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Title: The cotton-strip assay as an environmental surveillance tool for ecological integrity assessment of rivers affected by WWTP effluents

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Abstract: Environmental impact studies of rivers affected by wastewater treatment plant (WWTP) effluents have been greatly restricted by the difficulties associated with carrying out bioassays in the field and also by the complex interactions between the pollutants contained in the discharges. The cotton-strip assay (CSA) enables study of organic matter decomposition potential, an important ecosystem process in rivers, by taking into account all of the factors affecting this process. However, the CSA has never been used for assessment of WWTP effluents. In the present study, we selected six fluvial zones affected by discharges from small WWTPs and placed cotton strips at increasing distances from the discharge points in each zone. After 17 days, we evaluated decay of the strips by measuring cotton tensile strength loss (CTSL) and cotton mass loss (CML). We then determined the relationships between these parameters and various physico-chemical and biological properties in the water, as well as the $\delta^{15}\text{N}$ isotopic signal and metal contents of aquatic mosses transplanted in the same sampling sites and used as biomonitors. Although the WWTPs were similar, some of the discharges stimulated and others inhibited decomposition of the cotton strips. This was probably due to differences in the proportions of various types of pollutants (with trophic or toxic effects) in the discharges. The CSA proved to be a simple, practical and economic bioassay and suitable for evaluating the ecological integrity of fluvial ecosystems.

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25 October 2019

Dear Editor,

Please find attached the new version of our manuscript (ref. WR50241R2) "The cotton-strip assay as an environmental surveillance tool for ecological integrity assessment of rivers affected by WWTP effluents". (Authors: Carballeira, Villares, Mata-Rivas & Carballeira), which has been carefully revised in response to the reviewers' comments.

I look forward to hearing your response.

Yours sincerely,

Rubén Villares

Responses to reviewer #1:

Did you really only present these parts of the model, which have been significant? I hope I misunderstood your description. You need to present the full model, not only parts of it!

Response: we have now included all of the variables selected for inclusion in the models in Tables in 4 and 5, including the non significant variables. We previously did not include the non significant variables, simply in order to reduce the size of the tables.

... some revised paragraph are hard to read, because the inserted text disrupts the text flow. In these cases, the revision seems to be done in a hurry, i.e. you did not bother to connect the inserted sentences with the rest of the paragraph.

Response: we have revised the text again in order to address these problems.

I also found some spelling and grammar mistakes, but that can happen. Please check your manuscript carefully. Maybe you ask a native speaker for help

Response: the manuscript has been revised again by a native English speaker with experience in revising scientific articles.

But I do not like that you cite a manuscript in preparation (line 380). That is a no-go for international publications

Response: we have removed the sentence referring to the manuscript in preparation. We realise that this should be avoided, but sometimes there is no alternative, e.g. when there are few similar studies available.

I do not like your cheeky way to respond to the reviewer's comments, especially to the main concern of reviewer 2, but that is my personal opinion. Responses to reviewer need to be written in a polite way, because it can happen that the reviewer get send the revised manuscript for a second review.

Response: it was absolutely not our intention to be disrespectful and we were fully aware that our comments might be read by the same reviewers. We have re-read the original responses and do not understand why reviewer#1 thinks that we were "cheeky". We are sorry for this misunderstanding.

Responses to reviewer #2:

I suggest lines 303-309 should be incorporated into the Statistical Methods section.

Response: we have added the text to the Material and Methods section.

I suggest the authors revisit the graphical abstract as it is quite busy.

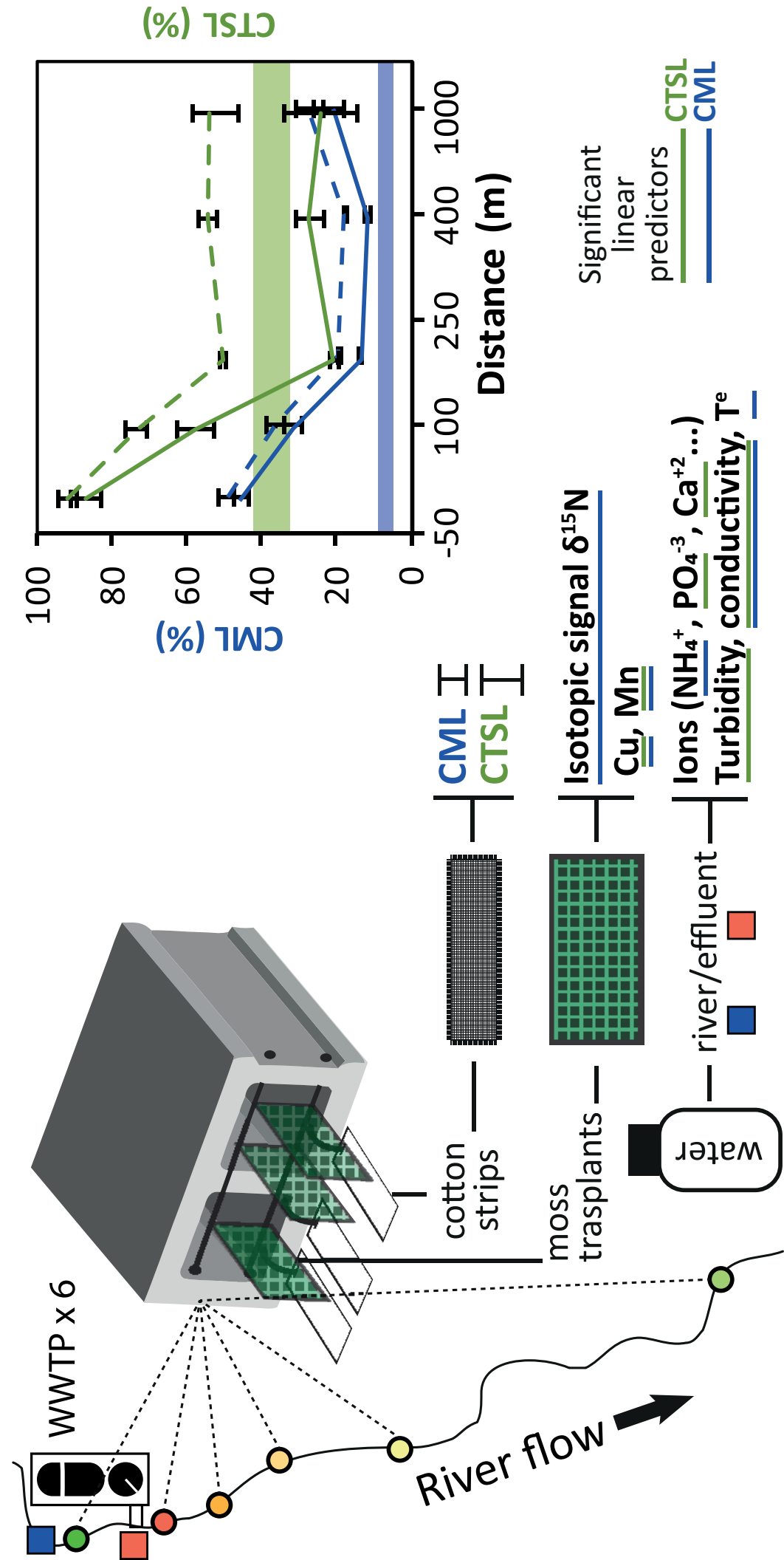
Response: We have removed the non-significant parameters from the graphical abstract so that it does not appear so cluttered.

WWTP effects on riverine organic matter decomposition were assessed

Cotton strip assay (CSA) was used

Mass loss of cotton strips was a better endpoint than tensile strength loss

CSA is a suitable assay for evaluating the ecological integrity of fluvial ecosystems



Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

**The cotton-strip assay as an environmental surveillance tool for ecological integrity
assessment of rivers affected by WWTP effluents**

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Abstract

Environmental impact studies of rivers affected by wastewater treatment plant (WWTP) effluents have been greatly restricted by the difficulties associated with carrying out bioassays in the field and also by the complex interactions between the pollutants contained in the discharges. The cotton-strip assay (CSA) enables study of the organic matter decomposition potential, an important ecosystem process in rivers, by taking all of the factors affecting this process into account. However, the CSA has never been used for assessment of WWTP effluents. In the present study, we selected six fluvial zones affected by discharges from small WWTPs and placed cotton strips at increasing distances from the discharge points in each zone. After 17 days, we evaluated decay of the strips by measuring cotton tensile strength loss (CTSL) and cotton mass loss (CML). We then determined the relationships between these parameters and various physico-chemical and biological properties in the water, as well as the $\delta^{15}\text{N}$ isotopic signal and metal contents of aquatic mosses transplanted in the same sampling sites and used as biomonitors. Although the WWTPs were similar, some of the discharges stimulated and others inhibited decomposition of the cotton strips. This was probably due to differences in the proportions of various types of pollutants (with trophic or toxic effects) in the discharges. The CSA proved to be a simple, practical and economic bioassay and suitable for evaluating the ecological integrity of fluvial ecosystems.

Keywords: ecosystem services; environmental monitoring plan; ecological integrity; biodegradation; monitoring; bioassay.

1. Introduction

Waste water treatment plant (WWTP) effluents comprise a complex mixture of organic matter, nutrients, metals, pesticides, pharmaceuticals, personal care products and other types of compounds. The nutrients derived directly from these effluents or from mineralization of the organic matter favour the autotrophic components of fluvial ecosystems, although excess

concentrations may lead to eutrophication processes. On the other hand, toxic pollutants can have negative effects that inhibit the development of aquatic organisms. Physico-chemical analysis of the discharges is of little value for predicting the effects on the functioning of fluvial ecosystems because of the potential additive, synergistic and antagonistic interactions in the complex mixture of substances (Kienzler et al., 2016), and in situ ecotoxicological assessment of this type of effects is scarce. Although structural indexes are often used to evaluate the health of fluvial ecosystems, the use of indicators of ecosystem processes such as organic matter decomposition and primary production is less common (Clapcott et al., 2010; Tiegs et al., 2013). However, these functional indicators provide useful information about organic matter processing and energy transfer that is not given by structural indexes (Niyogi et al., 2013). Decay of dead organic matter is a fundamental process in any ecosystem, as mineralized nutrients maintain primary production. A reduction in the breakdown of organic matter will affect primary productivity as well as the ecosystem service of water purification (Schäfer et al., 2012). The litter bag assay is the method most commonly used to evaluate litter decomposition, but the results vary depending on the mesh size, bag shape, litter species, litter mass, etc. (Lecerf and Chauvet, 2008; Ágoston-Szabó, 2016). Although many of these parameters can be standardized, it is almost impossible to standardize litter quality (Tiegs et al., 2013). Use of cotton strips as a standard substrate has therefore been proposed in order to enable comparison of decomposition rates in different types of studies, and the method has been demonstrated to be useful for studying the organic matter decomposition potential in both soils and fluvial environments (e.g. Trettin et al., 1996; Boulton and Quinn, 2000; Tiegs et al., 2007; Clapcott and Barmuta, 2010). The cotton-strip assay is ecologically relevant as it is based on the decomposition of cellulose, the most abundant polymer on earth. Loss of the tensile strength of the cotton strips is one of the parameters measured in the CSA; this process tends to occur much more rapidly than the loss of leaf litter mass from riparian trees, and the exposure times are short and operationally practical. Another advantage of the CSA is that the cotton strips lack nutrients, and they are therefore useful for studying how nutrients affect litter decay (Newman

et al., 2001). Moreover, cotton strips are less likely to be fragmented and lost than the litter in the litter-bag assay (Egglshaw, 1972).

The aim of the present study was to evaluate how the effluents from WWTPs serving small urban populations (less than 20,000 population equivalent) affect the rate of organic matter decomposition (estimated by the CSA) in the rivers receiving the discharges. We hypothesized that the decomposition rates will be positively affected by the effluents and will generally decrease with the distance from the discharge point, as the bioavailability of the nutrients decreases. This is because the effluents are mainly generated by small populations rather than by large industries, which would be more likely to discharge compounds with potentially toxic effects. This is the first time that this test has been used for in situ assessment of the impact of WWTPs. Studies of the environmental impacts of small WWTPs are scarce, despite the fact that small treatment plants in regions such as the study area discharge large amounts of effluents into rivers and are generally less efficient than the plants that serve large cities. We hypothesized that the decomposition rates will be positively affected by the effluents and that, in general, these rates will decrease with the distance from the discharge point as the bioavailability of the nutrients decreases. This is because the effluents mainly correspond to small populations rather than to large industries, which would be more likely to discharge compounds with potentially toxic effects.

2. Methods

2.1. Study areas

Six WWTPs in Galicia (NW Spain) were selected for study. The WWTPs each serve less than 20,000 population equivalent, are not close to other sources of contamination and discharge their effluents directly into fluvial ecosystems (Table 1). Some of the rivers in the study area have small tributaries or streams. However, these streams arise in areas that are not affected by pollution sources, and in all cases the flow rates are much lower than those of the main rivers.

Six sampling points were established at each WWTP: one point upstream of the discharge point, another immediately beside the discharge point (0 m) and points at 100, 200, 400 and 1000 m downstream of the discharge points (Fig. 1).

2.2. Preparing and exposing the cotton strips

Cotton strips were prepared by cutting unprimed 100% natural cotton canvas (12 oz natural cotton duck, 400 g m⁻²; manufacturer Russell and Chapple) into rectangles of length 8 cm and width 27 threads, following the procedure described by Tiegs et al. (2013). The strips were held in a forced air oven at 40 °C until exposure. Laboratory control strips were processed in the same way as the experimental strips but were kept in the oven (40 °C) during the exposure period.

The strips were weighed before being attached with plastic cable ties to concrete bricks, to minimise disturbance from the hydrological conditions and to maximise contact with the benthos in the exposure sites (Fig. 2). The bricks were placed in areas with similar substrates (granulometry, current speed, etc.) to minimise interference in the decomposition process. Pieces of mesh were placed in front of each hole in the brick (facing the direction of current) to protect the cotton strips and to prevent them being covered or moved by passing objects (Fig. 2). At each exposure site, the bricks were placed almost perpendicular to the current, at a slight angle to prevent obstruction of the mesh protectors (Fig. 2). Triplicate cotton strips were exposed at each sampling point for 17 days, in September 2016, as the flow rate of the rivers is usually minimal at this time of year in the study region (Table 1) and the impact of the WWTP effluents should therefore be maximal. The strips were collected after the exposure period, washed vigorously in situ and placed in 50 mL conical centrifuge tubes (Falcon™) containing 80 % ethanol to prevent any further decomposition before processing.

2.3. Processing the strips

In the laboratory, the cotton strips were removed from the tubes and dried at 40 °C for 48 h. The dried strips were weighed to determine loss of biomass. The tension of each replicate strip was measured immediately in a system consisting of two clamps, an extensometer (SF-500, Yueqing, China) and a manual test stand. The strips were manually stretched by fitting the ends to the clamps, with the aim of stretching the strips 2 cm every minute. The cotton tensile strength loss (CTSL) was calculated as the percentage loss for each sample (x), relative to the mean value for the control samples located upstream of each effluent discharge point (*fc*, field control) or to the mean value determined for the laboratory control (*lc*) samples.

$$CTSL_{fc} = \frac{CTS_{fc} - CTS_x}{TS_{fc}} 100$$

$$CTSL_{lc} = \frac{CTS_{lc} - CTS_x}{CTS_{lc}} 100$$

The cotton mass loss (CML) was calculated as the percentage loss for each sample (x) relative to the mean value for the control sample located upstream of each effluent discharge point (*fc*, field control) or to the mean value determined for the laboratory control (*lc*) samples.

$$CML_{fc} = \frac{CM_{fc} - CM_x}{CM_{fc}} 100$$

$$CML_{lc} = \frac{CM_{lc} - CM_x}{CM_{lc}} 100$$

2.4. Physico-chemical characterization of the study sites

The flow rate was estimated with a flowmeter (Flowatch, JDC Instruments, Yverdon-les-Bains Switzerland). The river was subdivided across its width into sections of side one metre, and the flow rate was calculated from the minimum, maximum and mean flow rates and the depth and width of all sections, in accordance with ISO 1088 (2007). The following parameters were also measured: electrical conductivity and temperature (XS COND 7+ conductivity meter, Carpi,

Italy; accuracy $\pm 2\%$ of full scale for conductivity ± 0.5 °C), pH (Mettler Toledo SevenGo pH meter, Columbus, Ohio, USA; accuracy ± 0.01) and turbidity (Lutron TU-2016 turbidimeter, Taipei, Taiwan; accuracy ± 0.5 NTU). At the same time, water samples were collected at the upstream sites (control) and at each discharge point for analysis of the following parameters: i) cations (Na^+ , NH_4^+ , K^+ , Mg^{+2} , Ca^{+2}) and anions (Cl^- , NO_2^- , NO_3^- , PO_4^{-2} , SO_4^{-2}) by ionic chromatography (Metrohm 861 Advanced Compact IC, Herisau, Switzerland); ii) total organic carbon by TOC-L CSN, with a Shimadzu NDIR detector; iii) total N by TOC-L CSN, with a Shimadzu CLD detector; iv, Kyoto, Japan), chemical oxygen demand (COD) by digestion with excess potassium dichromate and posterior titration with ferrous ammonium sulphate; v) biological oxygen demand (BOD_5) with an OXITOP manometric measuring device (Germany).

2.5. Moss transplants

Triplicate samples of the aquatic moss *Fontinalis squamosa* were exposed for 10 days beside the cotton strips in each exposure site, for determination the $\delta^{15}\text{N}$ isotopic signal and evaluation of metal accumulation. The moss samples were first devitalized, in order to prevent the moss metabolism interfering in the accumulation of metals (Fernández et al., 2009), by exposing them to different temperatures for several hours (8 h at 40 °C, 8 h at 80 °C and 8 h at 100 °C). The moss transplants were exposed in bags (10 x 10 cm) made from fibreglass mosquito netting (mesh size 2 mm) previously washed in a 2.5 % solution of sodium hypochlorite. Aliquots of 0.6 g (dry weight) of moss were placed in each bag. In the laboratory, the moss samples were removed from the bags, placed in paper bags and dried in an oven (48 h at 40 °C). The moss samples were then ground in a zirconium oxide tangential mill (Retsch MM400, Haan, Germany) and placed in glass vessels for storage at room temperature until chemical analysis. Subsamples of moss ($3 \text{ mg} \pm 0.1$ dry weight) were placed in tin capsules (Eurovector, Pavia, Italy) and processed in an elemental analyzer (Flash EA1112, ThermoFinnigan, Waltham, MA, USA) coupled to an isotopic ratio mass spectrometer (Deltaplus:ThermoFinnigan) in the Instrumental Techniques of Analysis Unit (UTIA) at the University of A Coruña (Carballeira et

al., 2013). The $\delta^{15}\text{N}$ isotopic signal was determined as the $^{15}\text{N}/^{14}\text{N}$ isotopic ratio of the sample (R_{sample}) relative to the isotopic composition of air, which is constant and used as standard (R_{standard}) (Robinson, 2001):

$$\delta^{15}\text{N} (\text{‰}) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 10^3$$

The metal analysis was conducted at the RIAIDT Instrumental Analysis Unit (University of Santiago de Compostela). The samples were digested with HNO_3 and H_2O_2 in a microwave oven (Milestone, Ethos1 Plus, Sorisole, Italy) and metal contents were determined in a mass spectrometer (ICP-MS, Agilent 7700x, Santa Clara, CA, USA).

2.6. Statistical analysis

The statistical analysis was carried out using IBM SPSS Statistics, Version 24. Automatic linear modelling analysis (Yang, 2013) was used to explore the main predictors of CTSL and CML, taking all variables measured in the water samples and in the moss transplants into account. As some variables were measured at all sampling sites and others were only measured at the discharge points and at the preceding sites, the two corresponding data sets were analysed separately. The forward stepwise variable selection method was used, and inclusion/exclusion was determined with the corrected Akaike information criterion (AICC). In addition to the usual parameters, the software estimates a parameter known as “predictor importance”, calculated as the residual sum of squares with the predictor removed from the model normalized to a sum of 1. The parameter thus varied from 0 (less importance) to 1 (greater importance). Collinearity was previously diagnosed by examining the Variance Inflation Factor (VIF), and in some cases variables were eliminated to prevent this problem.

3. Results

3.1. Spatial variation in CML and CTSL

Analysis of the laboratory control strips showed that the sensitivity of manual stretching was adequate, with low variability between control replicates, particularly for the mass loss parameter (Table 2).

The changes in CML and CTSL varied along the different stretches of river considered (Figs. 3 and 4). Thus, on some occasions the values of these parameters fluctuated, although with no clear increasing or decreasing trend, e.g. for WWTP III and CTSL (Fig. 3) and WWTP II and CML (Fig 4). In some cases, the values of CTSL and CML tended to increase with increasing distance from the discharge, as observed at WWTP V (Figs. 3 and 4). Decreasing trends were also observed, e.g. for CTSL and CML at WWTP IV (Figs. 3 and 4).

When both parameters were expressed relative to the laboratory controls (CTSL_{lc} and CML_{lc}), the values were always positive, i.e. the mass or tensile strength of the laboratory controls (not degraded) was always higher than in the field samples (Table 3). However, some negative values were obtained when CML and CTSL were expressed relative to the field control (i.e. the point situated upstream of the discharge), some negative values were obtained. These negative values sometimes corresponded to the points closest to the discharge point, e.g. for CTSL_{fc} and WWTP V (Fig. 3), a positive value was only obtained at the point furthest from the discharge. On other occasions, the most negative values corresponded to the points furthest from the discharge points, e.g. CTSL_{fc} and CML_{fc} at WWTP IV (Figs. 3 and 4).

The trends in CML values were similar for both types of control in all WWTPs (Fig. 4). By contrast, the values of CTSL were more variable, although the trends were still fairly similar (Fig. 3). The values of CML were less disperse (Fig. 4) than those of CTSL, e.g. for WWTP V at 200 m and 400 m from the discharge point (Fig. 3). The relationship between CML and CTSL was examined by regression analysis, and in both cases linear regression models provided adequate fits to the data. Considering all sampling points, the association between both parameters expressed relative to the laboratory control was highly significant ($p < 0.001$), with a coefficient of determination (R^2) of 0.718. The equivalent relationship for the values expressed

relative to the field controls was highly significant, although with a lower coefficient of determination ($R^2=0.654$).

3.2. Relationships between CML, CTSL and the physico-chemical characteristics of the water and chemical composition of the moss transplants

The ranges of values determined for each of the study variables are shown in Table 3. Conductivity was significantly related to both CML_{lc} and $CTSL_{lc}$, with a slightly higher coefficient of determination in the former case (Fig. 5). The coefficients of determination for the relationships between conductivity and the parameters expressed relative to the field controls were much lower (0.269 and 0.091 for CML_{fc} and $CTSL_{fc}$, respectively). CML and CTSL were not significantly related to pH, turbidity, total N, total organic C, BOD₅, COD or water temperature. Considering the anions and cations analysed, the strongest relationship was that between $CTSL_{fc}$ and phosphates ($R^2 = 0.924$, Fig. 6), although with a negative slope. Sulphate concentrations were related to both $CTSL_{lc}$ and CML_{lc} , and a significant relationship between Ca and $CTSL_{lc}$ was also observed. Neither CML or CTSL were significantly related to the other anions and cations (Cl^- , NO_2^- , NO_3^- , Na^+ , NH_4^+ , K^+ , Mg^{+2}). Regression analysis revealed that $\delta^{15}N$ in the transplanted moss was highly significantly related to CML_{lc} . A significant relationship was observed for $CTSL_{lc}$, although the coefficient of determination was much lower (Fig. 7). Regression analysis was also used to examine the relationships between loss of mass or tensile strength and the metal concentrations in the transplanted moss, although in all cases the coefficients of determination were very low, with values often close to zero.

3.3. Linear modelling

Linear modelling was used to search for the main predictors of CTSL and CML. taking into account all variables measured both in water samples and in the moss transplants. As many of the variables were correlated, they were removed from the analysis to prevent colinearity.

However, in almost all cases the final results were similar to that obtained without removing any of the variables. As some variables were measured at all sampling sites and others were only measured at the discharge points and at the preceding sites, the two corresponding data sets were analysed separately. For the variables measured at all sampling sites (Table 4), for both CTSL and CML relative to the field control, Cu was the most important variable selected, whereas conductivity was the variable selected in most cases (CTSL_{lc}, CML_{fc} and CML_{lc}); for CML relative to both controls, $\delta^{15}\text{N}$ was selected as the most important variable. The models for CML produced a better fit, with higher coefficients of determination. The analysis conducted with the parameters measured only at the discharge point and at the upstream site (Table 5) showed the importance of phosphates and calcium, as already indicated by the individual regressions for CTSL. The programme did not select any variable for CML_{fc}, whereas for CML_{lc}, the isotopic signal $\delta^{15}\text{N}$ in moss and ammonium in water were identified as explanatory variables. As the linear regression analysis detected collinearity in some cases, some variables were therefore excluded to avoid this problem. However, the final results were similar to those obtained before removal of any of the variables.

4. Discussion

The positive values of CTSL and CML expressed relative to the laboratory controls indicate greater degradation of the test cotton strips than of the control strips (Figs. 3 and 4). The negative values observed in some cases, when the degradation parameters were expressed relative to the field controls, indicate that the discharge had an inhibitory effect on the decomposition in these cases. When the most negative values corresponded to the samples close to the discharge points, this may indicate the presence in the effluents of immediately bioavailable toxic substances that inhibit the degradation of organic matter (Bastias et al., 2018). However, when the most negative values corresponded to the samples furthest from the discharge points, this may be attributed to the toxic substances not being immediately bioavailable. For example, toxins that display affinity for organic matter may pass into solution

once the organic matter (always present at high concentrations in the WWTP effluents considered) is degraded (Zaidi and Imam, 2008). However, the values of both $CTSL_{fc}$ and CML_{fc} at the most distant sampling point (located at 1000 m from the discharge) were almost always positive, indicating greater loss of mass or tensile strength than in the field control upstream of the discharge and suggesting that the effluent almost always affected the most distant sampling points included in the study.

We found that CML and CTSL were positively related. Tiegs et al. (2007) observed a curvilinear relationship between CML and CTSL, with tensile strength loss varying between 0 and 95 % within a narrow range of mass loss of 20 to 30 %. According to these authors the use of mass loss rather than tensile strength loss would reduce the sensitivity of the cotton-strip assay. In the present study, the relationship between these parameters was linear and also highly significant, suggesting that mass loss is a useful indicator of degradation. The lower dispersion of data is also an advantage relative to CTSL.

Among the relationships between the degradation parameters and the physico-chemical characteristics of the water, the general lack of association with water temperature is noteworthy. An association was only observed in the linear model for ML_{fc} , in fifth place and with a negative slope, which implies less loss of mass at higher temperature. Although a relationship between temperature and cotton tensile strength is not always observed (Niyogi et al., 2013), temperature is often reported to be one of the most important parameters affecting organic matter degradation (Trettin et al., 1996; Griffiths and Tiegs, 2016). However, the lack of correlation between temperature and cotton degradation in the present study may be due to the fact that the mean daily temperature or occasional temperature measurements do not realistically reflect how variations in temperature affect the decomposition rates (Vyšná et al., 2014). It is also possible that the cotton strips were exposed to a limited range of temperatures and that no temperature effect occurred. Slocum et al. (2009) reported that temperature clearly affected degradation of cotton strips, although these researchers used two very different temperatures (20°C and 35°C). Carballera et al. (in prep.) also observed that temperature affected the CSA carried out the laboratory at temperatures of 10°C and 20°C.

The significant, negative relationship (with a high coefficient of determination) between $CTSL_{fc}$ and phosphates implies that a higher concentration of these compounds in water will be associated with lower loss of tensile strength. This finding directly contrasts with those of other studies (e.g. Newman et al., 2001; Bierschenk, 2012; Tiegs et al., 2013). It is generally considered that microbial communities are stimulated by the presence of nutrients. However, Tiegs et al. (2013) suggested that the different microbial communities in different sites act as an inoculum for the communities colonizing the organic substrate and that the variation in microbial communities will influence the decomposition rate, independently of the direct effects of nutrients. It is also possible that the toxins present in the effluents will negatively affect the decomposition rate (Bastias et al., 2018) and that phosphates will be more available when decomposition rates are low.

Degradation of organic matter generates sulphates, which explains why discharge of urban sewage leads to increased concentrations of this anion (Antelo and Arce, 1996). In sites characterised by high concentrations of sulphates, the rate of degradation of organic matter (including cotton strips) will be high, which would explain the significant relationships between both $CTSL_{lc}$ and CML_{lc} and sulphate concentrations observed in the present study. The significant relationship between $CTSL_{lc}$ and Ca may indicate that high concentrations of Ca favour decomposition, as also indicated by the positive correlations between Ca in leaf litter and rates of decomposition observed in forest soils (Jacob et al., 2010). It is also possible that some of the Ca in the water will be derived from organic matter decomposition, so that higher concentrations will be found in sites characterised by high decomposition rates (Upadhyay, 1987).

Aquatic bryophytes can be used as a tool for biomonitoring water quality (Debén et al., 2015, 2017). The significant relationships between the parameters measured in the moss and those measured in the cotton strips appear to indicate an association between the composition of the WWTP effluents and the decomposition of the cotton strips. WWTP effluents are enriched in ^{15}N (Curt et al., 2004; Cole et al., 2005), particularly effluents subjected to secondary treatments (Hicks et al., 2017), such as those considered in the present study. As a result, macrophyte $\delta^{15}N$

has been proposed as an indicator of the presence of N of anthropogenic origin in aquatic ecosystems (Cole et al., 2005; Carballeira et al., 2012; 2013). Both the individual regressions and the linear modelling indicate a relationship between the isotopic signal $\delta^{15}\text{N}$ in moss transplants and the degradation rates, confirming the validity of both measures as descriptors of organic load exposure. Thus, although the result obtained for phosphates was unexpected, the importance of another nutrient, N, was clearly observed in this case. It is important to note that the physico-chemical characteristics of the water were determined in the present study by single measurements and may be poorly representative owing to the high temporal variability of physico-chemical characteristics and contaminant levels found in river waters and WWTP effluents (Meybeck et al., 1996; Salgado et al., 2011; Morosini et al., 2017). As a result, it is possible that the significant relationships for parameters measured in water were merely circumstantial. On the other hand, the chemical composition of the transplanted moss may be considered more representative as it reflects the concentrations in the water during exposure of the transplants (Debén et al. 2016, 2017; Carballeira et al., 2019).

Although no significant relationship between the parameters measured in the cotton strips and metal concentrations in mosses was initially observed, the metals were selected in the linear modelling. Cu was particularly important, although with a negative slope, which implies a toxic effect, so that the rate of degradation will decrease as the metal concentration increases (Chew et al., 2001). Mn was also included in the model, with a positive slope, possibly indicating that this element is associated with the discharges. Larger organic loads should imply larger amounts of microorganisms that help degrade the cellulose. Organic matter can have complex effects on metal toxicity and bioaccumulation, and the toxicity and bioaccumulation of elements may decrease due to the formation of complexes with dissolved organic matter (Luider et al., 2004; Ravichandran, 2004). By contrast, other studies have shown that the solubility and bioavailability of elements such as Rh, Hg and Zn may increase with the amount of dissolved organic matter in water (Rupa et al., 2003; Chen et al., 2004; Ravichandran, 2004; Sures and Zimmermann, 2007). Thus, the CSA will indirectly provide information about the bioavailability and accumulation of some metals. However, determining the origin of the

toxicity in sewage is very difficult due to the multiple overlapping factors affecting the process (Gartiser et al., 2010). Vasquez and Fatta-Kassinos (2013) indicated the weak correlation between physico-chemical and ecotoxicological data for treated wastewater from sewage treatment plants. Moreover, the stressors affecting the allochthonous organic matter breakdown rates are almost unknown (Schäfer et al., 2012).

In view of the scarcity of other similar studies using the CSA, the relationships between the rate of decay of the cotton strips and the concentrations of metals in the effluent and the river water are, in short, difficult to explain due to the complex interactions between the decomposer organisms (grazers, shredders, microbes, etc.), the type of material and stage of decomposition, the environmental conditions, the metals present in the environment, the metals released during the decomposition process and those bound to other substances, and the presence of other pollutants (Schaller et al., 2011; Van Nevel et al., 2014; Schaller and Brackhage, 2015; Ferreira et al., 2016).

Sewage treatment has advanced greatly in recent years, and the quality of the effluents discharged from modern WWTPs is such that several in situ studies have not detected any differences between control sites and sites affected by the effluents (Spänhoff et al., 2007). However, such findings can only be corroborated by integrated environmental monitoring involving various lines of evidence (LOE). The primary LOE in integrated monitoring plans adapted to WWTPs usually consists of the physico-chemical characterization of the discharges by conventional analysis of parameters that play an important role in the survival of aquatic species (Gosset et al., 2016), with the aim of verifying whether the treatments comply with the limits outlined in the prevailing legislation for the discharge of effluents in different types of water bodies. WWTPs were originally designed to reduce the amounts of biodegradable organic matter, solids and even nutrients in effluents, but not to totally remove harmful substances (Ormad et al., 2011). Therefore, in addition to this LOE, analysis of persistent organic pollutants that can potentially be accumulated should be conducted, while not overlooking emergent compounds that are ubiquitous in sewage effluent (Kümmerer, 2009; European Commission, 2012). However, physico-chemical characterization does not indicate how the biota may be

affected (Carballeira et al., 2012). The impacts on ecosystems due to the interactive effects between contaminants present in urban sewage waste on release into the environment are unclear and unpredictable (Filby et al., 2007). Thus, a second LOE should be included in order to evaluate the overall toxicity of effluents by the use of a battery of bioassays carried out under laboratory conditions in order to standardize the ecotoxicological assessment of WWTP discharges (ECETOC, 2004; OSPAR, 2007; EEA, 2011).

The complexity of WWTP effluents, which contain both stimulators (organic matter) and inhibitors (toxins), modifies the balance between autotrophic and heterotrophic processes and produces spatial discontinuities in ecosystem functioning (Aristi et al., 2015). Therefore, only bioassays carried out in field conditions (in situ) provide realistic information about the ecotoxicity of discharges (Gosset et al., 2016). ~~Bioassays carried out in~~ In situ bioassays could form part of a third LOE aimed at evaluating the ecological integrity. However, these types of bioassay are often difficult to develop and interpret due to the complex interactions between contaminants and between these and the effects of numerous physical factors (Filby et al., 2007). In this respect, the CSA can be considered a simple, rapid, economic and standardized protocol for characterizing the receptor ecosystem integrity from a functional perspective relative to conventional structural methods such as the study of epilithic diatoms or benthic macroinvertebrate communities.

For validation of the usefulness of the CSA as a routine monitoring tool, future studies should consider different scenarios and conditions in order to take into account the probable complex interactions between decomposers and mixtures of contaminants into account.

5. Conclusions

Despite the similarity in the WWTPs under study, the response measured by decomposition of cotton strips varied in the different sites and was more complex than originally hypothesized (i.e. a straightforward decrease in the decomposition rates with increasing distance from the discharge points), probably because the complex interactions between the contaminants present

in the effluents generate trophic and/ or toxic effects in variable proportions. For most of the WWTPs considered, the effects of the discharges extended more than 1 km downstream. Mass loss appears to be a suitable measure of decomposition potential, showing even lower between-replicate variability than tensile strength loss. We recommend that bioassays such as the cotton strip assay, which can be conducted in situ under field conditions, should be included in environmental monitoring plans for WWTPs in order to evaluate the ecological integrity of the fluvial ecosystems affected. Such bioassays provide information about the effects on the receptor ecosystems, where the organisms involved in the decomposition process are exposed to effluent discharges under natural environmental conditions.

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**The cotton-strip assay as an environmental surveillance tool for ecological integrity
assessment of rivers affected by WWTP effluents**

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Abstract

Environmental impact studies of rivers affected by wastewater treatment plant (WWTP) effluents have been greatly restricted by the difficulties associated with carrying out bioassays in the field and also by the complex interactions between the pollutants contained in the discharges. The cotton-strip assay (CSA) enables study of the organic matter decomposition potential, an important ecosystem process in rivers, by taking all of the factors affecting this process into account. However, the CSA has never been used for assessment of WWTP effluents. In the present study, we selected six fluvial zones affected by discharges from small WWTPs and placed cotton strips at increasing distances from the discharge points in each zone. After 17 days, we evaluated decay of the strips by measuring cotton tensile strength loss (CTSL) and cotton mass loss (CML). We then determined the relationships between these parameters and various physico-chemical and biological properties in the water, as well as the $\delta^{15}\text{N}$ isotopic signal and metal contents of aquatic mosses transplanted in the same sampling sites and used as biomonitors. Although the WWTPs were similar, some of the discharges stimulated and others inhibited decomposition of the cotton strips. This was probably due to differences in the proportions of various types of pollutants (with trophic or toxic effects) in the discharges. The CSA proved to be a simple, practical and economic bioassay and suitable for evaluating the ecological integrity of fluvial ecosystems.

Keywords: ecosystem services; environmental monitoring plan; ecological integrity; biodegradation; monitoring; bioassay.

1. Introduction

Waste water treatment plant (WWTP) effluents comprise a complex mixture of organic matter, nutrients, metals, pesticides, pharmaceuticals, personal care products and other types of compounds. The nutrients derived directly from these effluents or from mineralization of the organic matter favour the autotrophic components of fluvial ecosystems, although excess

concentrations may lead to eutrophication processes. On the other hand, toxic pollutants can have negative effects that inhibit the development of aquatic organisms. Physico-chemical analysis of the discharges is of little value for predicting the effects on the functioning of fluvial ecosystems because of the potential additive, synergistic and antagonistic interactions in the complex mixture of substances (Kienzler et al., 2016), and in situ ecotoxicological assessment of this type of effects is scarce. Although structural indexes are often used to evaluate the health of fluvial ecosystems, the use of indicators of ecosystem processes such as organic matter decomposition and primary production is less common (Clapcott et al., 2010; Tiegs et al., 2013). However, these functional indicators provide useful information about organic matter processing and energy transfer that is not given by structural indexes (Niyogi et al., 2013). Decay of dead organic matter is a fundamental process in any ecosystem, as mineralized nutrients maintain primary production. A reduction in the breakdown of organic matter will affect primary productivity as well as the ecosystem service of water purification (Schäfer et al., 2012). The litter bag assay is the method most commonly used to evaluate litter decomposition, but the results vary depending on the mesh size, bag shape, litter species, litter mass, etc. (Lecerf and Chauvet, 2008; Ágoston-Szabó, 2016). Although many of these parameters can be standardized, it is almost impossible to standardize litter quality (Tiegs et al., 2013). Use of cotton strips as a standard substrate has therefore been proposed in order to enable comparison of decomposition rates in different types of studies, and the method has been demonstrated to be useful for studying the organic matter decomposition potential in both soils and fluvial environments (e.g. Trettin et al., 1996; Boulton and Quinn, 2000; Tiegs et al., 2007; Clapcott and Barmuta, 2010). The cotton-strip assay is ecologically relevant as it is based on the decomposition of cellulose, the most abundant polymer on earth. Loss of the tensile strength of the cotton strips is one of the parameters measured in the CSA; this process tends to occur much more rapidly than the loss of leaf litter mass from riparian trees, and the exposure times are short and operationally practical. Another advantage of the CSA is that the cotton strips lack nutrients, and they are therefore useful for studying how nutrients affect litter decay (Newman

et al., 2001). Moreover, cotton strips are less likely to be fragmented and lost than the litter in the litter-bag assay (Egglshaw, 1972).

The aim of the present study was to evaluate how the effluents from WWTPs serving small urban populations (less than 20,000 population equivalent) affect the rate of organic matter decomposition (estimated by the CSA) in the rivers receiving the discharges. We hypothesized that the decomposition rates will be positively affected by the effluents and will generally decrease with the distance from the discharge point, as the bioavailability of the nutrients decreases. This is because the effluents are mainly generated by small populations rather than by large industries, which would be more likely to discharge compounds with potentially toxic effects. This is the first time that this test has been used for in situ assessment of the impact of WWTPs. Studies of the environmental impacts of small WWTPs are scarce, despite the fact that small treatment plants in regions such as the study area discharge large amounts of effluents into rivers and are generally less efficient than the plants that serve large cities.

2. Methods

2.1. Study areas

Six WWTPs in Galicia (NW Spain) were selected for study. The WWTPs each serve less than 20,000 population equivalent, are not close to other sources of contamination and discharge their effluents directly into fluvial ecosystems (Table 1). Some of the rivers in the study area have small tributaries or streams. However, these streams arise in areas that are not affected by pollution sources, and in all cases the flow rates are much lower than those of the main rivers. Six sampling points were established at each WWTP: one point upstream of the discharge point, another immediately beside the discharge point (0 m) and points at 100, 200, 400 and 1000 m downstream of the discharge points (Fig. 1).

2.2. Preparing and exposing the cotton strips

Cotton strips were prepared by cutting unprimed 100% natural cotton canvas (12 oz natural cotton duck, 400 g m⁻²; manufacturer Russell and Chapple) into rectangles of length 8 cm and width 27 threads, following the procedure described by Tiegs et al. (2013). The strips were held in a forced air oven at 40 °C until exposure. Laboratory control strips were processed in the same way as the experimental strips but were kept in the oven (40 °C) during the exposure period.

The strips were weighed before being attached with plastic cable ties to concrete bricks, to minimise disturbance from the hydrological conditions and to maximise contact with the benthos in the exposure sites (Fig. 2). The bricks were placed in areas with similar substrates (granulometry, current speed, etc.) to minimise interference in the decomposition process. Pieces of mesh were placed in front of each hole in the brick (facing the direction of current) to protect the cotton strips and to prevent them being covered or moved by passing objects (Fig. 2). At each exposure site, the bricks were placed almost perpendicular to the current, at a slight angle to prevent obstruction of the mesh protectors (Fig. 2). Triplicate cotton strips were exposed at each sampling point for 17 days, in September 2016, as the flow rate of the rivers is usually minimal at this time of year in the study region (Table 1) and the impact of the WWTP effluents should therefore be maximal. The strips were collected after the exposure period, washed vigorously in situ and placed in 50 mL conical centrifuge tubes (Falcon™) containing 80 % ethanol to prevent any further decomposition before processing.

2.3. Processing the strips

In the laboratory, the cotton strips were removed from the tubes and dried at 40 °C for 48 h. The dried strips were weighed to determine loss of biomass. The tension of each replicate strip was measured immediately in a system consisting of two clamps, an extensometer (SF-500,

Yueqing, China) and a manual test stand. The strips were manually stretched by fitting the ends to the clamps, with the aim of stretching the strips 2 cm every minute. The cotton tensile strength loss (CTSL) was calculated as the percentage loss for each sample (x), relative to the mean value for the control samples located upstream of each effluent discharge point (fc , field control) or to the mean value determined for the laboratory control (lc) samples.

$$CTSL_{fc} = \frac{CTS_{fc} - CTS_x}{TS_{fc}} 100$$

$$CTSL_{lc} = \frac{CTS_{lc} - CTS_x}{CTS_{lc}} 100$$

The cotton mass loss (CML) was calculated as the percentage loss for each sample (x) relative to the mean value for the control sample located upstream of each effluent discharge point (fc , field control) or to the mean value determined for the laboratory control (lc) samples.

$$CML_{fc} = \frac{CM_{fc} - CM_x}{CM_{fc}} 100$$

$$CML_{lc} = \frac{CM_{lc} - CM_x}{CM_{lc}} 100$$

2.4. Physico-chemical characterization of the study sites

The flow rate was estimated with a flowmeter (Flowwatch, JDC Instruments, Yverdon-les-Bains Switzerland). The river was subdivided across its width into sections of side one metre, and the flow rate was calculated from the minimum, maximum and mean flow rates and the depth and width of all sections, in accordance with ISO 1088 (2007). The following parameters were also measured: electrical conductivity and temperature (XS COND 7+ conductivity meter, Carpi, Italy; accuracy $\pm 2\%$ of full scale for conductivity ± 0.5 °C), pH (Mettler Toledo SevenGo pH meter, Columbus, Ohio, USA; accuracy ± 0.01) and turbidity (Lutron TU-2016 turbidimeter, Taipei, Taiwan; accuracy ± 0.5 NTU). At the same time, water samples were collected at the upstream sites (control) and at each discharge point for analysis of the following parameters: i)

cations (Na^+ , NH_4^+ , K^+ , Mg^{+2} , Ca^{+2}) and anions (Cl^- , NO_2^- , NO_3^- , PO_4^{-2} , SO_4^{-2}) by ionic chromatography (Metrohm 861 Advanced Compact IC, Herisau, Switzerland); ii) total organic carbon by TOC-L CSN, with a Shimadzu NDIR detector; iii) total N by TOC-L CSN, with a Shimadzu CLD detector; iv, Kyoto, Japan), chemical oxygen demand (COD) by digestion with excess potassium dichromate and posterior titration with ferrous ammonium sulphate; v) biological oxygen demand (BOD_5) with an OXITOP manometric measuring device (Germany).

2.5. Moss transplants

Triplicate samples of the aquatic moss *Fontinalis squamosa* were exposed for 10 days beside the cotton strips in each exposure site, for determination the $\delta^{15}\text{N}$ isotopic signal and evaluation of metal accumulation. The moss samples were first devitalized, in order to prevent the moss metabolism interfering in the accumulation of metals (Fernández et al., 2009), by exposing them to different temperatures for several hours (8 h at 40 °C, 8 h at 80 °C and 8 h at 100 °C). The moss transplants were exposed in bags (10 x 10 cm) made from fibreglass mosquito netting (mesh size 2 mm) previously washed in a 2.5 % solution of sodium hypochlorite. Aliquots of 0.6 g (dry weight) of moss were placed in each bag. In the laboratory, the moss samples were removed from the bags, placed in paper bags and dried in an oven (48 h at 40 °C). The moss samples were then ground in a zirconium oxide tangential mill (Retsch MM400, Haan, Germany) and placed in glass vessels for storage at room temperature until chemical analysis. Subsamples of moss (3 mg $\pm$ 0.1 dry weight) were placed in tin capsules (Eurovector, Pavia, Italy) and processed in an elemental analyzer (Flash EA1112, ThermoFinnigan, Waltham, MA, USA) coupled to an isotopic ratio mass spectrometer (Deltaplus:ThermoFinnigan) in the Instrumental Techniques of Analysis Unit (UTIA) at the University of A Coruña (Carballeira et al., 2013). The $\delta^{15}\text{N}$ isotopic signal was determined as the $^{15}\text{N}/^{14}\text{N}$ isotopic ratio of the sample (R_{sample}) relative to the isotopic composition of air, which is constant and used as standard (R_{standard}) (Robinson, 2001):

$$\delta^{15}\text{N} (\text{‰}) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 10^3$$

The metal analysis was conducted at the RIAIDT Instrumental Analysis Unit (University of Santiago de Compostela). The samples were digested with HNO₃ and H₂O₂ in a microwave oven (Milestone, Ethos1 Plus, Sorisole, Italy) and metal contents were determined in a mass spectrometer (ICP-MS, Agilent 7700x, Santa Clara, CA, USA).

2.6. Statistical analysis

The statistical analysis was carried out using IBM SPSS Statistics, Version 24. Automatic linear modelling analysis (Yang, 2013) was used to explore the main predictors of CTSL and CML, taking all variables measured in the water samples and in the moss transplants into account. As some variables were measured at all sampling sites and others were only measured at the discharge points and at the preceding sites, the two corresponding data sets were analysed separately. The forward stepwise variable selection method was used, and inclusion/exclusion was determined with the corrected Akaike information criterion (AICC). In addition to the usual parameters, the software estimates a parameter known as “predictor importance”, calculated as the residual sum of squares with the predictor removed from the model normalized to a sum of 1. The parameter thus varied from 0 (less importance) to 1 (greater importance). Collinearity was previously diagnosed by examining the Variance Inflation Factor (VIF), and in some cases variables were eliminated to prevent this problem.

3. Results

3.1. Spatial variation in CML and CTSL

Analysis of the laboratory control strips showed that the sensitivity of manual stretching was adequate, with low variability between control replicates, particularly for the mass loss parameter (Table 2).

The changes in CML and CTSL varied along the different stretches of river considered (Figs. 3 and 4). Thus, on some occasions the values of these parameters fluctuated, although with no clear increasing or decreasing trend, e.g. for WWTP III and CTSL (Fig. 3) and WWTP II and CML (Fig 4). In some cases, the values of CTSL and CML tended to increase with increasing distance from the discharge, as observed at WWTP V (Figs. 3 and 4). Decreasing trends were also observed, e.g. for CTSL and CML at WWTP IV (Figs. 3 and 4).

When both parameters were expressed relative to the laboratory controls (CTSL_{lc} and CML_{lc}), the values were always positive, i.e. the mass or tensile strength of the laboratory controls (not degraded) was always higher than in the field samples (Table 3). However, some negative values were obtained when CML and CTSL were expressed relative to the field control (i.e. the point situated upstream of the discharge). These negative values sometimes corresponded to the points closest to the discharge point, e.g. for CTSL_{fc} and WWTP V (Fig. 3), a positive value was only obtained at the point furthest from the discharge. On other occasions, the most negative values corresponded to the points furthest from the discharge points, e.g. CTSL_{fc} and CML_{fc} at WWTP IV (Figs. 3 and 4).

The trends in CML values were similar for both types of control in all WWTPs (Fig. 4). By contrast, the values of CTSL were more variable, although the trends were still fairly similar (Fig. 3). The values of CML were less disperse (Fig. 4) than those of CTSL, e.g. for WWTP V at 200 m and 400 m from the discharge point (Fig. 3). The relationship between CML and CTSL was examined by regression analysis, and in both cases linear regression models provided adequate fits to the data. Considering all sampling points, the association between both parameters expressed relative to the laboratory control was highly significant ($p < 0.001$), with a coefficient of determination (R^2) of 0.718. The equivalent relationship for the values expressed relative to the field controls was highly significant, although with a lower coefficient of determination ($R^2 = 0.654$).

3.2. Relationships between CML, CTSL and the physico-chemical characteristics of the water and chemical composition of the moss transplants

The ranges of values determined for each of the study variables are shown in Table 3. Conductivity was significantly related to both CML_{lc} and $CTSL_{lc}$, with a slightly higher coefficient of determination in the former case (Fig. 5). The coefficients of determination for the relationships between conductivity and the parameters expressed relative to the field controls were much lower (0.269 and 0.091 for CML_{fc} and $CTSL_{fc}$, respectively). CML and CTSL were not significantly related to pH, turbidity, total N, total organic C, BOD₅, COD or water temperature. Considering the anions and cations analysed, the strongest relationship was that between $CTSL_{fc}$ and phosphates ($R^2 = 0.924$, Fig. 6), although with a negative slope. Sulphate concentrations were related to both $CTSL_{lc}$ and CML_{lc} , and a significant relationship between Ca and $CTSL_{lc}$ was also observed. Neither CML or CTSL were significantly related to the other anions and cations (Cl^- , NO_2^- , NO_3^- , Na^+ , NH_4^+ , K^+ , Mg^{+2}). Regression analysis revealed that $\delta^{15}N$ in the transplanted moss was highly significantly related to CML_{lc} . A significant relationship was observed for $CTSL_{lc}$, although the coefficient of determination was much lower (Fig. 7). Regression analysis was also used to examine the relationships between loss of mass or tensile strength and the metal concentrations in the transplanted moss, although in all cases the coefficients of determination were very low, with values often close to zero.

3.3. Linear modelling

Linear modelling was used to search for the main predictors of CTSL and CML. For the variables measured at all sampling sites (Table 4), for both CTSL and CML relative to the field control, Cu was the most important variable selected, whereas conductivity was the variable selected in most cases ($CTSL_{lc}$, CML_{fc} and CML_{lc}); for CML relative to both controls, $\delta^{15}N$ was selected as the most important variable. The models for CML produced a better fit, with higher coefficients of determination. The analysis conducted with the parameters measured only at the discharge point and at the upstream site (Table 5) showed the importance of phosphates and calcium, as already indicated by the individual regressions for CTSL. The programme did not

select any variable for CML_{fc} , whereas for CML_{lc} , the isotopic signal $\delta^{15}N$ in moss and ammonium in water were identified as explanatory variables. As the linear regression analysis detected collinearity in some cases, some variables were therefore excluded to avoid this problem. However, the final results were similar to those obtained before removal of any of the variables.

4. Discussion

The positive values of CTSL and CML expressed relative to the laboratory controls indicate greater degradation of the test cotton strips than of the control strips (Figs. 3 and 4). The negative values observed in some cases, when the degradation parameters were expressed relative to the field controls, indicate that the discharge had an inhibitory effect on the decomposition in these cases. When the most negative values corresponded to the samples close to the discharge points, this may indicate the presence in the effluents of immediately bioavailable toxic substances that inhibit the degradation of organic matter (Bastias et al., 2018). However, when the most negative values corresponded to the samples furthest from the discharge points, this may be attributed to the toxic substances not being immediately bioavailable. For example, toxins that display affinity for organic matter may pass into solution once the organic matter (always present at high concentrations in the WWTP effluents considered) is degraded (Zaidi and Imam, 2008). However, the values of both $CTSL_{fc}$ and CML_{fc} at the most distant sampling point (located at 1000 m from the discharge) were almost always positive, indicating greater loss of mass or tensile strength than in the field control upstream of the discharge and suggesting that the effluent almost always affected the most distant sampling points included in the study.

We found that CML and CTSL were positively related. Tiegs et al. (2007) observed a curvilinear relationship between CML and CTSL, with tensile strength loss varying between 0 and 95 % within a narrow range of mass loss of 20 to 30 %. According to these authors the use of mass loss rather than tensile strength loss would reduce the sensitivity of the cotton-strip

assay. In the present study, the relationship between these parameters was linear and also highly significant, suggesting that mass loss is a useful indicator of degradation. The lower dispersion of data is also an advantage relative to CTSL.

Among the relationships between the degradation parameters and the physico-chemical characteristics of the water, the general lack of association with water temperature is noteworthy. An association was only observed in the linear model for ML_{fc} , in fifth place and with a negative slope, which implies less loss of mass at higher temperature. Although a relationship between temperature and cotton tensile strength is not always observed (Niyogi et al., 2013), temperature is often reported to be one of the most important parameters affecting organic matter degradation (Trettin et al., 1996; Griffiths and Tiegs, 2016). However, the lack of correlation between temperature and cotton degradation in the present study may be due to the fact that the mean daily temperature or occasional temperature measurements do not realistically reflect how variations in temperature affect the decomposition rates (Vyšná et al., 2014). It is also possible that the cotton strips were exposed to a limited range of temperatures and that no temperature effect occurred. Slocum et al. (2009) reported that temperature clearly affected degradation of cotton strips, although these researchers used two very different temperatures (20°C and 35°C).

The significant, negative relationship (with a high coefficient of determination) between $CTSL_{fc}$ and phosphates implies that a higher concentration of these compounds in water will be associated with lower loss of tensile strength. This finding directly contrasts with those of other studies (e.g. Newman et al., 2001; Bierschenk, 2012; Tiegs et al., 2013). It is generally considered that microbial communities are stimulated by the presence of nutrients. However, Tiegs et al. (2013) suggested that the different microbial communities in different sites act as an inoculum for the communities colonizing the organic substrate and that the variation in microbial communities will influence the decomposition rate, independently of the direct effects of nutrients. It is also possible that the toxins present in the effluents will negatively affect the decomposition rate (Bastias et al., 2018) and that phosphates will be more available when decomposition rates are low.

Degradation of organic matter generates sulphates, which explains why discharge of urban sewage leads to increased concentrations of this anion (Antelo and Arce, 1996). In sites characterised by high concentrations of sulphates, the rate of degradation of organic matter (including cotton strips) will be high, which would explain the significant relationships between both CTSL_{lc} and CML_{lc} and sulphate concentrations observed in the present study. The significant relationship between CTSL_{lc} and Ca may indicate that high concentrations of Ca favour decomposition, as also indicated by the positive correlations between Ca in leaf litter and rates of decomposition observed in forest soils (Jacob et al., 2010). It is also possible that some of the Ca in the water will be derived from organic matter decomposition, so that higher concentrations will be found in sites characterised by high decomposition rates (Upadhyay, 1987).

Aquatic bryophytes can be used as a tool for biomonitoring water quality (Debén et al., 2015, 2017). The significant relationships between the parameters measured in the moss and those measured in the cotton strips appear to indicate an association between the composition of the WWTP effluents and the decomposition of the cotton strips. WWTP effluents are enriched in ¹⁵N (Curt et al., 2004; Cole et al., 2005), particularly effluents subjected to secondary treatments (Hicks et al., 2017), such as those considered in the present study. As a result, macrophyte $\delta^{15}\text{N}$ has been proposed as an indicator of the presence of N of anthropogenic origin in aquatic ecosystems (Cole et al., 2005; Carballeira et al., 2012; 2013). Both the individual regressions and the linear modelling indicate a relationship between the isotopic signal $\delta^{15}\text{N}$ in moss transplants and the degradation rates, confirming the validity of both measures as descriptors of organic load exposure. Thus, although the result obtained for phosphates was unexpected, the importance of another nutrient, N, was clearly observed in this case. It is important to note that the physico-chemical characteristics of the water were determined in the present study by single measurements and may be poorly representative owing to the high temporal variability of physico-chemical characteristics and contaminant levels found in river waters and WWTP effluents (Meybeck et al., 1996; Salgado et al., 2011; Morosini et al., 2017). As a result, it is possible that the significant relationships for parameters measured in water were merely

circumstantial. On the other hand, the chemical composition of the transplanted moss may be considered more representative as it reflects the concentrations in the water during exposure of the transplants (Debén et al. 2016, 2017; Carballeira et al., 2019).

Although no significant relationship between the parameters measured in the cotton strips and metal concentrations in mosses was initially observed, the metals were selected in the linear modelling. Cu was particularly important, although with a negative slope, which implies a toxic effect, so that the rate of degradation will decrease as the metal concentration increases (Chew et al., 2001). Mn was also included in the model, with a positive slope, possibly indicating that this element is associated with the discharges. Larger organic loads should imply larger amounts of microorganisms that help degrade the cellulose. Organic matter can have complex effects on metal toxicity and bioaccumulation, and the toxicity and bioaccumulation of elements may decrease due to the formation of complexes with dissolved organic matter (Luider et al., 2004; Ravichandran, 2004). By contrast, other studies have shown that the solubility and bioavailability of elements such as Rh, Hg and Zn may increase with the amount of dissolved organic matter in water (Rupa et al., 2003; Chen et al., 2004; Ravichandran, 2004; Sures and Zimmermann, 2007). Thus, the CSA will indirectly provide information about the bioavailability and accumulation of some metals. However, determining the origin of the toxicity in sewage is very difficult due to the multiple overlapping factors affecting the process (Gartiser et al., 2010). Vasquez and Fatta-Kassinos (2013) indicated the weak correlation between physico-chemical and ecotoxicological data for treated wastewater from sewage treatment plants. Moreover, the stressors affecting the allochthonous organic matter breakdown rates are almost unknown (Schäfer et al., 2012).

In view of the scarcity of other similar studies using the CSA, the relationships between the rate of decay of the cotton strips and the concentrations of metals in the effluent and the river water are, in short, difficult to explain due to the complex interactions between the decomposer organisms (grazers, shredders, microbes, etc.), the type of material and stage of decomposition, the environmental conditions, the metals present in the environment, the metals released during the decomposition process and those bound to other substances, and the presence of other

pollutants (Schaller et al., 2011; Van Nevel et al., 2014; Schaller and Brackhage, 2015; Ferreira et al., 2016).

Sewage treatment has advanced greatly in recent years, and the quality of the effluents discharged from modern WWTPs is such that several in situ studies have not detected any differences between control sites and sites affected by the effluents (Spänhoff et al., 2007). However, such findings can only be corroborated by integrated environmental monitoring involving various lines of evidence (LOE). The primary LOE in integrated monitoring plans adapted to WWTPs usually consists of the physico-chemical characterization of the discharges by conventional analysis of parameters that play an important role in the survival of aquatic species (Gosset et al., 2016), with the aim of verifying whether the treatments comply with the limits outlined in the prevailing legislation for the discharge of effluents in different types of water bodies. WWTPs were originally designed to reduce the amounts of biodegradable organic matter, solids and even nutrients in effluents, but not to totally remove harmful substances (Ormad et al., 2011). Therefore, in addition to this LOE, analysis of persistent organic pollutants that can potentially be accumulated should be conducted, while not overlooking emergent compounds that are ubiquitous in sewage effluent (Kümmerer, 2009; European Commission, 2012). However, physico-chemical characterization does not indicate how the biota may be affected (Carballeira et al., 2012). The impacts on ecosystems due to the interactive effects between contaminants present in urban sewage waste on release into the environment are unclear and unpredictable (Filby et al., 2007). Thus, a second LOE should be included in order to evaluate the overall toxicity of effluents by the use of a battery of bioassays carried out under laboratory conditions in order to standardize the ecotoxicological assessment of WWTP discharges (ECETOC, 2004; OSPAR, 2007; EEA, 2011).

The complexity of WWTP effluents, which contain both stimulators (organic matter) and inhibitors (toxins), modifies the balance between autotrophic and heterotrophic processes and produces spatial discontinuities in ecosystem functioning (Aristi et al., 2015). Therefore, only bioassays carried out in field conditions (in situ) provide realistic information about the ecotoxicity of discharges (Gosset et al., 2016). In situ bioassays could form part of a third LOE

aimed at evaluating the ecological integrity. However, these types of bioassay are often difficult to develop and interpret due to the complex interactions between contaminants and between these and numerous physical factors (Filby et al., 2007). In this respect, the CSA can be considered a simple, rapid, economic and standardized protocol for characterizing the receptor ecosystem integrity from a functional perspective relative to conventional structural methods such as the study of epilithic diatoms or benthic macroinvertebrate communities. For validation of the usefulness of the CSA as a routine monitoring tool, future studies should consider different scenarios and conditions in order to take the probable complex interactions between decomposers and mixtures of contaminants into account.

5. Conclusions

Despite the similarity in the WWTPs under study, the response measured by decomposition of cotton strips varied in the different sites and was more complex than originally hypothesized (i.e. a straightforward decrease in the decomposition rates with increasing distance from the discharge points), probably because the complex interactions between the contaminants present in the effluents generate trophic and/ or toxic effects in variable proportions. For most of the WWTPs considered, the effects of the discharges extended more than 1 km downstream. Mass loss appears to be a suitable measure of decomposition potential, showing even lower between-replicate variability than tensile strength loss. We recommend that bioassays such as the cotton strip assay, which can be conducted in situ under field conditions, should be included in environmental monitoring plans for WWTPs in order to evaluate the ecological integrity of the fluvial ecosystems affected. Such bioassays provide information about the effects on the receptor ecosystems, where the organisms involved in the decomposition process are exposed to effluent discharges under natural environmental conditions.

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Table 1
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Table 1. Characteristics of the sewage treatment plants and flow rate of the rivers affected by the WWTP effluents, measured at the same time as the cotton strips were placed in the sites (see text for details)

Location	Code	Population equivalent design	Mean design flow rate (m ³ day ⁻¹)	Secondary treatment	Advanced treatment	Flow rate (L s ⁻¹) of the river
Sigüeiro	I	5000	1500	Activated sludge	Elimination of N Disinfection by UV light	1792.0
Barcia	II	16000	5530	Activated sludge	Elimination of N and P Disinfection by UV light	105.8
Faramello	III	14000	3857	Activated sludge	Elimination of N and P Disinfection by UV light	10.9
Bandeira	IV	2000	500	Activated sludge	Disinfection by UV light	123.5
Caldas de Reis	V	15000	3857	Activated sludge	Elimination of N and P Disinfection by UV light	634.3
Carballiño	VI	19000	4800	Activated sludge	Elimination of N	182.4

Table 2. Descriptive statistics for the laboratory control strips. n= number of samples, S.D.= standard deviation, C.V.= coefficient of variation.

	n	Mean	S.D.	C.V.
Tensile strength (kg)	12	34.13	2.55	7.47
Mass (g)	12	0.732	0.008	1.14

Table 3
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Table 3. Range of values for each study site where the cotton strips were exposed, physico-chemical characteristics of the water, isotopic signal, $\delta^{15}\text{N}$, and metal concentrations in transplants of the moss *Fontinalis squamosa*

		Site											
		I		II		III		IV		V		VI	
		Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
Cotton strips	Units												
CTSLfc	%	20.23	83.04	-87.37	68.68	54.05	74.73	-74.08	15.43	-30.16	71.70	20.92	87.12
CTSLlc	%	40.88	89.97	65.23	94.19	68.69	92.09	44.90	73.23	64.56	92.29	37.53	91.95
CMLfc	%	14.71	47.32	9.55	21.26	33.72	47.16	-22.48	-6.23	-0.16	37.27	11.66	45.65
CMLlc	%	5.91	50.43	28.76	43.91	14.33	54.73	13.48	29.36	23.99	52.39	6.79	49.34
Water													
Conductivity	μs cm ⁻¹	100.0	238.0	198.8	221.0	126.0	258.0	88.1	109.3	69.5	264.0	68.7	240.0
Temperature	°C	14.6	18.6	15.5	17.5	19.2	24.8	14.9	17.4	18.6	21.7	14.7	16.6
Turbidity	NTU	0	1.72	0	6.3	0	1.76	0	2.87	0	1.73	0	10.24
pH		7.70	8.60	6.76	6.95	6.75	6.85	6.33	6.63	6.76	7.52	6.71	7.43
COD	mg l ⁻¹	14.25	65.5	7.95	10.7	6.75	10.5	7.05	86.1	5.35	21.6	12.5	68.6
BOD ₅	mg l ⁻¹	4.0	20.0	3.5	3.5	3.0	3.0	4.0	8.5	3.0	7.0	4.0	9.0
Na ⁺	mg l ⁻¹	10.45	37.20	8.83	12.63	11.15	15.03	7.78	27.35	5.18	66.90	4.78	26.05
NH ₄ ⁺	mg l ⁻¹	0.05	22.28	0.05	0.19	0.05	0.05	0.05	0.35	0.05	5.90	0.05	3.63
K ⁺	mg l ⁻¹	1.30	8.50	1.03	4.35	1.93	4.33	1.13	6.73	0.63	8.30	1.10	8.33
Mg ⁺²	mg l ⁻¹	2.75	4.00	4.55	5.60	1.80	1.83	1.58	3.15	0.60	1.65	0.50	2.45
Ca ⁺²	mg l ⁻¹	5.45	11.50	12.35	12.90	7.93	8.25	2.48	11.83	3.55	6.45	3.50	11.55
Cl ⁻	mg l ⁻¹	14.50	37.05	11.25	18.98	15.03	18.10	9.93	26.08	7.25	67.90	6.85	27.93
NO ₂ ⁻	mg l ⁻¹	0.10	0.30	0.10	0.50	0.06	0.09	0.09	0.16	0.08	0.40	0.10	6.18
NO ₃ ⁻	mg l ⁻¹	0.70	9.43	8.13	29.03	7.50	13.23	8.85	51.90	1.00	3.33	2.63	11.08
PO ₄ ⁻²	mg l ⁻¹	0.20	0.30	0.25	0.35	0.25	1.50	0.25	3.20	0.30	14.70	0.20	0.30
SO ₄ ⁻²	mg l ⁻¹	5.35	41.88	16.50	21.68	8.05	11.88	3.00	17.75	2.70	12.30	2.35	18.00
TN	mg l ⁻¹	2.35	16.60	2.00	6.65	1.93	3.45	2.13	12.05	1.10	6.85	1.25	7.23
TOC	mg l ⁻¹	0.45	7.88	0.20	2.03	0.13	0.40	0.00	4.33	0.43	5.15	1.35	3.28
<i>F. squamosa</i>													
δ ¹⁵ N	‰	1.40	2.55	3.02	5.18	2.20	6.92	1.78	2.31	2.48	3.51	1.61	4.10
V	μg g ⁻¹	31.94	41.72	47.41	96.70	22.96	35.23	21.21	35.05	28.39	33.41	24.01	28.56
Cr	μg g ⁻¹	21.16	25.15	35.23	89.06	19.04	27.42	16.18	30.02	18.06	22.28	16.88	23.47
Mn	μg g ⁻¹	7332	11445	9971	12025	4041	10611	3786	6023	2918	10142	5180	7076
Co	μg g ⁻¹	57.50	87.95	102.89	123.04	30.01	73.63	35.59	58.11	34.23	80.01	46.49	62.95
Ni	μg g ⁻¹	21.87	31.63	106.92	117.31	12.40	20.30	23.31	29.61	8.88	13.70	9.12	12.66
Cu	μg g ⁻¹	23.24	41.63	299	339	29.41	67.39	15.23	219	19.44	33.73	18.89	69.49
Zn	μg g ⁻¹	186	213	607	680	215	408	85	239	130	179	158	249
As	μg g ⁻¹	18.56	27.30	21.45	33.93	11.77	24.90	15.99	22.24	24.51	62.64	28.68	59.28
Se	μg g ⁻¹	8.08	9.35	9.58	10.51	4.83	9.09	6.05	7.65	6.24	9.03	7.01	8.41
Rh	ng g ⁻¹	1.09	1.33	1.32	2.16	1.08	1.53	1.07	1.80	0.83	1.27	0.95	2.25
Cd	μg g ⁻¹	0.65	0.90	1.46	1.63	0.26	0.76	0.38	0.73	0.44	0.67	0.46	0.63
Hg	μg g ⁻¹	0.11	0.42	0.17	0.22	0.10	0.17	0.14	0.33	0.09	0.15	0.16	0.61
Pb	μg g ⁻¹	6.56	10.06	10.07	21.07	7.71	14.72	9.67	14.86	6.74	11.29	6.14	30.54

Table 4. Summary of the proposed models for exploring the main predictors of CTSL and ML by automatic linear modelling, taking into account only the variables measured at all sampling points: conductivity, pH, temperature and turbidity in water samples; total N, $\delta^{15}\text{N}$ and metal concentrations (Mn, Cu, As, Se, Rh, Hg) in transplants of the aquatic moss *F. squamosa*. In order to prevent colinearity, the following variables were excluded: V, Cr, Co, Ni, Zn, Cd and Pb in *F. squamosa*.

Source	Model R^2	F	df	Sig.	Importance	Slope
CTSL _{fc}	0.368	5.22	4	0.003		
Cu		11.13	1	0.003	0.407	-
Mn		6.86	1	0.015	0.250	+
Turbidity		6.71	1	0.016	0.245	+
$\delta^{15}\text{N}$		2.67	1	0.115	0.098	+
CTSL _{lc}	0.450	15.30	2	0.000		
Conductivity		6.26	1	0.018	0.611	+
$\delta^{15}\text{N}$		3.98	1	0.054	0.389	+
CML _{fc}	0.694	14.15	5	0.000		
Cu		38.86	1	0.000	0.507	-
$\delta^{15}\text{N}$		12.41	1	0.002	0.162	+
Mn		11.05	1	0.003	0.144	+
Conductivity		7.54	1	0.011	0.098	+
Temperature		6.84	1	0.015	0.089	-
CML _{lc}	0.606	14.47	4	0.000		

$\delta^{15}\text{N}$	12.09	1	0.002	0.456	+
Conductivity	7.98	1	0.008	0.301	+
Cu	2.65	1	0.114	0.242	-

Table 5
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Table 5. Summary of the proposed models for exploring the main predictors of CTSL and CML by automatic linear modelling only taking into account the variables measured at the sampling points corresponding to the discharge points and the upstream site: conductivity, pH, temperature, cations (NH_4^+ , K^+ , Ca^{+2}), anions (Cl^- , NO_2^- , NO_3^- , PO_4^{-3} , SO_4^{-2}), total organic carbon and total N in water samples; total N, $\delta^{15}\text{N}$ and metal concentrations (V, Mn, Cu, As, Se, Rh, Hg and Pb) in transplants of the aquatic moss *F. squamosa*. The following variables were excluded in order to prevent colinearity: turbidity, Na^+ , Mg^{+2} , COD and BOD_5 in water samples, and also Cr, Co, Ni, Zn, Cd in *F. squamosa*.

Source	Model R^2	F	df	Sig.	Importance	Slope
CTSL _{fc}	0.875	18.53	2	0.020		
	PO_4^{-3}	18.53	2	0.020	1.000	-
CTSL _{lc}	0.587	8.81	2	0.008		
	Ca^{+2}	5.88	1	0.038	0.568	+
	$\delta^{15}\text{N}$	4.47	1	0.064	0.432	+
CML _{fc}	No variables were significant predictors					
CML _{lc}	0.812	24.79	2	0.000		
	$\delta^{15}\text{N}$	37.84	1	0.000	0.707	+
	NH_4^+	15.65	1	0.003	0.293	+

Figure 1

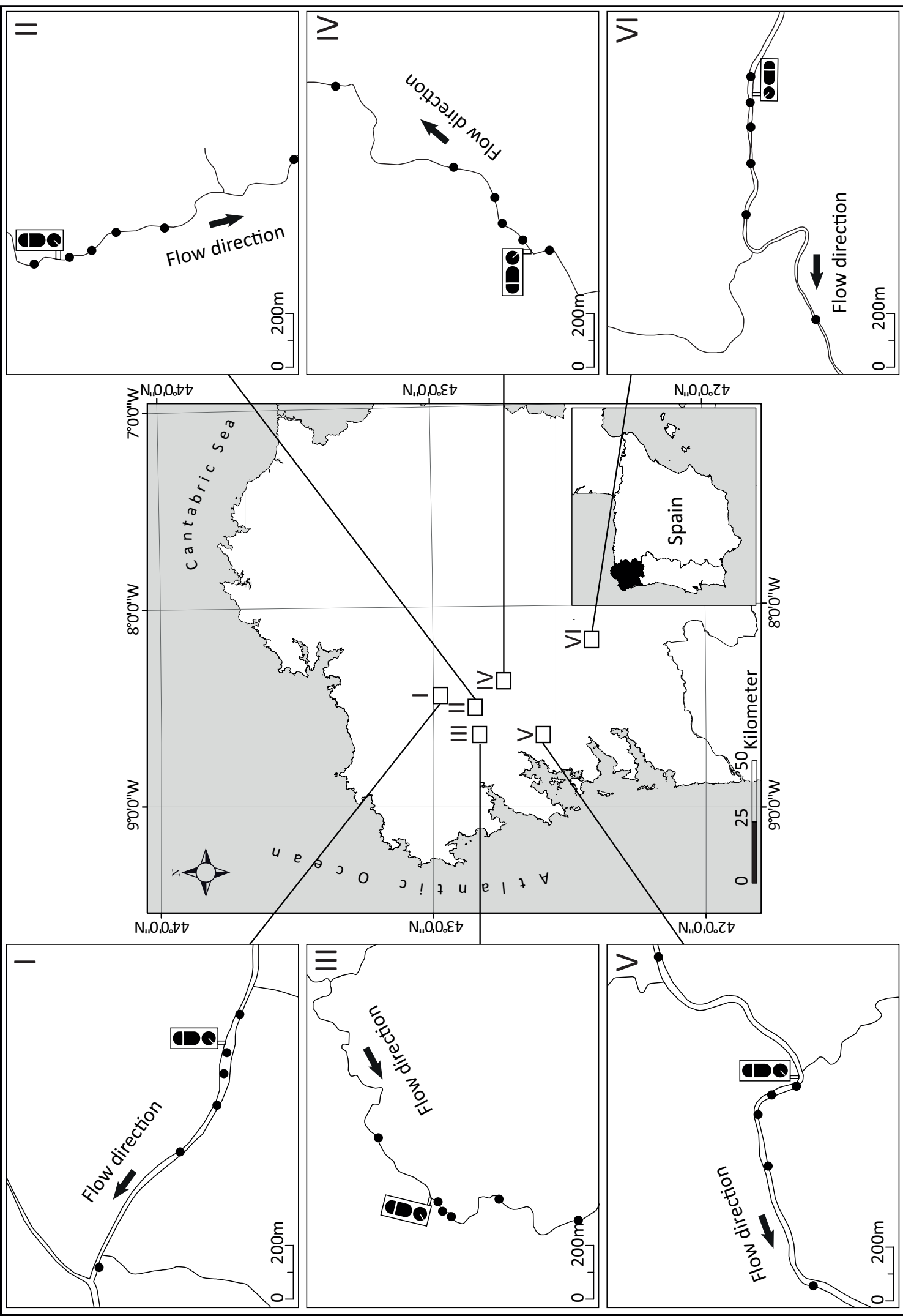


Fig. 1. Location of the sampling sites.

Figure 2

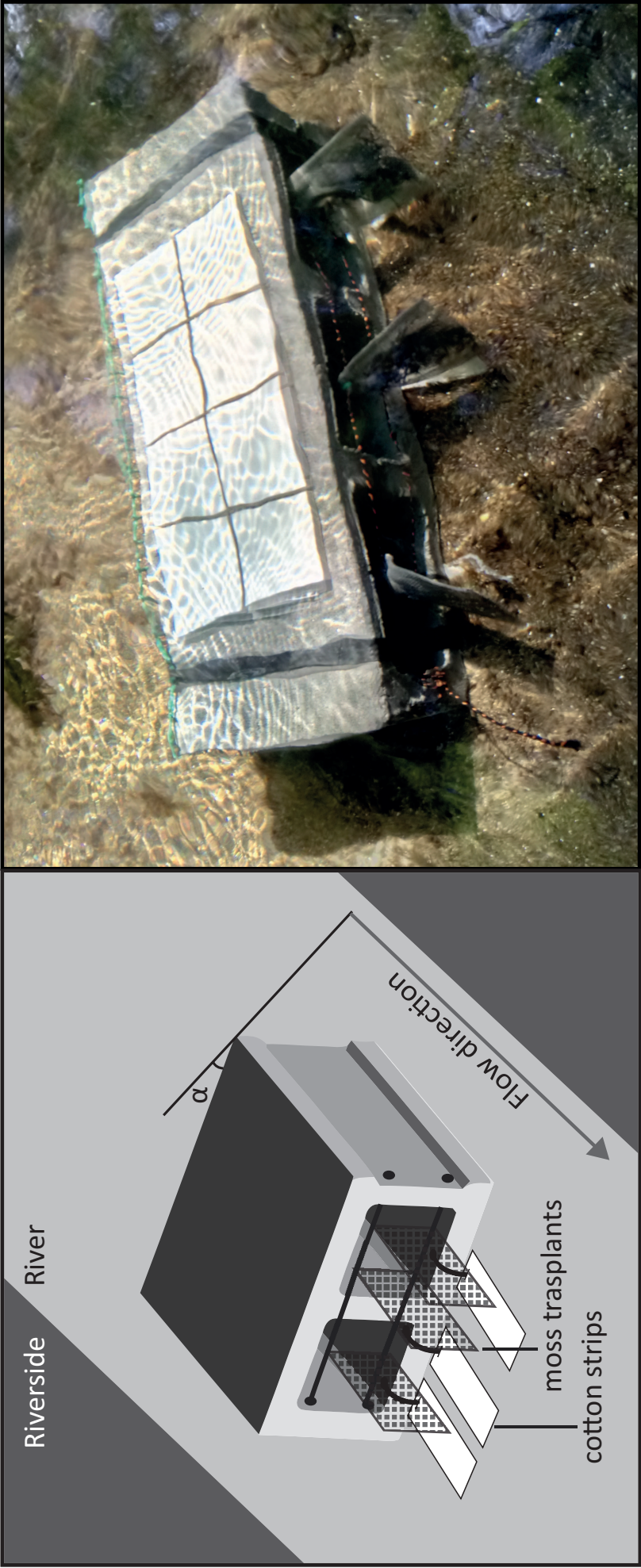


Fig. 2. Diagram and photograph showing how the cotton strips were attached to concrete bricks and exposed on the riverbed.

Figure 3

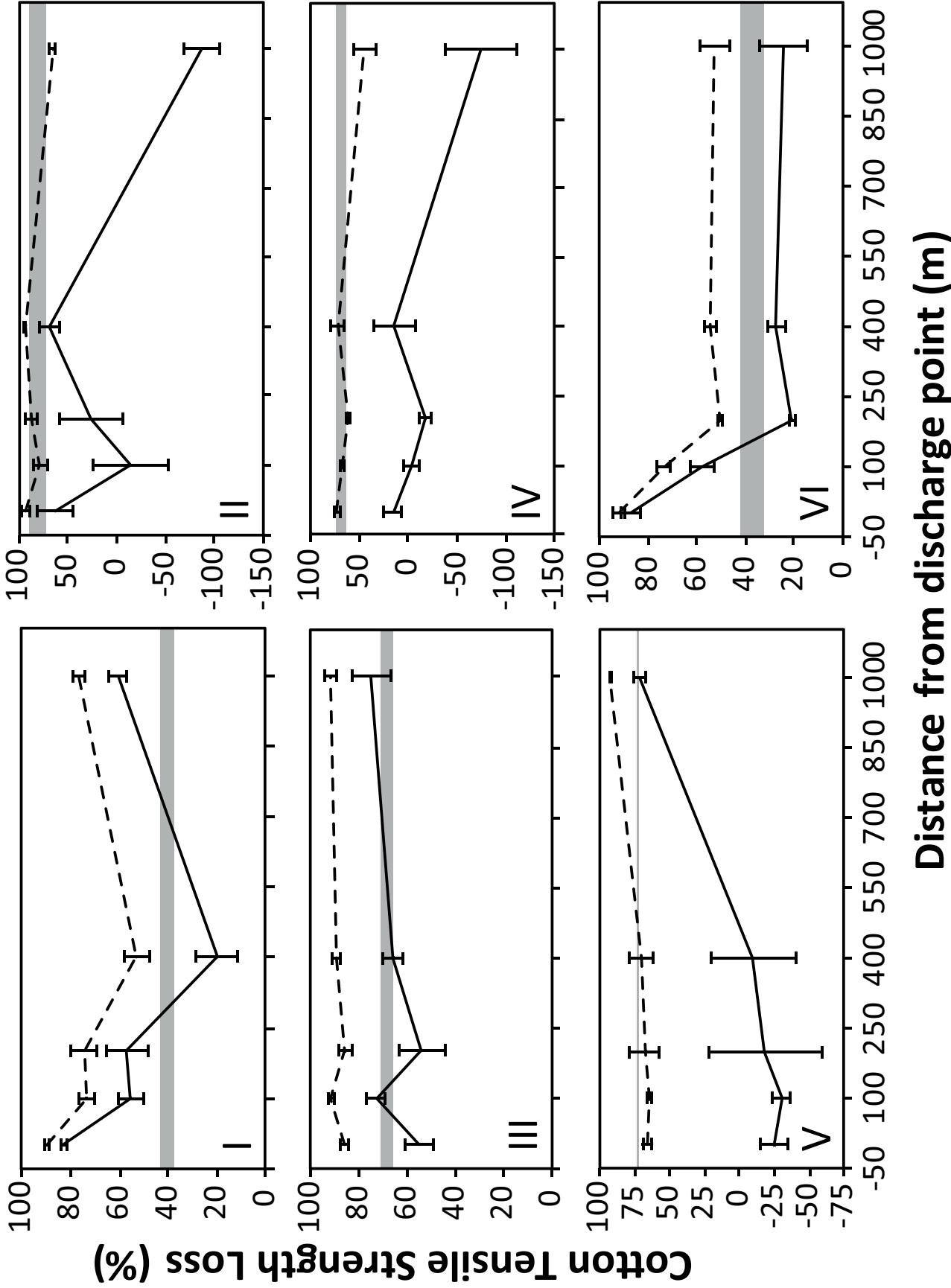


Fig. 3. Tensile strength loss from the cotton strips, expressed as a percentage of the field control (continuous line) and as a percentage of the laboratory control (dashed line). The plotted values are means of 3 replicates, and standard error bars are shown. The grey bands represent the mean values plus/minus the standard error for each site located upstream of the discharge point.

Figure 4

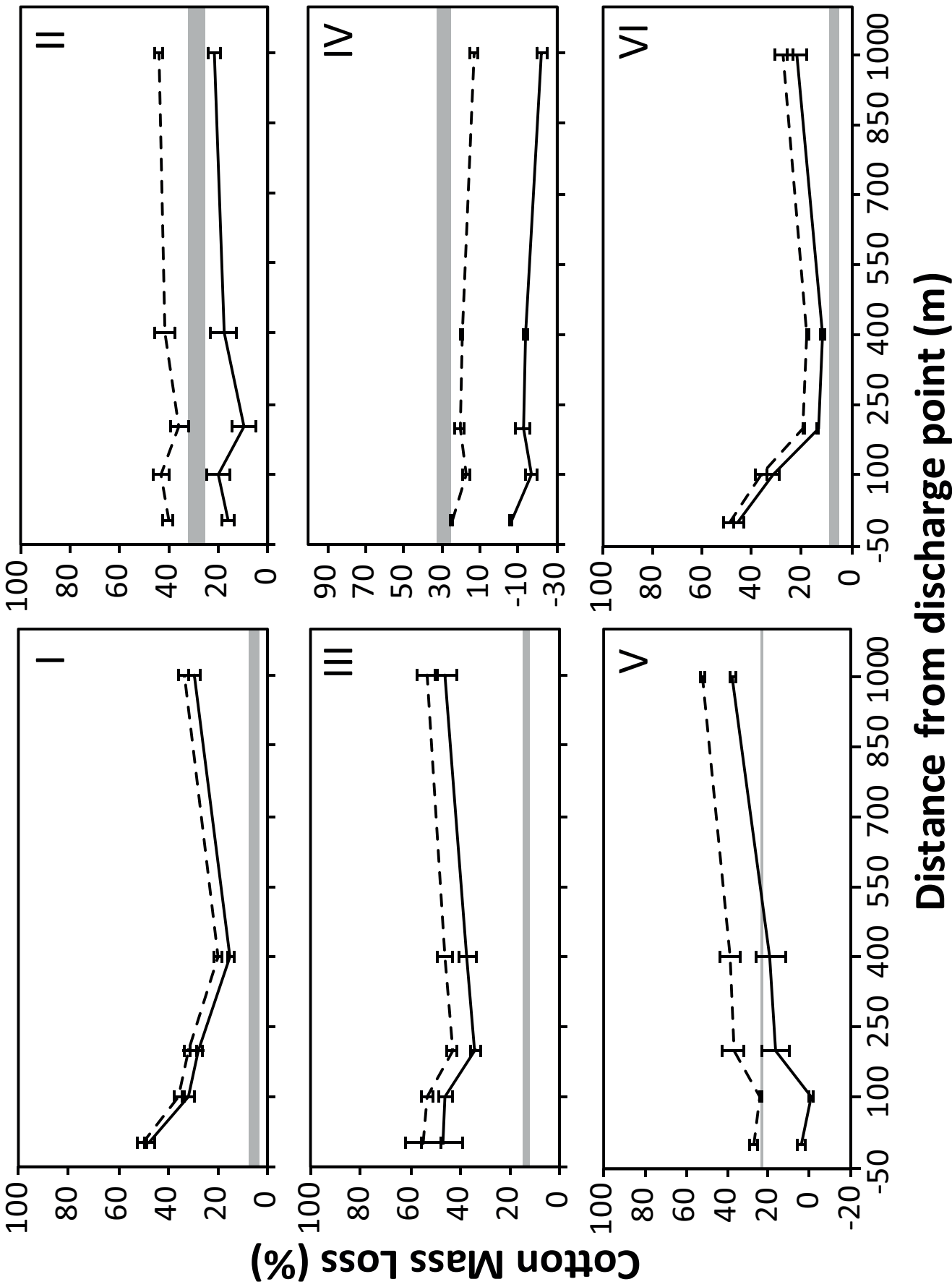


Fig. 4. Mass loss from the cotton strips, expressed as a percentage of the field control (continuous line) and of the laboratory control (dashed line). The values plotted are means of 3 replicates, and the standard error bars are shown. The gray bands represent the mean values plus/minus the standard error for each site located upstream of the discharge point.

Figure 5

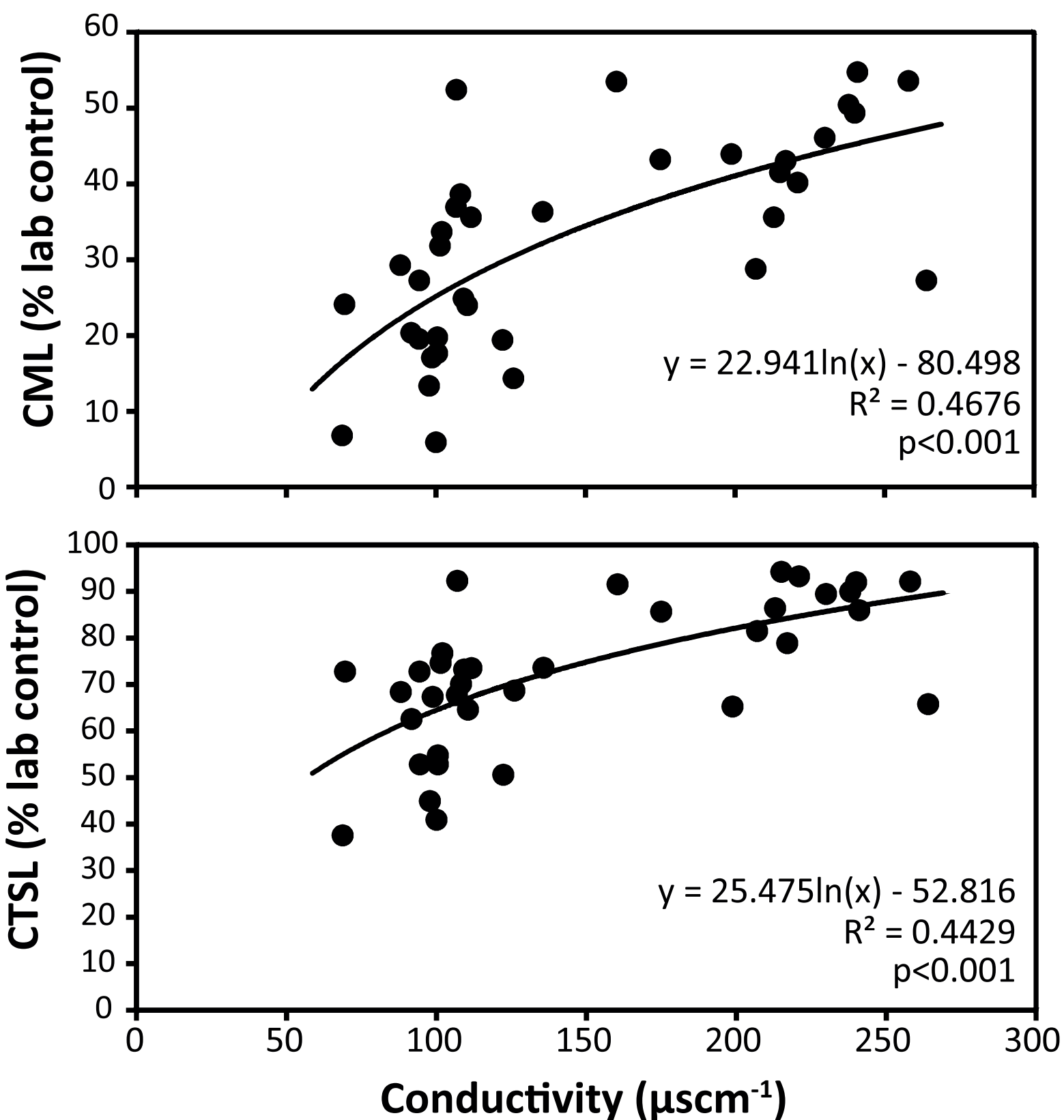


Fig. 5. Scatter plots of conductivity against cotton mass loss (CML) and cotton tensile strength loss (CTSL), expressed relative to the laboratory control and considering all samples, showing the best fit regression lines.

Figure 6

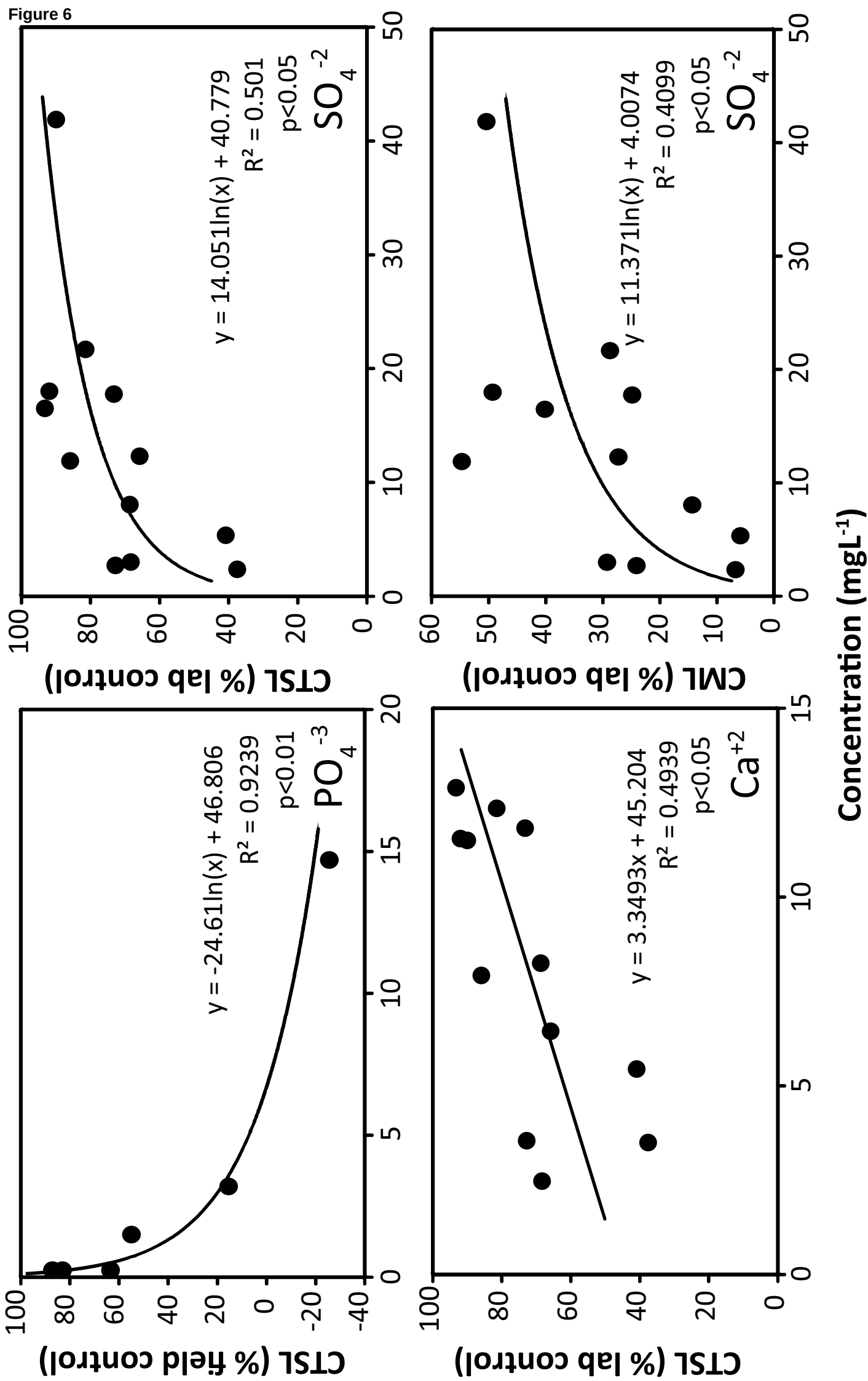


Fig. 6. Scatter plots of concentration of selected cations and anions against cotton mass loss (CML) and cotton tensile strength loss (CTSL). The best fit curves are shown in all plots (only significant regressions are shown).

Figure 7

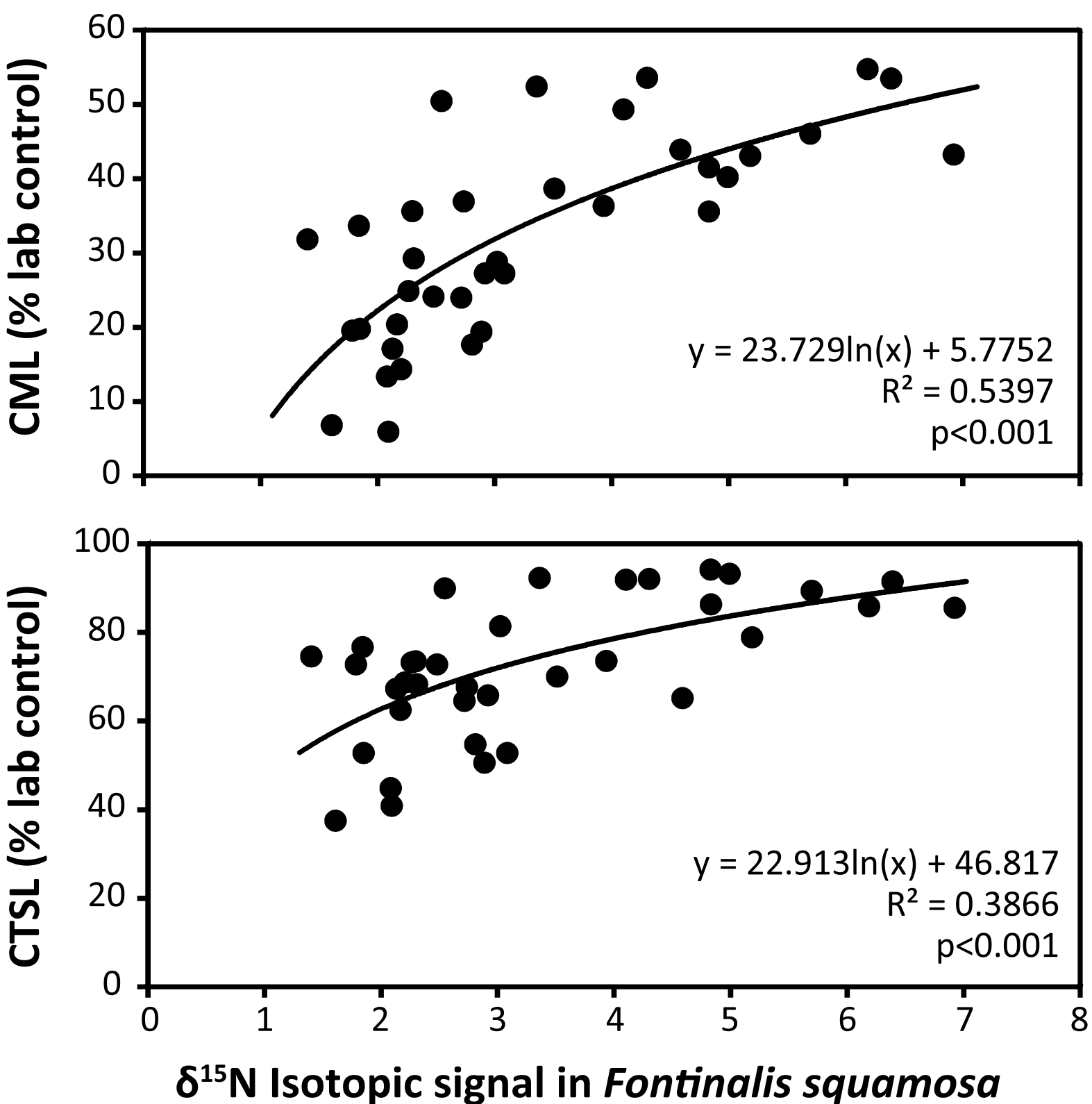


Fig. 7. Scatter plot of the $\delta^{15}\text{N}$ isotopic signal in transplants of the moss *Fontinalis squamosa* against cotton tensile strength loss (CTSL) and cotton mass loss (CML), expressed relative to the laboratory controls. The best fit curves are shown in both plots.