeroptera, Plecoptera and Trichoptera would be affected more than stress tolerant taxa like, for example, *Asellus aquaticus*. We also aimed to answer whether macroinvertebrate diversity is affected and how. We expected a decline in diversity since drainage related changes in streams have been shown to reduce macroinvertebrate diversity (Ramchunder et al., 2012; Brown et al., 2019; Rajakallio et al., 2021). Furthermore, we expected that those taxa whose food source is directly impacted by browning would show a more profound response than others. That is, the abundances of algal scrapers who rely on periphyton for nutrition would decline.

3. Thirdly, we were interested in the bacterial community responses to browning. Here, our objective was to answer the question whether community composition is affected by the browning gradient and if the response differs in the active and the total community. Again, if a response occurs, we wanted to see if the change was gradual or in the form of an abrupt shift, and whether it is similar to the macroinvertebrate community response.Furthermore, we explored if increased DOC causes phylogenetic clustering, that is, if the taxa present in brownified streams are more phylogenetically similar.

3 Materials and methods

3.1 Study sites

First to second order headwater streams with a broad range of DOC concentrations were selected for the study. All three papers included 45 tributaries of River Iijoki in north-central Finland. These sites represent a gradient of DOC concentrations from 4.7 mg DOC L^{-1} to 26.2 mg DOC L^{-1}. For papers II and III, ten more sites from the same catchment were included, bringing the high DOC end of the gradient up to 27 mg L^{-1} and the number of sites to 55. For paper II, an additional eight sites from the River Koutajoki watershed in north-eastern Finland were selected to extend the DOC gradient on the low end to 3.6 mg L^{-1} (overall n=63). The catchments of both rivers are characterised by peatlands and mixed forests. Sampling was conducted at a continuous 50 m long riffle section at each stream.

3.2 Biological sampling

3.2.1 Benthic macroinvertebrates

Benthic macroinvertebrate samples were collected in late September 2019 (53 sites) or 2020 (10 sites). A 2-min kick-net (Ø 500-µm) sample was taken at each site, which is known to cover about 1.3 m^2 of the stream bed and captures about 75% of all species present in a riffle (Mykrä et al., 2006). The samples were placed in jars and preserved with 70% ethanol immediately after sampling in the field. All macroinvertebrates (excluding chironomids and simulids) were identified to species or genus level.

3.2.2 Bacterial biofilm

To collect the biofilm samples, we incubated a set of unglazed, ceramic, 10 × 10 cm tiles (n=8) at each study site for five weeks from mid-August to late September 2019 (45 sites) and 2020 (10 sites, Paper III). The tiles were attached to metal plates with silicone and secured on the stream bed with a rebar at four random locations in the riffle section. Each plate had two 10 × 10 cm tiles attached and one 10 × 5 cm tile for periphyton sampling (see below). One rebar was hammered into the stream bed at each random location in the riffle, and each rebar secured one metal plate with tiles to the stream bed.

The biofilms were collected by wiping the top surface of the tiles with a sterile Speci-Sponge® (Whirl-Pak, Nasco). The biofilm from four tiles (one randomly selected tile from each metal plate) was pooled across the four plates into one sponge for fatty acid analysis (Paper I); and the biofilm from the four other tiles (again one from each metal plate) was pooled into another sponge for bacterial community analysis (Paper III). The sponges were placed into sterile 50 ml Eppendorf tubes. These were immediately placed into a cold box with dry ice in the field and transferred to a -80°C freezer at the laboratory.

3.3 Environmental measurements

At the time of sampling, water samples and environmental measurements were taken at all sites. From the water samples, dissolved organic carbon (DOC, mg L^{-1}), water colour (mg Pt L^{-1}), total phosphorus (TP, µg L^{-1}), nitrate-N (NO_3, µg L^{-1}), and pH were measured following national standards (National Board of Waters, 1981). For the DOC concentration measurements, the water was filtered (Ø 0.45 µm Whatman GF/F) and analysed by infrared spectrometry with a Shimadzu TOC-VCPH analyser (Shimadzu Scientific Instruments, Kyoto, Japan). A Lambda 650 (Perkin Elmer) spectrophotometer was used to measure absorbance spectra (190-800 nm; 1 nm intervals) from filtered water samples (Ø 0.2 µm PES membrane filter). To measure the quality of dissolved organic matter (DOM), excitation-emission matrices (EEM) of fluorescence were measured for 55 sites using a 1 cm quartz cuvette in a Varian Cary Eclipse fluorometer (Agilent). Bandwidths were set to 5 nm for excitation (ex) and 4 nm for emission (em). A series of emission scans (290–700 nm) were collected over excitation wavelengths ranging from 230 to 455 nm in 5 nm increments. Milli-Q (Millipore) water was used as the reference for absorbance and EEM measurements. DOM quality was characterized with two univariate indices: i) fluorescence index (the ratio of em470/520 nm, obtained at ex370) and ii) freshness index (the ratio of em380 nm and max[em420-435 nm] at ex310 nm). The former indicates the relative contribution of terrestrial versus microbial sources to the DOM pool, and the latter describes the contribution of recently produced DOM, with higher values representing a higher proportion of fresh DOM (see Hansen et al., 2016). As another measure of DOC quality, we calculated specific UV absorbance (SUVA) by dividing raw absorbance at 254 nm

(A254) by the DOC concentration (mg L^{-1}), with higher values indicating a greater proportion of aromatic and humic substances in the water (Weishaar et al., 2003).

Water temperature was measured during the incubation period on an hourly basis by attaching a HOBO Pendant logger (Onset, Massachusetts) to one of the rebars in every stream. The resulting temperature data was averaged across the whole study period for each stream. Five transects were randomly placed along the riffle, perpendicular to the flow. Current velocity (cm s^{-1}, Schiltknecht® MiniAir20) and water depth (cm) were measured at three locations along these transects. Stream width (m) was also measured at the transects.

Moss cover (%) was estimated in 20 0.25 m^2 plots distributed randomly across the study section. Substratum size was also estimated in ten of these plots. The mean substratum size was calculated as the weighted average of particle size classes using a modified Wentworth scale from 1 (clay/silt 0.001–0.07 mm) to 10 (large boulder to bedrock >512 mm). The Simpson diversity index was calculated to estimate substrate diversity using the estimated relative cover of each Wentworth class in each quadrat. Mean riparian canopy cover (%) was estimated from fisheye-lens upward photographs taken at ten random locations along the study site using the GLAMA mobile phone application (Tichý, 2016).

Periphyton samples were also collected from 5 × 10 cm tiles (n = 4 per site), which were incubated together with the other tiles (see above). After collection, these tiles were placed in zip-lock bags and frozen at -20°C. They were further processed in the laboratory by scraping the upper surface into 100 ml of water using a toothbrush and then immediately filtering onto a 0.7 µm Whatman GF/F glass fibre filter. The filters were wrapped in aluminium foil and frozen at -20°C. Ethanol (90%) was used to extract algal pigments, and chlorophyll-α concentration was measured using a spectrophotometer (Shimadzu UV-1601PC) with fluorescence readings at 665 and 750 nm.

3.4 Fatty acid sample processing

The sponges with the fatty acid samples were freeze-dried for 48 hours prior to analysis. Lipids were extracted using the Folch method (Folch et al., 1957): chloroform: methanol 2:1 mixture was sonicated for 10 min, after which 0.75 mL of distilled water was added. Fatty acids of total fraction were methylated in acidic conditions. Toluene and sulfuric acid were used for the transesterification of fatty acid methyl esters (FAMEs) at 50°C for 16 h, which is an optimal method for methylation of PUFA. FAMEs were analysed with a gas chromatograph (Shimadzu

Ultra, Kyoto, Japan) equipped with a mass detector (GC–MS), using helium as a carrier gas (linear velocity = 36.3 cm s^{-1}). The temperature of the injector was 270°C, and we used a splitless injection mode (for 1 min). Temperatures of the interface and ion source were 250°C and 220°C, respectively. Phenomenex® (Torrance, California, USA) ZB-FAME column (30 m × 0.25 mm × 0.20 µm) with 5 m Guardian was used with the following temperature program: 50°C was maintained for 1 min, then the temperature was increased by 10°C min^{-1} to 130°C, followed by 7°C min^{-1} to 180°C, and 2°C min^{-1} to 200°C, then held at that temperature for 3 min, and finally heated at 10 °C min^{-1} to 260 °C. Total program time was 35.14 min, and the solvent cut time was 9 min.

Fatty acids were identified by the retention times using specific ions (Taipale et al., 2016), which were also used for quantification. Fatty acid concentrations were calculated using calibration curves based on known standard solutions (15, 50, 100 and 250) of a FAME standard mixture (GLC standard mixture 566c, Nu-Chek Prep, Elysian, Minnesota, U.S.A.) and using recovery percentage of internal standards. The Pearson correlation coefficient was > 0.99 for each individual fatty acid calibration curve. Additionally, we used 1,2-dinonadecanoyl-sn-glycero-3-phosphatidylcholine (Larodan, Malmö, Sweden) and free fatty acid of C23:0 (Larodan, Malmö, Sweden) as internal standards and for calculation of the recovery percentages.

3.5 Nucleic acid sample processing

Both DNA and RNA were extracted from the Speci-Sponges® using ZymoBIOMICS DNA/RNA miniprep kits following the manufacturer's recommendations. Residual DNA was removed from the RNA samples using the ZymoBIOMICS DNase I treatment during the extraction process. RNA was converted to cDNA using Quantabio's qScript cDNA synthesis kit for amplification. The V4-V5 region of the 16S rRNA gene was amplified using 519F and R926 primers for both DNA and RNA (cDNA) samples (Quince et al., 2011). Polymerase chain reaction was performed following Malazarte et al. (2022), and included the following steps: initial denaturation at 98°C for 3 min, 30 cycles of denaturation at 98°C for 10 s, annealing at 64 °C for 30 s, extension at 72°C for 20 s and a final extension at 72°C for 5 min. AMPureXP PCR purification reagent (Agencourt Bioscience, CA, USA) was used to clean the PCR amplicons and Bioanalyzer DNA-1000 chips (Agilent Technologies, Palo Alto, CA, USA) were used to quantify them. An equivalent amount of amplicon was pooled for each sample and purified with AmpureXP purification reagent. Each sample was quantified with the PicoGreen dsDNA assay kit (ThermoFisher, Carlsbad, CA, USA) and, based on the quantity, diluted to an appropriate concentration. Ion Torrent sequencing was applied using the Ion Torrent Hi-Q OT2 kit, Ion Torrent Hi-Q View Sequencing kit and 316 v2 chip with a sequencing length of 400 bp (ThermoFisher, Carlsbad, CA, USA).

3.6 Bioinformatics

The sequences were processed using the QIIME2 (v.2023-5) microbiome bioinformatics pipeline (Bolyen et al., 2019). Short reads (< 100 bp) were removed and demultiplexed with sample-specific barcodes using the q2-cutadapt plugin (Martin, 2011). Single-end demultiplexed sequences were processed with DADA2 using the denoise-pyro option with a truncate length of 250 nts (Callahan et al., 2016). Amplicon sequence variants (ASVs; Callahan et al., 2017) were aligned to the SILVA 16S Gene Database (version 138.1; Quast et al., 2013) using a pre-trained *classify-sklearn* naïve Bayes taxonomy classifier (via q2-feature-classifier; Bokulich et al., 2018). Singletons, mitochondria, chloroplasts, and unassigned sequences were removed prior to further analyses. Aligned sequences of ASVs were used to construct a maximum-likelihood tree in Fast-Tree (Price et al., 2010) to include the phylogeny of the bacteria in later analyses.

3.7 Data analyses

The streams were classified into three groups based on DOC concentration: low (< 10 mg L^{-1}; n = 19), moderate (10-15 mg L^{-1}; n =19) and high DOC (> 15 mg L^{-1}; n =25), roughly corresponding to 20%, 75% and 98% quantiles of water colour monitoring data for ca. 4500 streams in Finland during 2010–2017 (Finnish Environment Institute, HERTTA database). Principal component analysis (PCA) was used to visualise environmental gradients for both water chemistry and habitat variables (Paper II). Permutational multivariate analysis of variance (PERMANOVA) was performed to test differences among the DOC groups using the *adonis* function of the vegan R package (Oksanen et al., 2020). It was run with 9999 permutations using Euclidean distances on z-standardized data with zero mean and unit variance (Paper II).

3.7.1 Fatty acids (Paper I)

Fatty acid data were sorted according to their chemical structure (see Taipale et al., 2013) into four groups: (1) ω-3 polyunsaturated fatty acids, (2) ω-6 polyunsaturated fatty acids, (3) long-chain saturated fatty acids of mainly terrestrial origin (LSAFA) and (4) bacterial fatty acids (BFA), of which (1), (3) and (4) were used as response variables in data analyses. To assess the nutritional quality changes in the stream biofilm, eicosapentaenoic acid (EPA; 20:5 ω-3) content and the ratio of ω-3 and ω-6 fatty acids were also used as response variables.

We used ordinary least squares regressions with algal biomass (Chl-α, mg m^{-2}), LSAFA, ω-3, EPA fatty acids and the ratio of ω-3 and ω-6 fatty acids as the response variables, and the environmental measurements as the potential explanatory variables. The "all-possible-subsets" approach was used to select the most parsimonious regression model for each response variable, using the *regsubsets* function from the leaps R package (Lumley, 2020). The Bayesian information criterion (BIC; Neath & Cavanaugh, 2012) was used to assess parsimony, with the lowest value considered the most parsimonious. We used log10 transformation for all predictor variables (except pH) in the analysis to improve the normality of model residuals.

3.7.2 Benthic macroinvertebrates (Paper II)

The macroinvertebrate taxa in our data were characterised by their feeding habits using a European trait database (Schmidt-Kloiber & Hering, 2015). An affinity score ranging from 0.1 to 1.0 was assigned to each taxon for feeding traits. Four of these feeding trait categories were selected for further analyses: scrapers, shredders, gatherer-collectors and predators. This was done using a fuzzy-coding approach (Chevenet et al., 1994). The taxa abundances were weighted by the affinity scores.

The relationships between environmental variables and univariate community metrics (species richness and the abundances of each of the four feeding groups) were again analysed using least-squares multiple linear regressions. The abundance-based metrics were log10 transformed for the analysis. We used the variance inflation factor (VIF) to assess multicollinearity among explanatory variables. The values ranged from 1.5 to 2.7, suggesting some degree of multicollinearity, but were well below 5, which has been suggested as the threshold for alarming multicollinearity (Hair et al., 2016). The *regsubsets* function of the leaps R package (Lumley, 2020) was used to select the most parsimonious model based on the empirical variable selection (REVS) approach (Goodenough et al., 2012). The model with the lowest BIC was selected as the most parsimonious. The variable importance (% of R^2) was calculated for the model predictors using the 'lmg' method in the relaimpo R-package (Grömping, 2006) for further assessment of the relative importance of each predictor variable. The values were rescaled to sum up to 100%. We obtained confidence intervals (95%) for variable importance values by bootstrapping (n=9999).

Threshold Indicator Taxa Analysis (TITAN 2.4.1 R package; Baker et al., 2015) was used to investigate community change along the DOC-gradient. This method combines indicator species analysis (Dufrêne & Legendre, 1997) and non-parametric change-point analysis (Baker & King, 2010). Change points in the frequency and abundance of individual taxa are identified, as well as synchronous responses of multiple taxa along the gradient. Indicator values (IndVal) are calculated for all taxa and all possible change points along the gradient. Permutation tests are used to assess the level of uncertainty in these scores. The permuted IndVal scores are standardised as Z-scores and summed for positive ('Z+') and negative ('Z-') response values for each possible change point. Potential community thresholds are indicated by peaks in Z-values where a disproportionate number of taxa exhibit changes in occurrence and relative abundance (Baker & King, 2010). Uncertainty around the change points for each indicator taxon was determined with bootstrapping (n=1000). A taxon was assigned as a positive or negative responder if: (i) ≥95% of bootstrapped runs were significantly different from random distribution (at $P \leq 0.05$, high reliability), and (ii) if the change in frequency and abundance of the taxon was in the same direction for ≥ 95% of the bootstrapped runs (at $P < 0.05$, high purity).

3.7.3 Bacteria (Paper III)

Analyses of both active (RNA-based data) and total (DNA-based data) community

The relationship between DOC concentration and bacterial diversity (amplicon sequence variant [ASV] richness and Shannon diversity) was investigated using simple linear regressions, separately for the total (DNA) and the active (RNA) community. PERMANOVA was used to test differences in taxonomic composition among the three DOC groups, using relative abundance data with the Bray-Curtis dissimilarity coefficient (9999 permutations). We performed a global test and examined pairwise differences among site groups with separate PERMANOVAs. This was done separately for both the total and the active community.

We tested the significance of the environmental variables in structuring bacterial communities with distance-based redundancy analysis (dbRDA) based on Bray-Curtis dissimilarity using square root transformed relative abundance data. The permutation-based *ordistep* function from the vegan R package was used to select only those environmental variables with a significant contribution ($P < 0.05$) to the ordination configuration.

To assess the level of concordance between the bacterial communities and the macroinvertebrate communities from the same 55 sites, a Procrustes rotation analysis was performed. This scales, rotates and dilates one ordination solution and superimposes it onto a second ordination to maximise the fit between corresponding observations of the two ordination configurations (in this case, the dbRDA ordinations of the bacteria and macroinvertebrate data). The most frequently used method for Procrustean fitting is based on the least-squares criterion that minimizes the sum of the squared residuals (m^2) between the two configurations; the m^2 statistic is thus a measure of association (i.e., concordance) between the two ordinations (Digby & Kempton, 1987). Low values of m^2 indicate strong concordance. The *ProTest* function (Peres-Neto & Jackson, 2001) provides a measure of the significance of the procrustean fit by permutation procedure, which randomly permutes (n=9999 for this analysis) the original observations of one matrix so that each site can be assigned any of the values attributed to other sites (Jackson, 1995). The m^2 statistic is then recalculated for each permutation, and the proportion of the statistics smaller than or equal to the observed value provides the significance level of the test.

Analyses of active community (RNA-based data) only

Gradient forest (GF) analysis was used to test for any threshold responses of compositional turnover across environmental gradients. This method describes the magnitude of change in community composition along environmental gradients in a flexible, non-parametric and non-linear way while also accounting for potential other explanatory variables (Ellis et al., 2012; Pitcher et al., 2012). A GF model creates an aggregation of random forest (RF) models, each describing the environmental responses of individual species.

Information in these models about the relative importance of different predictors, and the position and magnitude of the greatest changes in the abundance of each taxon along the gradient is aggregated so as to maximize the correspondence of each predictor with species turnover. ASV relative abundances were arc sin square root transformed to meet statistical requirements, while predictors do not need to be transformed for random forest models (Wagenhoff et al., 2017). First, RF models consisting of 1000 regression trees were built for each ASV. The importance of each predictor variable, measured as R^2, is assessed by the degradation in performance when each predictor is randomly permuted using a conditional approach that accounts for collinearity between predictors (Ellis et al., 2012). GF aggregates all individual split points from the RF models with positive fits ($R^2 > 0$) to construct empirical distributions that represent species turnover along each environmental gradient. The importance of each predictor to overall compositional turnover is calculated by taking a weighted average of the species-specific conditional predictor importances. This means that species with highly predictive RF models have the greatest influence on the turnover functions (Ellis et al., 2012). The shape of the cumulative distribution curves describes the magnitude of compositional change along the gradient, with the standardized ratio of split density > 1 indicating the highest manifestation of a threshold (Pitcher et al., 2012). The steepest parts of the curve indicate rapid assemblage turnover, while flatter parts of the curve indicate more homogenous regions. The GF models were developed using packages extendedForest (Liaw & Wiener, 2002) and gradientForest (Ellis et al., 2012) in the statistical computing software R (R Core Team, 2022).

We performed a Threshold Indicator Taxa Analysis (TITAN 2.4.1 R package; Baker et al., 2015) in the same way as described above for the macroinvertebrate data. Here, we used it to identify indicator taxa that either increase or decrease along the DOC and nitrate gradients.

We characterised the phylogenetic community composition by calculating the mean nearest taxon distance (α-MNTDobs), which describes the mean phylogenetic distance to the closest relative for each ASV at each site (Fine & Kembel, 2011; Stegen et al., 2012). A null expectation for MNTD was created by placing ASVs randomly across the tips of the phylogeny using “taxa.labels” in the *ses.mntd* function of the R package picante (version 1.8.2; Kembel et al., 2010) and recalculating MNTD to provide a null MNTD value (MNTDnull). This was repeated 999 times, creating a distribution of MNTDnull values to which MNTDobs could be compared. The nearest taxon index (NTI), which quantifies the difference between MNTDobs and mean MNTDnull in units of standard deviation, was calculated with 999 randomisations ([Obs-Exp]/SDExp). For each sample, an NTI value of less than -2 indicates that taxa within the sample are more closely related than expected by chance (phylogenetically clustered), while an NTI value greater than +2 indicates that taxa are more distantly related than expected by chance (phylogenetic overdispersion). These values were then visualised in a linear regression against DOC concentration.

4 Results and discussion

4.1 The DOC gradient and environmental conditions

The water chemistry PCA confirmed multicollinearity of browning related variables, with DOC concentration, absorbance and nutrients having a strong correlation with axis 1 (see Paper II, Fig. 1b). The first two principal components explained 68% of the total variance. PERMANOVA indicated differences among the DOC groups ($F_{2,60} = 14.27$, $P < 0.001$), and all pairwise differences were also significant ($P < 0.001$).

The PCA of the instream variables revealed a size gradient among all the study sites (n=63), with water depth, stream width and substrate size increasing along the first axis and the first two principal components explaining 56% of the total variance (see Paper II, Fig. 1a). PERMANOVA showed that the DOC groups did not significantly differ in instream characteristics ($F_{2,60} = 1.01$, $P = 0.396$).

4.2 Effects of browning on benthic biofilm nutritional quality (Paper I)

4.2.1 Algal biomass

Algal biomass in the 45 study streams was controlled primarily by dissolved inorganic nitrogen (DIN) concentration, which, as the sole significant predictor, provided the lowest BIC value and explained 11.7% of algal biomass (Paper I; Table 3, Fig. 4a). DOC (or any other environmental variable measured) was not an important factor. Phytoplankton in boreal lakes has been shown to be largely nitrogen limited (e.g. Bergström, 2010; Isles et al., 2020; Lau et al., 2021), and a study conducted on northern Swedish streams found that benthic algal biomass was driven by an interaction between DIN availability and incident light levels, while DOC had a minor role (Burrows et al., 2021).

Fork et al. (2020) demonstrated a unimodal response of benthic algal production along a gradient of DOC in northern Swedish lakes, similar to that observed in lake pelagic zones (e.g. Bergström & Karlsson, 2019; Kelly et al., 2018). The unimodal pattern in pelagic primary productivity in lakes reflects a shift from nutrient-limitation to light-limitation, with a highly variable threshold from around five (Seekell et al., 2015) to 15 mg DOC L^{-1} (Bergström & Karlsson, 2019), or even up to 20 mg DOC L^{-1} (Olson et al., 2020). Fork et al. (2020) observed a corresponding shift at 8–9 mg DOC L^{-1} for lake periphyton primary production. They suggested that future benthic primary production will likely show mainly positive responses to increasing DOC in their study lakes, as most of them currently have very low DOC concentrations.

4.2.2 Fatty acids and nutritional quality

Biofilm nutritional quality responded strongly to the DOC-gradient. DOC was the key predictor of the proportion (as % of all fatty acids) of both LSAFAs and ω-3 fatty acids, as well as the ω-3/ ω-6 ratio. For LSAFAs, the relationship was positive (Paper I, Fig. 4b), while for ω-3 fatty acids and the ω-3/ ω-6 ratio, the relationship was negative (Paper I; Table 3, Fig. 4c and d). EPA was related to a combination of DOC (negative relationship), pH, water temperature and DIN (all positive), which together explained 41.9% of the variation in EPA content (Paper I, Table 3). Although the DOC gradient was not the chief predictor for EPA, two of the other factors (pH and nitrogen) are also associated with water browning.

The ω-3/ ω-6 ratio is frequently used as an indicator of resource quality. The strong response to our DOC gradient indicates that the nutritional quality of biofilms suffers substantially with increased DOC concentrations in streams. This is concerning since it has been suggested that quality is much more important than quantity when it comes to trophic transfer in both lakes (Gladyshev et al., 2010) and streams (Guo, Kainz, Sheldon, & Bunn, 2016; Lau et al., 2009; Torres-Ruiz et al., 2007). The aforementioned stream studies also indicate that algal PUFA content is a reliable indicator of basal food nutritional quality.

It has been shown that leaf litter on its own is a relatively poor nutrient resource but is enhanced by the presence of algal biofilm on the leaf surfaces (Guo, Kainz, Valdez et al., 2016). This challenges the traditional view that leaf litter and the associated decomposers (bacteria and fungi), which lack EPA and DHA, form the foundation of lotic food webs in forested landscapes. Instead, it seems that algae, although they may be present only in modest amounts, provide the nutritional base to support secondary production by reducing the EPA limitation of lotic consumers (Crenier et al., 2017; Guo et al., 2021). The algal PUFAs are effectively retained by invertebrate consumers, passing them onto predatory invertebrates and fish in lakes (Lau et al., 2014) and streams (Guo et al., 2018). This trophic support further extends beyond the aquatic environment, enhancing the breeding success of, for example, insectivorous birds feeding on emerging aquatic insects (Twining et al., 2016, 2019). In streams, diatoms appear to take a lead role in providing this basal nutritional support since other key components of stream periphyton, like green algae and cyanobacteria, do not produce long-chain PUFAs that are physiologically important for aquatic invertebrates (Hill et al., 2011; Torres-Ruiz et al., 2007).

4.3 Benthic macroinvertebrate community responses to browning (Paper II)

4.3.1 Benthic macroinvertebrate diversity along the browning gradient

Macroinvertebrate richness showed a gradual reduction in response to DOC and a positive relationship with water temperature (see Paper II, Table 1 and Fig. 2), with a range of between 8 and 40 taxa per site. DOC explained 76% of all variation in the multiple regression model (Paper II, Fig. S2a).

4.3.2 Compositional changes along the browning gradient

An abrupt shift in benthic macroinvertebrate community composition was detected in the threshold indicator taxa analysis (TITAN) when DOC concentrations reached around 12-13 mg L^{-1} (Paper II, Fig. 4a). Only four taxa increased along the DOC gradient (Paper II, Fig. 4b), while 13 sensitive taxa decreased (Paper II, Fig. 4c). The taxa that increased are known generalists that cope well with environmental stressors, e.g., the isopod *Asellus aquaticus* (see O'Callaghan et al., 2019).

These results reiterate the findings of some previous studies that found that drainage-induced changes to streams reduce invertebrate diversity (Ramchunder et al., 2012; Brown et al., 2019; Rajakallio et al., 2021; Koivunen et al., 2023). Jonsson et al. (2017) concluded that DOC and pH were the main drivers of benthic invertebrate richness and community composition in their study of 18 streams in northern Sweden. However, a direct comparison with our results is not possible as their sites only included one stream with a DOC concentration lower than the threshold we detected. With the exception of Koivunen et al. (2023), none of these studies focused directly on browning and were therefore not conducted along an anthropogenically caused browning gradient. Thus, our finding of both a gradual biodiversity loss and an abrupt biodiversity change along a browning gradient are novel, providing a benchmark against which forthcoming changes to stream biodiversity relative to the predicted browning trend can be gauged.

Long-term studies have shown a continuing browning trend in freshwaters (e.g., Asmala et al., 2019; Lapierre et al., 2021), and this is predicted to continue, with absorbance in Swedish lakes set to increase on average by a factor of 1.3 until 2030, in the worst-case scenario (Weyhenmeyer & Karlsson, 2009). With this in mind, our results suggest that a continuing loss of stream macroinvertebrate diversity is inevitable in areas impacted by land drainage and the accompanying water browning. Of course, species loss is not random but depends on species' traits in relation to the type of disturbance (Jonsson et al., 2002). Here, we have demonstrated that only a very small number of species benefitted from browning, while more vulnerable taxa were impacted negatively, many of which are algal scrapers.

4.3.3 Functional feeding groups

Algal scrapers were the only feeding group that showed a strong negative response to DOC, with 33.6% of the variation in scraper abundance being explained by DOC (Paper II, Fig. 3a). The other explanatory factors, to a lesser extent, were water temperature and stream width (Paper II, Table 1). In the multiple regression model, DOC was also very clearly the predominant predictor (75% of explained variation) for this feeding group (Paper II, Fig. S2b). This response was likely linked to an indirect effect of DOC via the key food source of scrapers, benthic biofilm, which is of lower nutritional quality at higher DOC levels, as was discussed above (Paper I). This potential link is further supported by the observation that other feeding groups showed only a weak response to DOC concentration or none at all.

Gatherer-collector abundance was explained almost equally by NO_3-N, DOC and stream width (Paper II; Table 1, Fig. S2c). While the univariate relationship of predator abundance with DOC concentration bordered significance (Paper II, Fig. 3c), only stream width and depth had significant effects when all environmental variables were included (Paper II; Table 1, Fig. S2d). Shredders did not respond to the DOC gradient (Paper II, Fig. 3d).

These observations, combined with those from the fatty acid data above (Paper I), suggest that the loss of benthic biodiversity was likely caused by impairment of the nutritional base of invertebrates as a result of browning.

4.4 Benthic biofilm bacterial community responses to browning (Paper III)

4.4.1 Taxonomic diversity

Univariate regressions showed that ASV richness (Paper III, Fig. 1) and diversity (Paper III, Fig. S1) increased with increasing DOC concentration (Paper III, Fig. 1b), however, this pattern was weak and only significant ($p < 0.05$) for the Shannon diversity of the active community (Paper III, Fig. S1). The overall trend in diversity was similar among the DOC site groupings: high-DOC sites (DOC > 15 mg L^{-1}) supported the highest average number of ASVs per site (total community: mean ± SE: 132 ± 9.7; active community: 88 ± 7.2), followed closely (122 ± 8.7 and 77 ±7.0, respectively) by the moderate-DOC (10-15 mg L^{-1}) sites. Taxa number was the lowest and most variable (107 ± 19.2 and 65 ± 14.1) in the low-DOC (< 10 mg L^{-1}) sites.

This response to the DOC gradient is much weaker and in the opposite direction to the response of macroinvertebrate richness described above (Paper II). Muscarella et al. (2016) observed a decrease in bacterial richness along a DOC gradient in a pond mesocosm experiment, but only in the metabolically active part of the community. This indicates that DOC acted as a strong selective force, favouring taxa able to consume terrestrial-derived DOC, an abundant but low-quality, highly recalcitrant resource. Taxa that lack the metabolic pathway that allows the use of DOC largely disappeared as DOC concentration increased. It is important to note here that Muscarella et al. (2016) studied water-column bacteria in experimental ponds while our focus was on benthic biofilm in small streams. Biofilm bacteria and bacterioplankton are known to exhibit widely differing environmental responses even when occupying physically adjacent habitats within the same water body (Gwoen et al., 2021; Ezzat et al., 2022; Malazarte et al., 2022). In our stream study, DOC had a minor impact on taxa numbers but greatly modified bacterial communities.

4.4.2 Community composition

Pseudomonata (previously *Proteobacteria)* and *Bacteroidota* were the dominant bacterial phyla in all three DOC site categories. *Pseudomonata* was the most abundant phylum in all instances except in the active community of the low-DOC sites (Paper III, Fig. S2), where *Bacteroidota* were slightly more abundant (38%)

than *Pseudomonata* (35%). *Bacteroidota* became relatively less abundant and *Pseudomonata* more abundant in the active community with increasing DOC concentration, while their relative abundances remained almost unchanged in the total community as DOC increased. Of the other bacterial phyla, only *Cyanobacteria* were consistently present and relatively abundant across the gradient. They ranged from 4% in the total community at high-DOC sites to 15% in the active community at low-DOC sites (Paper III, Fig. S2).

The dbRDA ordination of the active bacterial community showed distinct differences between the DOC groups (Paper III, Fig. 2a), which was confirmed by significant global PERMANOVA ($F_{2,52} = 1.768$, $p < 0.001$) and significant pairwise PERMANOVAs (low vs intermediate DOC: $F = 1.554$, $p = 0.006$; low vs high: $F = 2.043$, $p < 0.001$; intermediate vs high: $F = 1.454$, $p = 0.016$). Water temperature $F_{1,47} = 2.700$, $p < 0.001$), DOC ($F = 2.162$, $p < 0.001$), and pH ($F = 1.602$, $p = 0.003$) were the strongest correlates of the ordination solution (Paper III, Fig. 2a).

Results for the total community were similar (PERMANOVA, global test: $F_{2,52} = 1.760$, $p < 0.001$ [Paper III, Fig. 2b]). The low and high-DOC site groups still differed strongly ($F = 1.892$, $p < 0.004$), and the other differences were also significant (both $p < 0.04$) but less distinct. Water temperature ($F_{1,49} = 3.464$, $p < 0.001$), total phosphorous concentration ($F = 2.537$, $p < 0.001$), pH ($F = 2.510$, $p < 0.001$) and specific ultraviolet absorbance (SUVA; $F = 1.811$, $p < 0.002$) were the strongest drivers of community differentiation (Paper III, Fig. 2b).

Concordance between the ordination configurations of benthic bacterial and invertebrate communities was very strong, indicating that water browning exerted substantial control over both bacteria and macroinvertebrates (see Paper III, Fig. S3). The concordance was more distinct for the active bacterial community (ProTest: $m^2 = 0.209$; $r = 0.889$, $p < 0.001$) than for the bulk community ($m^2 = 0.573$; $r = 0.654$, $p < 0.001$).

4.4.3 Compositional turnover along environmental gradients

The overall importance measure in the gradient forest analysis identified nitrate-N and DOC concentrations as the most important predictors of the compositional turnover in the bacterial communities (Paper III, Fig. 3). Thus, all further analyses focused on these two variables.

For nitrogen, the peaks in the standardized split density plot showed that the greatest change in bacterial community composition occurred between 75 and 100 $\mu g\ NO_3\text{-}N\ L^{-1}$, with a smaller secondary peak at very low concentrations around 25 µg NO_3-N L^{-1} (Paper III, Fig. 4a). For DOC, we saw a major change in highly brownified waters (> 20 mg L^{-1}; Fig. 4b), and a second change point at very low DOC concentrations at 4-7 mg L^{-1}. Many species had major changes in relative abundance at 45 to 90 µg NO_3-N L^{-1} (Paper III, Fig. 4c) and at very high DOC concentrations (Paper III, Fig. 4d). The overall compositional turnover functions, which average the individual curves for the bacterial community, indicate the same ranges of the key predictors where the individual ASVs responded strongly (Paper III; Fig. 4e and Fig. 4f).

TITAN included 263 ASVs and identified 22 significant indicators of increasing DOC concentration, while only seven decreased significantly along the DOC gradient. The greatest increasers had median occurrence at > 20 mg DOC L^{-1}, although some had a lower limit as low as 12 mg L^{-1}. Five out of six of these taxa were *Pseudomonata*, with one ASV from phylum *Bacteroidota* (Fig. S4b). The three strongest decreasers, with a median occurrence at 8 to 12 mg L^{-1} and an upper limit at < 14 mg L^{-1}, represented the orders *Planctomycetales* (phylum *Plantomycetota*) and *Rhizobiales* (*Pseudomonata*), and the family *Saprospiraceae* (*Bacteroidota*) (Fig. S4a).

For nitrate-N, TITAN identified 11 ASVs, which decreased in relative abundance with increasing NO_3-N, with median occurrence at 3.5 to 8 µg L^{-1}. All of these taxa disappeared completely when the NO_3-N concentration reached 15 µg L^{-1} (Fig. S5a). Ten of these were *Pseudomonata* (mostly *Gamma*-, with one *Alphaproteobacteria*), and only one was from the phylum *Bacteroidota*. TITAN reported six significant increasers, but these did not show well-defined peaks of occurrence and were all rather widely distributed (Fig. S5b).

The initial community shift at 3 to 7 mg L^{-1} we see in our results is similar to the community change observed by Roiha et al. (2016) in their study of bacterial communities in a subarctic watershed, where DOC quantity and quality exerted strong control over community composition. Judd et al. (2006) also found that the experimental addition of terrestrial-derived DOC caused a significant change in both bacterial production and community composition. The second, much larger community shift in our data occurred in highly brownified streams. Many bacterial taxa are known to remain metabolically active in such brown-coloured waters (e.g., Berggren et al., 2010; Jones & Lennon, 2015), although with greatly reduced productivity. This is mainly because of the high energetic costs of producing extracellular enzymes required for the decomposition of terrestrial organic matter (Fitch et al., 2018). As bacterial community composition changes with water browning, stream ecosystem functioning, particularly the degradation of terrestrially derived DOM, likely changes concomitantly (see Logue et al., 2016).

Rofner et al. (2017) conducted a lake microcosm study, where the addition of soil organic matter caused a community shift in bacterioplankton. They attributed this change to initially rare taxa gaining dominance with increased soil organic matter. Species sorting was also operating in our study system, but the regional species pool seemed to be dominated by taxa well adapted to high-DOC environments, reflecting the fact that streams in the Iijoki basin drain extensive peatlands where even pristine streams have relatively high DOC concentrations (see Paper II).

Nitrogen concentration has frequently been reported as a key determinant of bacterial community composition in stream biofilm and sediment bacteria (e.g., Roberto et al., 2018; Pilgrim et al., 2022; Smucker et al., 2022). These findings come mostly from urban or agricultural watersheds where nutrient enrichment is usually much higher than in our streams; accordingly, threshold values for community change in these studies were also much higher. Pilgrim et al. (2022) detected significant changes in bacterial communities between 462 and 695 µg TN L^{-1}, and Smucker et al. (2022) over a range of 275–855 µg L^{-1}. The fact that these authors only reported total nitrogen complicates a direct comparison between their values and ours. However, Lehosmaa et al. (2021) also found an abrupt community shift in southern Finnish urban springs at around 400 µg NO_3-N L^{-1}. In our data, a major community shift occurs at concentrations below 100 µg L^{-1}. This value is well below the threshold concentration of 450 µg L^{-1} for nitrate-N, considered indicative of reference conditions in Finland (Aroviita et al., 2012). Nutrient enrichment and browning usually come hand in hand in drainage impacted streams. Drainage increases the transportation of both organic carbon and nutrients from managed peatlands (Marttila et al., 2018; Härkönen et al., 2023). Drained peatland forests may even create hot spots for a further increase in nutrient and carbon loading of boreal watercourses (Marttila et al., 2020).

For the macroinvertebrate community, we detected an abrupt shift at around 12-13 mg L^{-1} of DOC. At the same time, we observed a rapid change in biofilm bacterial communities at very low levels of DOC and another major shift in highly brownified streams (DOC > 20 mg L^{-1}). Although the range of DOC where the greatest shift in community composition occurred differed between these two taxonomic groups, both highlight the integral role of terrestrial-derived organic carbon in causing a biodiversity change in headwater streams.

4.4.4 Phylogenetic clustering and turnover of bacterial communities along the browning gradient

The mean nearest taxon index remained below 0 across the whole DOC gradient, increasing towards the highest DOC concentrations (Paper III, Fig. 5). This indicates that bacterial communities were phylogenetically more similar than expected by chance at very low DOC concentrations and became even more clustered as the water colour continued to darken.

The ability of bacteria to degrade high molecular weight organic material is thought to be taxonomically restricted (Bertilsson et al., 2007; Fitch et al., 2018). For example, it has been shown that functional dissimilarity among freshwater bacterioplankton communities was lowest at sites characterized by a high input of terrestrial DOM (Ruiz-González et al., 2015). In our data, almost all (21 out of 22) of the significant indicators for high-DOC sites were *Pseudomonata*, while the seven low-DOC indicators included three different phyla.

5 Conclusions and future directions

Browning had a strong impact on both macroinvertebrate and bacterial communities. While the shifts we observed differed between the macroinvertebrate and bacterial data, it was clear that DOC acted as a strong selective force in both communities. For macroinvertebrates, I detected a clear threshold at around 12-13 mg DOC L^{-1}. For bacteria, I found two shifts: one at very low DOC concentrations (~ 6 mg L^{-1}) and one at relatively high concentrations (> 20 mg L^{-1}). Furthermore, the availability of essential fatty acids was heavily compromised with increasing DOC concentrations.

With these clear changes occurring in stream ecosystems caused by drainage-induced browning, it raises the question of better protection for our freshwater systems from these impacts. In a country like Finland, it might be easy to take freshwater ecosystem services like high-quality drinking water and recreation for granted, but the browning trend is putting these at risk (Albrecht et al., 2023). From a management perspective, such research-based thresholds can be used in monitoring and bioassessment of a stream's ecological status, which can further inform management actions like restoration or prioritisation for conservation.

Peatland restoration can be expected to reduce the DOC loads in boreal streams in the long term or at least halt the increasing exposure. There is some evidence that peatland restoration by means of blocking drainage ditches causes a short-term increase in nutrient loads (Koskinen et al., 2017). This may have some initial negative impacts on stream communities and food webs, but the long-term consequences remain to be thoroughly investigated. Since peatland restoration is becoming an increasingly common activity (Humpenöder et al., 2020), there is a need to address these critical questions.

While the connectivity between streams and forestry practices reaches much further than just the immediate vicinity of the stream, riparian zones have been shown to be important drivers of stream ecosystem health (Suurkuukka et al., 2014). It is also known that riparian buffer zones protect streams from the influx of DOC associated with forest harvesting (Jyväsjärvi et al., 2020). It is no secret that headwater streams and riparian forests are inextricably linked in terms of functioning and biodiversity in both systems. It would be interesting to further investigate the effects of browning beyond the brown waters to see how the effects might cascade through to terrestrial consumers at the water's edge. For example, by means of fatty acid analysis of not only the communities within the streams, but also consumers in the riparian zone that feed on emerging invertebrates from the water. If stream macroinvertebrates, their food resources, and their riparian consumers are analysed for nutritional quality within a similar browning gradient, one could elucidate whether the nutritional degradation we saw in the periphyton translates further up the food web.

Another factor to consider when investigating browning is how it might interact with other changes occurring in these ecosystems. Climate change is predicted to alter the flow regimes of boreal streams, with flow-induced stress expected to become prolonged and more frequent, meaning longer periods of drought and flooding (Mustonen et al., 2016). Stressors can interact and have unexpected effects on ecosystems when acting in concert (e.g. Matthaei et al., 2010; Nuy et al., 2018; Tolkkinen et al., 2015; Townsend et al., 2008). There might be synergies or antagonistic interactions between the stressors or even reversals of the individual effects (Jackson et al., 2016). Thus, another important direction of future work would be to examine the cumulative effects of browning and climate change, and to test, for example, if browning affects the resilience of stream communities and ecosystem functions to drought.

Finally, this work adds to the mounting evidence of the detrimental effects of current land use practices and it informs future decisions about policies regarding land use, the protection of boreal freshwater systems and the goods and services they provide.

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I Jyväsjärvi, J., Rajakallio, M., Brüsecke, J., Huttunen, K.-L., Huusko, A., Muotka, T., & Taipale, S. J. (2022). Dark matters: Contrasting responses of stream biofilm to browning and loss of riparian shading. *Global Change Biology, 28*, 5159–5171.

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III Brüsecke, J., Muotka, T., Huttunen, K.-L., Lehosmaa, K., & Jyväsjärvi, J. (manuscript). Biofilm bacterial communities along a gradient of browning in boreal headwater streams.

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