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PRIMARY RESEARCH PAPER

Influence of storage method on the content of photosynthetic pigments of the aquatic moss *Fontinalis antipyretica*

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Abstract Aquatic bryophytes are frequently used as bioindicators of water quality in rivers. Poor water quality increases physiological stress in moss, and stress levels can be estimated by measuring the concentrations of photosynthetic pigments and by calculating pigmentary indices. However, studies collecting many samples in a short time may need to store them until analysis. In the meantime, the pigments may suffer some degradation. Given the lack of studies on this problem for aquatic bryophytes, this one investigates the effect of three storage methods (refrigerated at 6 °C, frozen at − 20 °C and dried at room temperature) on the concentrations of chlorophyll *a*, chlorophyll *b*, pheophytin *a* and the following ratios: chlorophyll *a*/chlorophyll *b*, D430/D410, D665/D665*a*, D430/D665 and D480/D665 (where Dx is the absorbance at x nm) in the aquatic moss *Fontinalis antipyretica*. The results showed that refrigeration was the most suitable method, freezing might be suitable for some parameters, and drying was inadequate for this purpose. We recommend that all studies on photosynthetic pigments detail the time elapsed from collection to sample analysis, as well as the storage method used.

Keywords Chlorophyll · Pheophytin · Pigment ratios · Bioindicators · Bryophytes · Rivers

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Introduction

Aquatic bryophytes are excellent pollutant accumulators (Philips, 1980; Zechmeister et al., 2003) and are commonly used as biomonitors of river water quality (e.g. Vázquez et al., 2007; Villares et al., 2016; Gecheva et al., 2020). *Fontinalis antipyretica* is one of the most frequently used mosses (e.g. Debén et al., 2017; Bolsunovsky et al., 2020; Alaoui, 2021). Pollutant accumulation induces changes in the physiological state of the moss, and the measurement of such changes can also be used for biomonitoring. In particular, physiological stress may damage the photosynthetic pigments, resulting in the accumulation of their degradation products in the cell. Previous studies showed that trace elements (e.g. López et al., 1997; Kosior et al., 2010), fluorine (e.g. Aboal et al., 2008), pharmaceutical drugs (e.g. Aloui, 2021) and organic matter (e.g. Vázquez et al., 2013) can change the concentration of pigments. The resistance of each pigment to stress varies, therefore both pigment

concentrations and their ratios (pigmentary indices) can be used as stress indicators.

Chlorophyll *b*, present in the light-harvesting complexes of the photosystems, takes longer to degrade than chlorophyll *a*, present both in the light-harvesting complexes and in the core complexes of the photosystems (Arróniz-Crespo et al., 2008); therefore, Chl*a*/Chl*b* ratios decrease under stress conditions or in senescent plants (López & Carballeira, 1989; Agathokleous et al., 2018; Alaoui, 2021). Chlorophyll degrades into pheophytin (by losing the complexed Mg atom). So pheophytin is also an indicator of physiological stress. After extraction, chlorophylls and pheophytins can be quantified spectrophotometrically, as they have absorption maxima at different wavelengths: pheophytins at 410–415 nm and chlorophylls at 430–435 nm. Thus, the relationship between the optical densities (absorbances) at 430 and 410 nm (D430/D410) is an index of the relative abundance of chlorophylls and pheophytins (Martínez-Abaigar & Núñez-Olivera, 1998).

Another useful index is the ratio of absorbances measured before and after the acidification of a pigment extract. Acidification of an acetone extract transforms each chlorophyll molecule into its corresponding pheophytin. Both pigments absorb around 665 nm but pheophytins are weaker absorbers. If chlorophyll were absent in the extract, the acidification would not change the absorbance, so the ratio D665/D665*a* (absorbances at 665 nm before and after acidifying the sample) would be 1. Otherwise, the ratio D665/D665a should be > 1 because chlorophyll becomes transformed into pheophytin. Both pheopigment indices (D430/D410 and D665/D665a) decrease in stress situations (Ah-Peng, 2003; Megateli et al., 2009; Hu et al., 2013).

Carotenes resist physiological stress better than chlorophylls (Martínez-Abaigar & Núñez-Olivera, 1991). Therefore, the relative quantities of chlorophylls and carotenes are also useful for bioindication. At 430 nm, both of them absorb radiation, but only chlorophyll absorbs at 665 nm. Therefore, D430/D665 ratios show the relationship between the combined absorbances of chlorophylls and carotenes and that of chlorophylls alone (Núñez-Olivera et al., 2004). Carotenes also absorb radiation at 480 nm, so the D480/D665 ratio also estimates the relative abundances of carotenes and chlorophylls (Martínez-Abaigar & Núñez-Olivera, 1998). Both carotene indices (D430/D665 and D480/D665) increase with stress level.

A difficulty that arises in studies that collect large numbers of samples in a short time is that the samples cannot be processed immediately and must be stored for some time. Samples can be frozen, stored in a refrigerator, or left to dry at room temperature. Storing aquatic mosses out of their natural environment is a stressful situation that could affect the pigment contents and ratios. Determining the storage method that causes the least stress to the samples is necessary for the correct design of biomonitoring studies. However, to the best of our knowledge, there is no published work on the effect that different storage methods exert on the pigment composition of aquatic bryophytes.

For all these reasons, this study investigates the effect of three conservation methods on the composition of photosynthetic pigments and pigmentary indices in the aquatic bryophyte species *Fontinalis antipyretica* Hedw., to find the method that causes the least variation of their initial values. The methods tested were as follows: room temperature at 20ºC, refrigerator at 6ºC, and frozen at -20 °, in all three cases for 14 days.

Material and methods

Sampling

In July 2016, samples of the aquatic moss *F. antipyretica* Hedw. were collected in three rivers in Galicia (NW of Spain). The rivers, of different sizes and with different land uses in their basins, were selected to introduce heterogeneity in the sampling, as would be expected in regional-scale biomonitoring studies. The study area has a temperate and humid climate. The yearly average temperatures oscillate between 12 °C and 13 °C. Rainfall exceeds 1000 mm per year, although with a marked summer decrease, that results in low water levels. The sampling stations (one in each river) were as follows:

SS1, located in the Rio Tinto (42° 48′ 56.6″ N, 8° 37′ 37.30″ W (datum: WGS84); altitude: 77 m a.s.l.), a small river about 5 m wide at the sampling site, surrounded by agricultural and forestry crops.

SS2, Ladra River (43° 9′ 48.30″ N, 7° 43′ 5.80″ W; 395 m a.s.l), about 20 m wide, located in a

well-developed riparian forest area, although there are extensive meadows and crops upstream.

SS3, Madalena River (43° 17′ 13.00″ N, 7° 41′ 17.00″ W; 425 m a.s.l.), about 10 m wide, located 600 m downstream of the wastewater treatment plant the town of Vilalba (14,000 inhabitants).

Each sampling station comprised a stretch of river about 100 m long. The moss grew abundantly, albeit irregularly distributed, forming clumps on solid substrates such as rocks or tree roots. Each 100 m stretch was divided into 3 contiguous sections of equal length for collection of one replicate moss sample from each. These were composed samples gathered by collecting material from 10 to 15 clumps of submerged moss in each section. The moss samples were shaken in the river water to remove loosely attached sediment, debris and invertebrates and excess water was then gently squeezed out. They were transported to the laboratory in refrigerated containers.

We did not collect the moss at a standard depth because of its irregular distribution, but we avoided mosses growing too close to the water surface. We collected them in July, but the minimum flow of the rivers in the study area usually occurs in September. Therefore, we do not believe that any collected material were exposed to the air in the previous 10 months.

Storage procedures

At the laboratory, each sample was divided into 6 sub-samples, which were stored as follows:

(a) No storage: processed within 24 h of collection. These were the control samples and the other treatments were compared against them.
(b) Fresh: samples kept in a refrigerator at 6 °C inside closed polyethylene bags for 2, 7 or 14 days.
(c) Frozen: samples kept at – 20 °C in closed polyethylene bags for 14 days. The samples were left in a refrigerator at 6 °C overnight to thaw.
(d) Dry: The samples were spread out in plastic trays and kept for 2 days at room temperature to dry. They were then kept for 14 days in plastic containers covered with a sheet of filter paper and at room temperature. Before further processing, the samples were re-hydrated by spraying distilled water over them (Fernández et al., 2010).

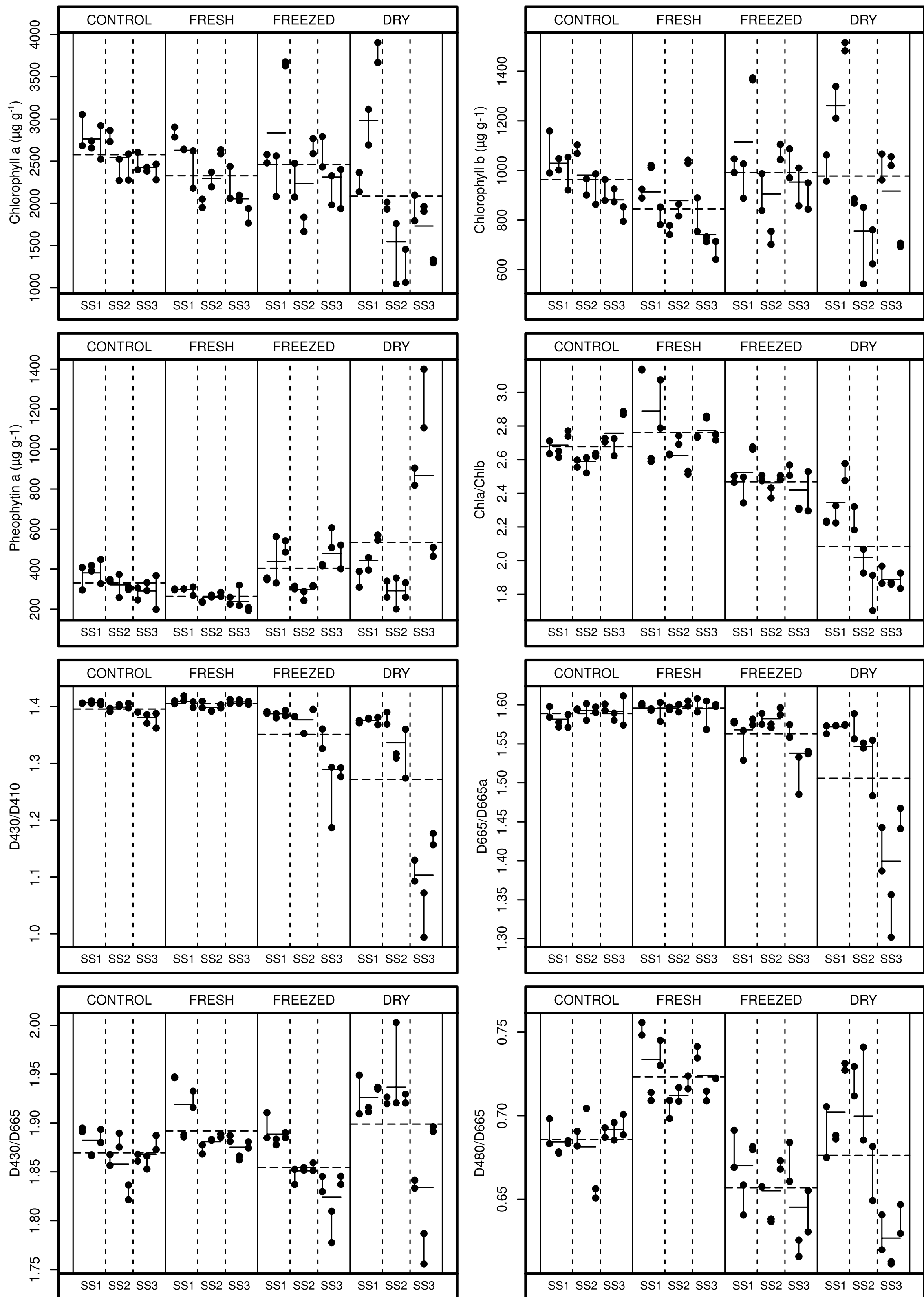
Pigment analysis and indices calculation

After the storage time was completed, 5-cm fragments were separated from the apices of randomly selected branches until two replicates of 100 mg of fresh weight were collected from each subsample. Pigment extraction was done with 90% acetone, manually grinding the samples in a mortar using quartz sand as abrasive, transferring the extract to 25 ml volumetric flasks, completing the volume with 90% acetone (Vollenweider, 1974), and subsequently centrifuging the extract at 3000 rpm for 3 min to remove suspended particles. 3 ml of extract was introduced in glass cuvettes with a 1 cm optical path and absorbance was determined with a T70 + spectrophotometer (PG Instruments, Lutterworth, UK). Absorbances were measured at 410, 430, 480, 647, 664, 665 and 750 nm (denoted as D410, etc. hereafter) with a bandwidth of 2 nm. D750 nm was always negligible, confirming the absence of turbidity in the extracts that could interfere with the other measurements. The extracts in the cuvettes were acidified with 30 µl of 1N HCl, and the absorbance at 665 nm (D665*a*) was measured (Lorenzen, 1967; Hendry et al., 1987; Zapata, 1988).

The concentrations of chlorophyll *a* and *b* were estimated from the absorbance data using the equations of Jeffrey & Humphrey (1975), and the pheophytin *a* concentrations with the equation of Baird et al. (2017). The ratio Chl*a*/Chl*b* was estimated from the concentration values. The following absorbance ratios (pigmentary indices) were also calculated: D430/D410 and D665/D665*a* as pheopigment indices (they decrease with increasing stress); D430/D665 and D480/D665 as carotene indices (increase with stress).

Statistical analyses

A first set of statistical analyses were aimed at identifying significant differences in the contents or ratios of pigments between the control samples (measurements done the same day as collection) and the samples stored for 14 days. The experiment design included three factors: storage (fixed, four levels) and SS (random, three levels) were the main factors and were orthogonal to each other. The third, stretch (three levels), was random and nested inside SS. For each combination of these factors, there were two pigment determinations. The data


CONTROL
FRESH
FREEZED
DRY
Chlorophyll a (μg g-1)
Chlorophyll b (μg g-1)
Pheophytin a (μg g-1)
Chla/Chlb
D430/D410
D665/D665a
D430/D665
D480/D665
SS1 SS2 SS3

◂**Fig. 1** Photosynthetic pigment concentrations and pigmentary indices in *F. antipyretica* stored in different ways: in a refrigerator at 6 °C for 1 (control) or 14 days (fresh), frozen at – 20 °C for 14 days (freezed) and dried at room temperature for 14 days (dry)

were analysed with ANOVA, but the Shapiro–Wilk normality test applied to the error data of each variable ($n = 72$) showed that the distributions of the pigment concentrations were normal but those of the pigmentary indices were not. Probability density distributions of the errors of the pigmentary indices showed symmetric distributions that had higher densities near the mode and longer tails than the Normal distribution. They were compatible with the shape of Cauchy's distribution, which is the expected distribution for the quotient of two normally distributed variables (we did not test this formally). We considered that the influence of the distributions on the performance of the ANOVA was small because they were symmetric and the experimental design was fully equilibrated (Underwood, 1997).

We also tested the data for homoscedasticity using Levene's test, which rejected this hypothesis for all variables, except for the concentrations of chlorophyll *b*. The effects of heteroscedasticity on the results of the ANOVA will be discussed later.

A second set of analyses investigated whether the samples stored fresh showed any temporal pattern in their pigment contents and indices (measured at days 1, 2, 7 and 14). Curvilinear regression analyses (linear, logarithmic, inverse, power, exponential) were used in this case. In all cases in which the fit of a model was significant, the logarithmic model was also significant. Therefore, in order to simplify the presentation of the results, only the significant logarithmic models will be discussed later. Note that we were interested in confirming the existence of temporal changes, not in accurately modelling them.

All these analyses were performed with IBM's SPSS Statistics software, version 28 and R (R Core Team, 2020).

Results

Comparison between conservation methods after 14 days of storage

Figure 1 shows the complete data set grouped by the different combinations of factors (SS $\times$ storage), the means of each combination (short horizontal lines) and the overall means for each level of the storage factor (long horizontal lines). As a visual aid, the pairs of replicate data were joined by a line.

Table 1 summarizes the ANOVA results: it contains the *p*-values resulting from the *F*-tests. We explained above that the data were heteroscedastic. Heteroscedasticity could cause *F*-tests to yield *P*-values that are too low, increasing the probability of Type I errors (Underwood, 1997). To compensate for this bias, we reduced the rejection probability from the usual $P < 0.05$ to $P < 0.01$. Note that even if the rejection probability were reduced to $P < 0.005$, most of the tests would still be significant (see Table 1). Therefore, we considered the ANOVA results sufficiently reliable. Full ANOVA tables are available as supplementary material.

The effect of storage was significant for all the variables except for chlorophyll *a* and chlorophyll *b*, while the effect of SS was significant for all variables except for D430/D410. However, the interaction between these two factors was significant (except for chlorophyll *a* and chlorophyll *b*), as was the interaction between storage and stretch, i.e. the effect of storage on the pigment concentrations and pigmentary indices detected by the ANOVA was not homogeneous across the combinations of SS and stretch. Therefore, the usual post hoc tests to identify which treatments caused significant effects on the variables cannot be applied. The effects must be considered for each combination of SS and stretch.

Fortunately, relevant patterns can be readily observed in Fig. 1. The means and dispersion of control and fresh data for all variables were similar. Dry data, when compared to the other two methods, showed different means, larger dispersion, or both. In particular, the combination dry + SS3 produced the most disperse data for most variables. The frozen samples showed an intermediate situation.

The overall means of Chl*a*/Chl*b*, D430/D410 and D665/D665*a* varied in the order control ≈ fresh > freezed > dry, and pheophytin *a* increases in the same order. The means of the carotene indices (D430/D665 and D480/D665) varied in a less consistent way, and those of the chlorophyll contents did not change significantly.

For the purposes of this study, changes in means are not the only relevant effect of the storage. A method is inadequate if it increases the variability

Table 1 Summary of the ANOVAs applied to the data of this study

Source of variation	D. f	Chlorophyll *a*	Chlorophyll *b*	Pheophytin *a*	Chl *a*/Chl *b*	D665/D665*a*	D480/D665	D430/D665	D430/D410
Treatment	3	0.070	0.110	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
SS	2	< 0.001	0.002	0.003	0.003	< 0.001	0.006	< 0.001	0.156
Stretch in SS	6	0.251	0.237	0.122	0.174	0.077	0.132	0.010	< 0.001
Treatment x SS	6	0.168	0.455	0.001	0.038	< 0.001	0.003	0.001	< 0.001
Treatment x stretch in SS	18	< 0.001	< 0.001	< 0.001	< 0.001	0.009	< 0.001	< 0.001	0.002

It contains the *P*-values produced by the F-tests for the components treatment (levels: control, rresh, freezed and dry), SS (sampling site: SS1-SS3), stretch (three stretches per SS, nested in SS) and the interactions of treatment with SS and stretch. The same analysis was applied to each of the variables considered

of the data, even if the mean values do not change. Variability increased in the sequence control ≈ fresh < freezed < dry. The differences were more pronounced for the pheophytin-related indices (D430/D410 and D665/D665*a*).

Changes over time in the refrigerated samples

The temporal evolution of the refrigerated samples could be studied in more detail because there were measurements at intermediate times (2 and 7 days). As was explained in the methods section, we used logarithmic regressions to detect significant changes in the variables over time. The concentrations of chlorophyll *a*, chlorophyll *b* and pheophytin *a* in all SSs were similar on the first and second days, diminished after 7 days and remained more or less stable from 7 to 14 days (Fig. 2). As to the pigmentary indices, the indices D430/D410 and D665/D66*a* increased significantly in a single SS each. Carotene indices showed significant increases over time, D480/D665 in the three SSs and D430/D665 in SS1 and SS2. In these cases, the increase was fast, from the first to the second day, remaining stable the following days. The ratio Chl*a*/Chl*b* did not show temporal changes in any of the SSs.

In summary, the concentrations of the three pigments showed a downward trend in all cases. The indices did not show significant trends consistently, but when they existed, the values increased along time.

Discussion

Samples stored in refrigerator

Although the ANOVA did not find significant differences in the means of Chl*a* and Chl*b* between control and fresh treatments, the regression analysis showed that the contents of both pigments diminished along the storage period (Fig. 2). However, the ratio Chl*a*/Chl*b* did not show significant temporal changes, indicating that the behaviour of both types of chlorophyll was similar (Fig. 2). This contrasts with the observation that chlorophyll *a* degrades more quickly in stress situations than chlorophyll *b* (Hendry et al., 1987; Arróniz-Crespo et al., 2008). The absence of light in the refrigerator did not affect this index, although previous studies showed that Chla/Chl*b* ratios become reduced in conditions of low light intensity (Martínez-Abaigar & Núñez-Olivera, 1998). It is possible that 14 days in the dark were not enough to change the chlorophyll contents.

The indices related to pheophytin (D430/D410 and D665/D665*a*) did not show convincing evidence of storage-induced stress. The control and fresh overall means were quite similar (Fig. 1) and each index showed a significant trend in only one SS (Fig. 2). The D430/D410 values were within the range that Martínez-Abaigar & Núñez-Olivera (1998) determined for healthy aquatic bryophytes (1.26–1.38 or higher), indicating good condition. Similarly, the D665/D665*a* values were within the ranges given by these authors (1.50–1.74) for healthy individuals.

The indices involving carotenoids (D430/D665 and D480/D665) showed an increasing trend, most notably in the first 48 h, which can be attributed to an increase in the physiological stress of the plants (Otero et al., 2006). The trend is consistent, as only D430/D665 in SS3 did not show it. According to Martínez-Abaigar & Núñez-Olivera (1998), the carotenoid/chlorophyll ratio diminishes in situations of low incident radiation (the samples were in total darkness inside the refrigerator), but after 14 days, the values of D430/D665 remained within the range given by Martínez-Abaigar & Núñez-Olivera (1998) for healthy aquatic bryophytes (1.72–2.89) and around the mean value (1.91) established by López & Carballeira (1989) for the best class in their ecophysiological quality classification. There are few published data on the D480/D665 index to make an effective comparison, but Martínez-Abaigar & Núñez-Olivera (1998) set 0.77–1.20 as the range for this index. Peñuelas (1984) and Martínez-Abaigar et al. (1994) indicated that the narrow range of these indices in aquatic bryophytes limits their ecological significance. Moreover, there are various environmental factors affecting carotenoid contents (photoadaptation, for example) that further limit the use of these indices as indicators of physiological stress (Capblancq & Catalan, 1994; Spitale, 2009). In short, even in the cases in which the temporal evolution of the carotene indices was significant, its meaning remained ambiguous. In fact, to the naked eye, the moss still seemed to be in good state after 14 days in the fridge. In general, the differences found between control and freshly stored moss were not as large as those observed for the other two methods.

Freezed samples

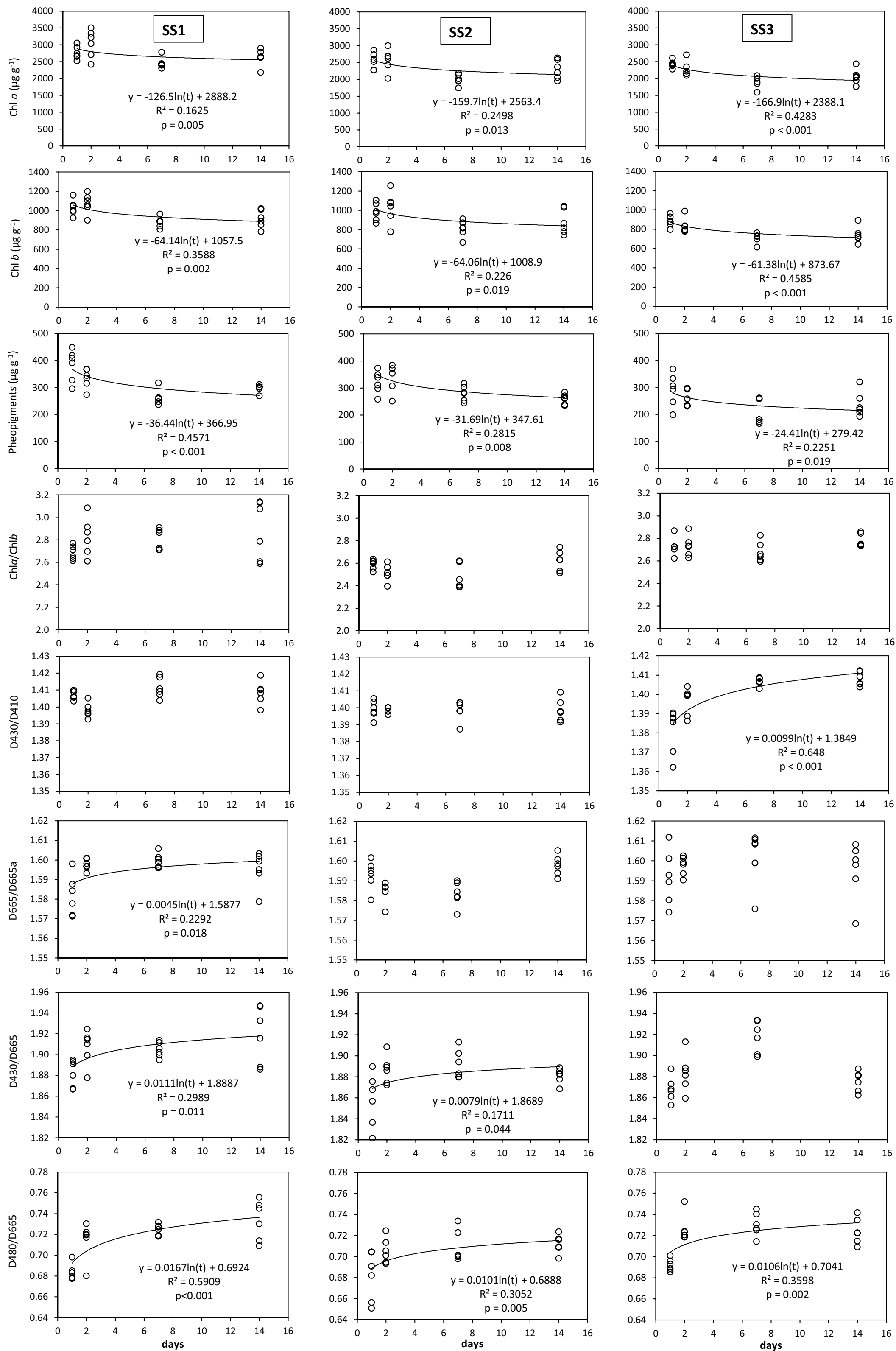
The changes in mean concentrations of chlorophylls and pheophytins after 14 days of freezing were small. All the indices showed reduced means when compared to the control samples, but the differences depended on both the SS and the stretch being considered. Pihakaski & Pihakaski (1979) found that freezing induced a reduction in the Chl*a*/Chl*b* ratio in the liverwort *Pellia epiphylla*. The mean of D480/D665 was smaller for freezed than for control samples, suggesting that carotenes degraded faster than chlorophylls, but this result is in disagreement with previous studies (Martínez-Abaigar & Núñez-Olivera, 1991). In short, freezing slightly changes the concentrations of pigments and pigmentary indices. For some variables, this effect was not constant, however, as SS and stretch changed their mean values.

Dried samples

The changes observed in the dried samples were qualitatively similar, but sometimes more intense, to those observed in the freezed samples. This is not surprising as in most cases aquatic mosses are more sensitive to desiccation than terrestrial mosses (Martínez-Abaigar & Núñez-Olivera, 1998). However, *Fontinalis antipyretica* can be temporarily exposed to the air under natural conditions and dry out without the emersion period affecting its vitality, especially if dehydration is gradual (Cruz de Carvalho et al., 2019). The Chl*a*/Chl*b* ratios were the lowest in the dried samples, which may be a consequence of the greater relative resistance of chlorophyll *b* to dessication. For the contents of pheophytin *a* and the indices D430/D410 and D665/D665*a*, the means of SS1 and SS2 were comparable to the values of the freezed samples but were lower for SS3. The samples from SS3 behaved differently from the other SSs for most variables (except chlorophylls *a* and *b*). We do not know the cause, but it was the most polluted SS, as a wastewater treatment plant discharges upstream.

Implications for biomonitoring

Our results showed that drying changed the contents and pigmentary ratios in the moss and therefore it is an inadequate storage technique. Furthermore, they showed that the other factors increased the variability of the data for all storage methods. The origin of the samples influenced significantly the outcome of the experiment. Even the stretch where the mosses were collected changed the effect of the storage method. This was true for the dried samples from SS3 (and to a lesser extent for the freezed ones), but not for those from SS1, which showed small changes when compared to the control. A storage method that inconsistently changes pigment contents and their ratios in the samples is not adequate for studying these variables. If changes during storage were consistent, the samples would remain comparable, but this was not the case.


SS1
SS2
SS3
Chl a (µg g-1)
y = -126.5ln(t) + 2888.2
R² = 0.1625
p = 0.005
y = -159.7ln(t) + 2563.4
R² = 0.2498
p = 0.013
y = -166.9ln(t) + 2388.1
R² = 0.4283
p < 0.001
Chl b (µg g-1)
y = -64.14ln(t) + 1057.5
R² = 0.3588
p = 0.002
y = -64.06ln(t) + 1008.9
R² = 0.226
p = 0.019
y = -61.38ln(t) + 873.67
R² = 0.4585
p < 0.001
Pheopigments (µg g-1)
y = -36.44ln(t) + 366.95
R² = 0.4571
p < 0.001
y = -31.69ln(t) + 347.61
R² = 0.2815
p = 0.008
y = -24.41ln(t) + 279.42
R² = 0.2251
p = 0.019
Chla/Chlb
D430/D410
y = 0.0099ln(t) + 1.3849
R² = 0.648
p < 0.001
D665/D665a
y = 0.0045ln(t) + 1.5877
R² = 0.2292
p = 0.018
D430/D665
y = 0.0111ln(t) + 1.8887
R² = 0.2989
p = 0.011
y = 0.0079ln(t) + 1.8689
R² = 0.1711
p = 0.044
D480/D665
y = 0.0167ln(t) + 0.6924
R² = 0.5909
p<0.001
y = 0.0101ln(t) + 0.6888
R² = 0.3052
p = 0.005
y = 0.0106ln(t) + 0.7041
R² = 0.3598
p = 0.002
days

◂**Fig. 2** Temporal trends of photosynthetic pigment concentrations and pigmentary indices in samples stored in a refrigerator at 6 °C for 14 days. The line was plotted for significant regressions only

Fresh storage produced quite consistent and predictable trends, although some differences were detected in the concentrations and indices over time. Therefore, we consider this storage method superior to the other methods tested, which produced more variable results.

Conclusions

Fresh storage at low temperature was the most adequate method to store the moss before analysis of their pigmentary contents and derived indices, at least for 14 days. Freezing might be a suitable method of storage for some parameters. Drying is not advisable, as this method can alter the contents and pigment ratios in unpredictable ways. In any case, it is highly recommended that studies analysing pigments and/or pigmentary indices report the storage method and the time passed between sample collection and their analysis.

Author contributions Rubén Villares: Conceptualization; methodology; investigation; formal analysis; writing—original draft; writing—review and editing. Carlos Real: Conceptualization; methodology; investigation; formal analysis; writing—review and editing. María D Vázquez: Conceptualization; methodology; investigation; writing—review and editing.

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Data availability Data are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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