

Investigating tools for biomonitoring browning in streams: chlorophyll content of *Fontinalis antipyretica*

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Abstract – The chlorophyll content of the bryophyte species *Fontinalis antipyretica* was studied in seven small boreal streams of varying water quality. The aim was to determine whether the chlorophyll content of *F. antipyretica* responds to increasing water colour, and if the bryophyte could thus be used as a bioindicator for browning in stream environments. Significant variation in the CHL contents (CHL $a+b$, CHL $a:b$) of *F. antipyretica* was observed between streams of differing water colour. Despite this, no significant effect of water colour or the dissolved organic carbon (DOC) concentration on the CHL contents (CHL $a+b$, CHL $a:b$) was observed. However, the CHL $a:b$ ratio decreased significantly with increasing light attenuation coefficient, thus suggesting that light is an important factor causing physiological changes in *F. antipyretica*. The DOC concentration explained one-third of the light attenuation coefficient, whilst no significant effect of water colour was found. Our findings suggest that whilst the CHL $a:b$ of *F. antipyretica* can indicate changes in light conditions, it does not directly show a clear pressure-response association with increased water colour or DOC concentration.

Keywords: Bioindicator / bryophyte / lotic waters / brownification / Water Framework Directive

Highlights

- CHL content of *F. antipyretica* varied between streams of varying water colour.
- Variation in CHL content was traced to differences in light availability.
- Water colour or DOC concentration did not affect CHL content of *F. antipyretica*.
- CHL content of *F. antipyretica* is probably not suitable indicator for browning.

1 Introduction

Throughout the Northern Hemisphere an increase in water colour, a phenomenon called browning, has been observed in freshwaters during recent decades (Monteith *et al.*, 2007; Weyhenmeyer *et al.*, 2016). Browning is caused by increased loading of dissolved organic carbon (DOC) and iron (Fe) from the catchment (Monteith *et al.*, 2007; Kritzberg and Ekström,

2012), and it is strongly driven by climate change-induced phenomena, such as increased precipitation and elevated temperature (Weyhenmeyer and Karlsson 2009; de Wit *et al.*, 2016). The role of land-use activities, especially ditching and forestry operations, is also prominent (Estlander *et al.*, 2021), whilst decreased atmospheric sulphate deposition also impacts browning due to the increased solubility of organic matter from the soils (Evans *et al.*, 2012; Finstad *et al.*, 2016).

Browning has several detrimental effects on aquatic ecosystems. It decreases light penetration in the water column and changes its spectral composition by increasing the absorption of short-wavelength light (Eloranta, 1999). The decrease in photosynthetically important short-wavelength light consequently decreases primary production, changing ecosystems from autotrophic to more heterotrophic (Thrane *et al.*, 2014). Particularly in lakes, thermal stratification becomes more steep as the darker water absorbs more solar irradiance (Eloranta, 1999).

The increased DOC concentration decreases water pH, which can be especially harmful to vulnerable stream ecosystems (Petrin *et al.*, 2007). Sedimentation rate increases as more organic matter is descending into streams from catchments, reducing macrophyte growth surfaces and burying deadwood, making the bottom an unsuitable habitat for many species (Kuglerová *et al.*, 2021).

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Although browning has several negative effects on both lake and stream ecosystems, it has not been sufficiently taken into account in water monitoring programmes (Sepp *et al.*, 2018; Albrecht *et al.*, 2024; Horppila *et al.*, 2024). For example, the monitoring programmes of the European Water Framework Directive (WFD) have come under scrutiny because the biological parameters currently used in the WFD are mainly targeted for monitoring eutrophication and are not suitable for monitoring browning (Sepp *et al.*, 2018; Horppila *et al.*, 2024). The need for new bioindicators is urgent especially in streams, because their rapidly changing hydrology makes physical and chemical monitoring methods insufficient for reflecting long-term environmental changes (Li *et al.*, 2010). Even though biological monitoring methods—such as the use of diatoms, zoobenthos, and fish—have been developed for streams and rivers for example in Finland, they face the same issue as those used in lakes: the indicators are primarily designed to monitor eutrophication (Sepp *et al.*, 2018; Horppila *et al.*, 2024).

Due to human activities, many small streams are also very disturbed ecosystems whose higher land-water interface make them more vulnerable to changes in catchment land-use than lakes (Tolkkinen *et al.*, 2020; Kuglerová *et al.*, 2021). Since disturbances in small streams also accumulate into downstream bodies of water (Asmala *et al.*, 2019), no restoration methods can be done in these downstream areas without having to monitor the status of smaller streams (Gomi *et al.*, 2002; Räike *et al.*, 2016).

One of the important biological quality elements (BQEs) in the status assessment of waterbodies is macrophytes (Penning *et al.*, 2008). In contrast to macrofauna, which can to some extent migrate to more suitable habitats, macrophytes are sessile organisms that cannot escape environmental stressors, making them particularly important as bioindicators of environmental change (Bhatta and Patra, 2020). In reference to tracking the effects of browning, one of the macrophyte-based bioindicators suggested is the chlorophyll *a* to *b* ratio (CHL *a*:*b*) of the floating-leaved macrophyte species yellow water-lily (*Nuphar lutea* (L.) Sm.) (Horppila *et al.*, 2022; Rajala *et al.*, 2024, 2025). The potential of this bioindicator arises from the adaptation mechanism of plants as primary producers to decreasing light (Kume *et al.*, 2018). With decreasing short-wavelength light, a phenomenon that is specifically linked to browning, macrophytes adapt to changing light conditions by increasing their CHL *b* concentration in relation to CHL *a* concentration (Barko and Filbin, 1983; Horppila *et al.*, 2022; Rajala *et al.*, 2024).

In comparison to the macrophyte BQEs currently employed in the WFD, which rely on the macrophyte community composition analysis, utilizing a macrophyte physiology-based indicator would offer several advantages. Species community composition analyses can be vulnerable to methodological variation arising from biases in taxonomical identification (Lyche-Solheim *et al.*, 2013). In addition, due to the spatial variability in macrophyte abundance, several sampling stations are needed for macrophyte community composition analysis (Lyche-Solheim *et al.*, 2013). For the macrophyte physiology-based monitoring method, CHL *a*:*b*, the spatial variability within lakes is low, and the method expresses a consistent seasonal variation across lakes with

differing water quality, thus enabling a uniform standardization of the sampling process (Rajala *et al.*, 2024, 2025).

A species commonly used for stream biomonitoring is the bryophyte greater water-moss (*Fontinalis antipyretica* Hedw.) (Alaoui *et al.*, 2021). *Fontinalis antipyretica* exhibits wide ecological tolerance, is widely distributed, long-lived, tolerant of pollution and has a high accumulation capacity, which is why it has been considered as a good bioindicator *e.g.* for metal pollution (Vuori and Muotka, 1999; Alaoui *et al.*, 2021). Due to its excellent biomonitoring properties, it could also serve as a good indicator for browning. However, since *F. antipyretica* shows several physiological differences compared with vascular plants, the impact of increasing water colour on their CHL contents (CHL *a*+*b*, CHL *a*:*b*) remains obscure. Bryophytes lack conductive systems, which changes the way they regulate nutrients and light, as well as capture CO₂ (Glime, 2014). In addition, *F. antipyretica* is a shade-adapted plant, which can also impact its responses to decreasing light (Proctor and Smirnov, 2011). Therefore, the effects of changing light conditions on *F. antipyretica* were examined in this study. The aim of the study was to investigate, if the water colour of the stream effects chlorophyll content (CHL *a*+*b*/*a*:*b*) of *F. antipyretica*, and if the CHL content could thus be used as an indicator of browning in streams. The specific research questions were: 1) Does the CHL content (CHL *a*+*b*/*a*:*b*) of *F. antipyretica* differ between streams? and 2) Does the water colour, DOC concentration or light attenuation coefficient (*K_d*) affect the CHL content (CHL *a*+*b*/*a*:*b*) of *F. antipyretica*.

2 Materials and methods

2.1 Selected study sites

The study was conducted in July 2023 in seven small streams located near the Evo district (61° 13'N, 25° 12'E) (Fig. 1). Due to the limited knowledge on the ecological state of small streams, we selected the streams with catchment areas less than 100 km². In order to study the effect of different water colour on the CHL content of *F. antipyretica*, we selected streams with as wide water colour range as possible. Sampling sites were chosen to ensure minimal shading of the overlying canopy and by selecting open areas. Other vegetation within the sampling areas was limited, and it was mainly composed of *F. antipyretica* and *N. lutea*, except in Heinäjoki, Mustajoki and Ormijoki, where *N. lutea* was absent in the sampling site. The wetted width at the sampling site varied between 1.3 m and 9 m, and all the streams were characterized with a coarse-grained bottom.

2.2 Macrophyte sampling and analysis

Submerged macrophytes (*F. antipyretica*) were collected from the running water area of each stream. Three replicate samples were collected from a 10 cm water depth, placed in dark plastic bags, and stored in cool, dark conditions (+4 °C) until they were taken to the laboratory on the same day. In the laboratory, the *F. antipyretica* samples were washed with tap water to remove periphyton. For the CHL *a*+*b* analysis, 1 cm × 1.5 cm of phyllids were cut, wrapped in aluminium foil



Fig. 1. Locations of the study lakes.

and stored in a freezer (-18°C) until analysis. According to recent studies, freezing is the most adequate method to preserve CHL samples prior analysis (Villares *et al.*, 2024). After freezing, the samples were macerated, and then weighted samples were extracted in 95% ethanol at 75°C for 5 min, following ultrasound treatment (Horppila *et al.*, 2022). The extract was also filtered using Whatman GF/C filters (pore size $1.2\ \mu\text{m}$), to remove particulate matter. The CHL *a* and *b* concentrations from the *F. antipyretica* samples were calculated with the equation of Lichtenthaler and Wellburn (1983):

$$C_{\text{Chla}} = 13.95 \times A_{665\text{nm}} - 6.88 \times A_{649\text{nm}}$$

$$C_{\text{Chlb}} = 24.96 \times A_{649\text{nm}} - 7.32 \times A_{665\text{nm}}$$

where *C* is the chlorophyll $\mu\text{g ml}^{-1}$ plant extract, and *A* is the measured absorbance at different wavelengths.

2.3 Physicochemical sampling

Water samples for water colour and DOC analyses were taken with a bottle sampler from the same location as the bryophyte samples. The water colour analysis was conducted with a Shimadzu UV-1800 spectrophotometer (Shimadzu

Table 1. Water quality parameters and CHL content of *Fontinalis antipyretica* of study streams.

	Evojoki	Haarajoki	Heinäjäki	Luutajoki	Majajoki	Mustajoki	Ormijoki
Water colour (mg Pt l ⁻¹)	117	94	60	79	151	158	37
DOC (mg l ⁻¹)	13	11	10	7	17	12	9
K_d (m ⁻¹)	1.6	3.0	0.7	0.2	0.8	1.8	0.7
pH	6.6	6.8	6.3	6.5	6.1	6.7	6.4
Temperature (°C)	21	15	20	16	21	15	20
CHL $a+b$ (µg mg ⁻¹)	0.78	1.26	0.83	1.01	1.66	0.91	1.27
CHL $a:b$	2.00	1.85	2.10	2.26	2.10	2.17	1.98

Table 2. Results from the analysis of variance: interstream differences in water colour, DOC concentration and CHL $a+b$ concentration and CHL $a:b$ of *Fontinalis antipyretica*.

Variable	df	Sum of squares	Mean square	F	<i>p</i>
Water colour	6	36983	6164	1250	<0.001
DOC	6	1.1828	0.19714	1446	<0.001
CHL $a+b$	6	1.3327	0.22212	4.626	0.009
CHL $a:b$	6	0.0684	0.01140	6.114	0.003

Corporation, Kyoto, Japan) as the absorbance of light at a 410 nm wavelength. After the spectrophotometric analysis, the results were converted to mg Pt l⁻¹, following the standard SFS-EN ISO 7887 (Finnish Standards Association, 2011). The DOC concentration was measured with a Shimadzu TOC 5000A analyser, following the standard SFS-EN 1484 (Finnish Standards Association, 1997).

In addition to the water samples, the water temperature and pH were measured with a YSI 6600 V2 sonde (YSI Inc., Yellow Springs, OH, USA), and the light intensity in the photosynthetically active radiation (PAR) wavelengths (400–700 nm) at the water column was measured with an LI-1500 data logger equipped with an LI-192SA Quantum Sensor (LI-COR Biosciences, Lincoln, NB, USA). The K_d was calculated with the equation:

$$K_d = \frac{\ln \frac{I_0}{I_Z}}{Z}$$

where I_0 and I_Z are light intensities just below the surface and at depth Z (0.1 m), respectively (Scheffer, 1998).

2.4 Statistical analysis

The interstream differences in the water colour, DOC concentration and CHL $a+b/a:b$ of *F. antipyretica* were studied with a one-way analysis of variance (ANOVA) and with Bonferroni t-tests. Prior to the ANOVA, the Shapiro–Wilk normality test and Levene's test for homogeneity of variance were computed. Based on these analyses, all data except for water colour were ln-transformed to improve normality.

Although DOC is usually the main determinant of water colour, the effects of DOC and water colour on light attenuation may differ as a result of Fe bound to organic substances (Horppila *et al.*, 2023). Therefore, the effect of water colour, DOC concentration and K_d on the CHL $a+b/a:b$

content of *F. antipyretica* was studied with a linear regression analysis. Additionally, linear regression analysis was used to investigate, whether the water colour and DOC concentration affect K_d in the streams. For the linear regression analysis, all data were ln-transformed prior analysis. All data analyses were computed with RStudio version 4.4.0, using the packages rstatix, PMCMRplus and ggplot2.

3 Results

3.1 Physicochemical characteristics of streams

During the sampling period, the water temperature in the streams varied between 15 °C and 21 °C, with the lowest temperature observed in Haarajoki and Mustajoki, and highest in Evojoki and Majajoki (Tab. 1). On average, the water temperature in the streams was 18 °C. Streams were characterized with a slightly acidic pH, ranging from 6.1 to 6.8 (Tab. 1).

Both the water colour and DOC concentration varied significantly between streams (Tab. 2), with the lowest water colour in Ormijoki (37 mg Pt l⁻¹) and the highest in Mustajoki (158 mg Pt l⁻¹) (Tab. 1). DOC concentration was lowest in Luutajoki (7 mg l⁻¹) and highest in Majajoki (17 mg l⁻¹) (Tab. 1). The K_d at 10 cm water depth was on average 1.3 m⁻¹, with lowest K_d observed in Luutajoki (0.2 m⁻¹) and highest in Haarajoki (3.0 m⁻¹) (Tab. 1). The DOC concentration explained 30% of the K_d ($F_{1,19}=8.183$, $R^2=0.301$, $p=0.01$), but surprisingly, the effect of water colour on K_d was not quite significant ($F_{1,19}=3.353$, $R^2=0.15$, $p=0.083$).

3.2 Chlorophyll content (CHL $a+b$, CHL $a:b$) of *F. antipyretica*

The CHL $a+b$ concentration of *F. antipyretica* varied significantly between streams (Tab. 2), showing the highest

Table 3. Results from the linear regression analysis: the effect of water colour, DOC concentration and K_d on CHL $a+b$ concentration and CHL $a:b$ of *Fontinalis antipyretica*.

Dependent	Independent	$F_{1,19}$	R^2	p
CHL $a+b$	Water colour	0.010	0.001	0.9215
	DOC	1.110	0.055	0.3054
	K_d	0.048	0.003	0.8283
CHL $a:b$	Water colour	0.823	0.042	0.3757
	DOC	0.551	0.028	0.4670
	K_d	10.654	0.359	0.0040

concentration in Majajoki ($1.66 \mu\text{g mg}^{-1}$) and the lowest in Evojoki ($0.78 \mu\text{g mg}^{-1}$) (Tab. 1). No effect of water colour, DOC concentration or K_d on the CHL $a+b$ concentration was observed (Tab. 3, Fig. 2).

The CHL $a:b$ ratio of *F. antipyretica* also varied significantly between streams (Tab. 2), with the highest ratio observed in Luutajoki (2.26) and the lowest in Haarajoki (1.85) (Tab. 1). Neither water colour nor DOC concentration influenced the CHL $a:b$ ratio of *F. antipyretica*. Instead, the ratio decreased significantly as the K_d increased ($F_{1,19} = 10.654$, $R^2 = 0.359$, $p = 0.0040$) (Tab. 3, Fig. 2).

4 Discussion

The CHL content of *F. antipyretica* varied between streams, and the variation in light intensity was reflected in the CHL content, since the CHL $a:b$ significantly decreased with increase in K_d . Whilst the total CHL $a+b$ concentration of *F. antipyretica* showed significant interstream variation, this variation was not significantly affected by water colour or DOC concentration, thus contradicting with previous studies done with other macrophyte species (Barko and Filbin, 1983; Valladares and Niinemets, 2008). The increase in the CHL $a+b$ concentration can be an adaptation mechanism of macrophytes to decreased light (Barko and Filbin, 1983; Valladares and Niinemets, 2008), but the CHL $a+b$ concentration also expresses higher background variation – *i.e.* variation that is not traced to differences in water quality – due to *e.g.* plant age (Wood and Bachelard, 1969; Kok *et al.*, 1990), or the changing depth distribution of photosynthetic pigments (Horppila *et al.*, 2022). Therefore, we did not find a significant relationship between CHL $a+b$ of *F. antipyretica* and the water colour, DOC or K_d .

Corroborating several previous studies (Pintado *et al.*, 1997; Klimenko, 2012; Horppila *et al.*, 2022; Rajala *et al.*, 2024), the CHL $a:b$ of *F. antipyretica* decreased with decreasing light availability. As sessile organisms, macrophytes have developed several mechanisms for adapting to changing light conditions, with adjusting of CHL pigments being one of the most profound (Pintado *et al.*, 1997; Zhao *et al.*, 2020). Under low light, an increase in the CHL b concentration enables macrophytes to be more efficient in capturing photosynthetically important short-wavelength light (Yamazaki *et al.*, 2005). In contrast, macrophytes under high light intensity decrease the overall CHL concentration to avoid cellular damage (Kume *et al.*, 2018).

Contrary to our expectations, water colour or the DOC concentration were not significant regulators of the CHL $a:b$ of *F. antipyretica*. The CHL $a:b$ of *F. antipyretica* decreased significantly only with increasing K_d between streams. DOC concentration significantly explained the variation in K_d ; however, unexpectedly, no significant relationship was found between water colour and K_d in our study. This difference most likely arises from the methodological differences. Whilst water colour is determined spectrophotometrically based on the absorbance of 410 nm wavelength of light, K_d is calculated based on the entire light spectrum in the water column. Unlike DOC and K_d , water colour and K_d did not show a significant relationship, which suggests that factors influencing light attenuation outside the 410 nm wavelength affected the light attenuation in the water column. This other factor is most likely Fe which, when bound to DOC, has a strong effect on the light attenuation of short-wavelength light (Xiao *et al.*, 2013; Horppila *et al.*, 2023). The effect of DOC-bound Fe on the light attenuation of short-wavelength light is not observed in water colour measurements because it is only based on the light absorption at 410 nm. Because K_d considered the whole light spectrum, the effect of DOC-bound Fe can be seen as significant. This phenomenon can be particularly prominent in high-coloured streams, where the average Fe concentrations have been shown to reach up to 1.4 mg l^{-1} (Turunen and Aroviita, 2024), being almost twice as high as in lakes (0.76 mg l^{-1}) (Horppila *et al.*, 2023). In addition, suspended solids can influence K_d especially in small streams, where the samples are taken closer to the bottom substrate than in lakes.

The differing responses in the CHL $a+b/a:b$ content of *F. antipyretica* compared to previous studies done on vascular plants (Horppila *et al.*, 2022; Rajala *et al.*, 2024, 2025), were likely due to physiological differences in these plant groups. Bryophytes are shade-adapted plants, and the light saturation in bryophytes already occurs at moderate light levels (Glime, 2014). Compared to vascular plants, bryophytes generally have higher CHL $a+b$ concentration and lower CHL $a:b$ ratio (Dar *et al.*, 2013). This trend was also observed in the current study: *F. antipyretica* had an average CHL $a+b$ concentration of $0.78 \mu\text{g mg}^{-1}$, which is 2.69-fold higher than that of *N. lutea* ($0.29 \mu\text{g mg}^{-1}$) reported by Rajala *et al.* (2024). Similarly, the CHL $a:b$ ratio of *F. antipyretica* was approximately 25% lower compared to *N. lutea* (2.83) (Rajala *et al.*, 2025). This refers to an adaptation mechanism of submerged species that enables their survival under lower light conditions (Dar *et al.*, 2013; Estlander *et al.*, 2025). These physiological adaptations can change the way *F. antipyretica* reacts to changing light conditions.

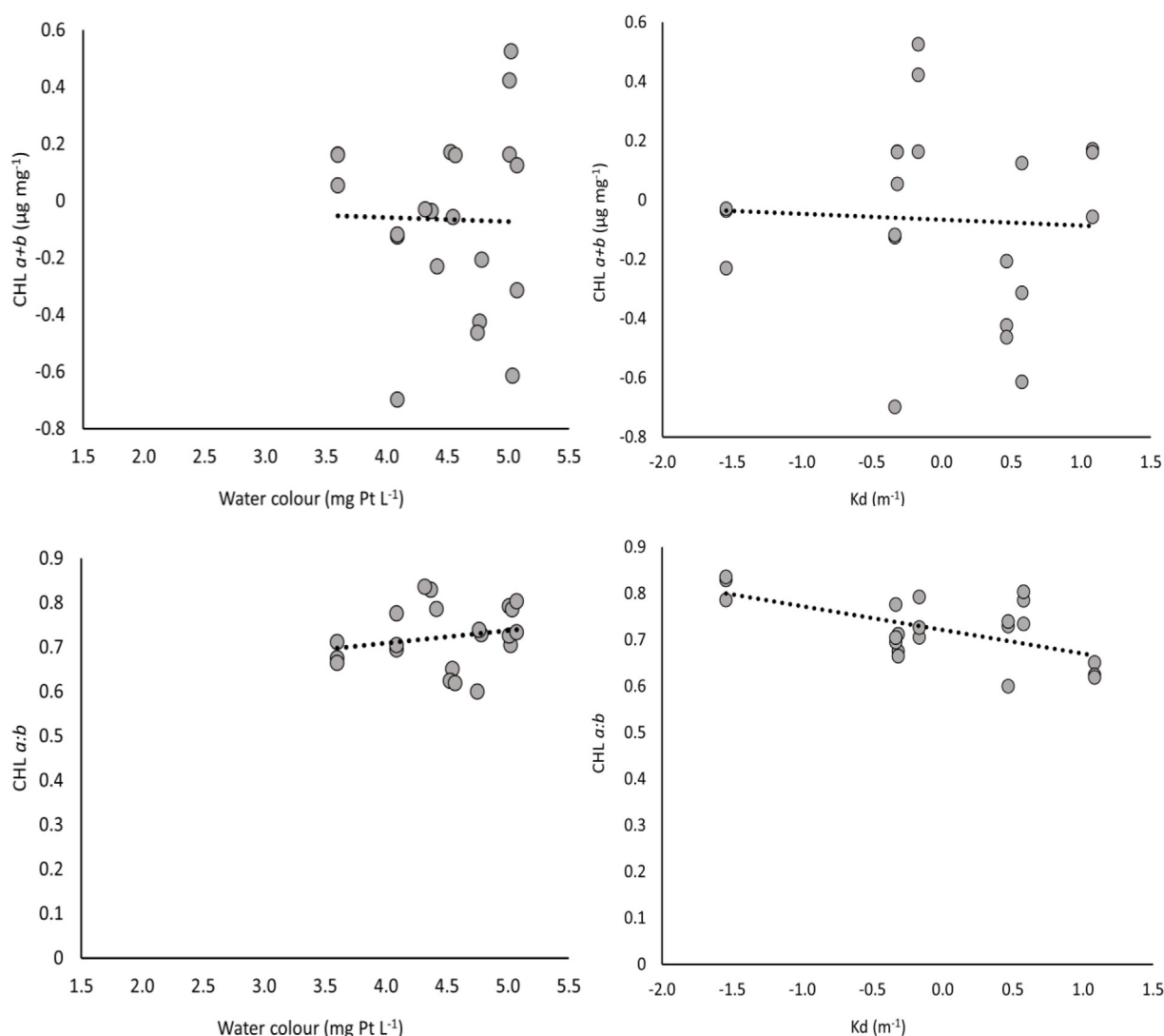


Fig. 2. Effect of water colour (left-side panels) and K_d (right-side panels) on CHL *a+b* concentration (upper panels) and CHL *a:b* (lower panels) of *Fontinalis antipyretica*. All data are ln-transformed.

Developing a new bioindicator to monitor browning of streams is necessary because, compared to lakes, running waters are even more vulnerable, *e.g.* to land-use changes inducing browning (Kuglerová *et al.*, 2021). The bioindicator should thus show a clear response specific to the environmental problem, such as increasing water colour, that it has been chosen to monitor (Cooper *et al.*, 2009; Poikane *et al.*, 2018). In the current study, a clear pressure-response association between CHL *a:b* and water colour or DOC concentration was not detected. CHL *a:b* of *F. antipyretica* indicated changes in K_d but did not reflect changes in the water colour. This hampers the feasibility of CHL *a:b* of *F. antipyretica* as a reliable indicator of browning. Instead of *F. antipyretica* CHL content, one suggested indicator for monitoring stream browning could be the photosynthetic pigment content of periphyton. As primary producers, the function of periphyton is controlled by light availability, and they are currently used as a biological quality element in the WFD (Sabater *et al.*, 2007). Periphyton is considered to be a good early warning indicator of environmental changes due to acknowledged rapid response to

environmental change (Montuelle *et al.*, 2010), and therefore it would be a suitable bioindicator in streams with rapidly changing water quality.

5 Conclusions

In the current study, we did observe a significant difference in the CHL content (CHL *a+b/a:b*) between streams. However, neither water colour nor DOC concentration affected CHL *a+b/a:b* of *F. antipyretica*. Light attenuation coefficient (K_d) did not affect CHL *a+b* concentration of *F. antipyretica*, but the CHL *a:b* ratio decreased significantly with increasing K_d . DOC concentration explained one-third of the K_d , but no significant effect of water colour on K_d was found. This was most likely due to methodological differences between the water colour measurement and K_d calculation. Water colour measurements at 410 nm do not capture the effect of DOC-bound Fe, which absorbs short wavelengths but is reflected in K_d that considers

the full light spectrum. As no clear pressure-response relationship between water colour or DOC concentration and CHL content of *F. antipyretica* was found, more research is needed to develop specific bioindicators to detect browning responses in stream environments. A potential bioindicator for stream browning could be the photosynthetic pigment content of periphyton as periphyton communities are known for rapid responses to water quality changes.

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Conflicts of interest

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

Data availability statement

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

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