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In situ and laboratory non-additive litter mixture effect on C dynamics of *Sphagnum rubellum* and *Molinia caerulea* litters

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Abstract

Purpose. The accumulation of carbon in peatlands originates from the slow rate of *Sphagnum* litter decomposition. Vegetation shifts can alter the *Sphagnum* decomposition rate through a litter mixture effect. This is rarely studied in peatlands. In a site colonized by vascular plants, we examined the effect of mixing litters of *Sphagnum* species with those of *Molinia caerulea* and *Betula* spp on litter C dynamics. We tested that water content and pH may explain the potential non-additive litter mixing effect.

Materials and methods. Litter bags with *S. cuspidatum* or *S. rubellum* and *M. caerulea* or *Betula* spp were placed in situ, in a *Sphagnum* decomposing environment and retrieved after one year of incubation. In the laboratory, the specific interaction between *S. rubellum* and *M. caerulea* was investigated. Solid, soluble and gaseous forms of C were studied in addition to the fluorescence of the dissolved organic matter (FDOM).

Results and discussion. Laboratory and field experiments showed that there is a non-additive effect of mixing *S. rubellum* and *M. caerulea* litter on C dynamics. The analyses of FDOM suggested a relatively higher living biomass in the laboratory measured mixture than in the expected one. The in situ *S. rubellum* moist environment could stimulate the decomposition of *M. caerulea* that experiences much drier conditions in its native environment. In the laboratory experiment, *M. caerulea* were kept moist, and no significant difference in water content between the measured and expected mixture was found. Also, pH decreased in the measured mixture, ruling out any direct effect on microbial activity.

Conclusions. The non-additive mixture effect observed in the laboratory may be triggered by an increase of the microbial biomass. This increase was not explained by direct moisture or a pH effect. The suggested hypothesis that a lower pH could affect the availability of labile organic substrate through increased OM hydrolysis and thus stimulate microbial growth has to be further studied.

Keywords Aromaticity • Fluorescence • Litter-mixture effect • Peatland invading species • Water extractable organic matter

1 Introduction

Temperature and water regime perturbations are provoking shifts in the limits of plant distribution and changes in plant abundances (Weltzin et al. 2003; IPCC 2007; Limpens et al. 2008). These changes will modify the functioning of ecosystems through the occurrences of new interactions (as some plant distribution limits move northward), or the strengthening of previously weak interactions (increased abundances of previously sparse species; Cornelissen et al. 2001). Among ecosystem processes, litter decomposition will be affected by vegetation changes especially because of litter mixture effects (Gartner and Cardon 2004). Litter decomposition is an important component in the functioning of ecosystems as it influences the carbon (C) and nutrient cycles (Berg and McClaugherty 2003). At the local scale, litter decomposition influences primary productivity through the mineralization of nutrients, and at the global scale, it affects the C cycle through soil organic matter (SOM) stabilization and accumulation (Limpens et al. 2008; Bardgett and Wardle 2010). When a vegetation change occurs, new litter mixture effects are expected to occur. These effects can be detected by comparing the measured values of two or more litters in mixture to theoretical values calculated from the same litters incubated in monoculture (Gartner and Cardon 2004). An additive effect is identified when there is no difference between the measured and expected decomposition rate. When a difference is observed, the litter mixture effect is non-additive. It can be synergistic, i.e. stimulation of the decomposition, or antagonistic, i.e. inhibition of the decomposition. Hoorens et al. (2010) showed that the litter mixture effect may be additive at the plant functional level, because these opposite interactions may take place at the same time. However, such an "averaging" effect may be limited in low biodiversity ecosystems. Identifying such a litter mixture effect in ecosystems and understanding the mechanisms behind it is essential to specify which vegetation change can enhance (increased SOM mineralization) or buffer (increased SOM accumulation) greenhouse gas emissions and/or dissolved organic carbon export.

To better understand soil C dynamics, Cotrufo et al. (2010) suggested that simultaneous measurements of (i) CO_2 production, (ii) remaining mass and (iii) water extractable organic carbon (WEOC) should be undertaken. As suggested by Schimel and Weintraub (2003), this latter compartment is thought to be the intermediate pool between remaining mass (solid C) and CO_2 production (gaseous C). Following the suggestions of Cotrufo et al. (2010) and after adapting the model of Schimel and Weintraub (2003) to make it experimentally testable, Gogo

et al. (2014) showed in laboratory incubation of peatland plant litters that the WEOC compartment is accurately accounted for when it is assumed that it results from the balance between solid OM decomposition and soluble C respiration, and is quantitatively the most sensitive pool to changes in solid OM decomposition and respiration rates. In the context of the litter mixture effect, quantities as well as the chemical characteristics of WEOC are probably affected. Creamer et al. (2015) showed that litter WEOC is more sensitive to temperature change than WEOC from the mineral soil. This fraction may also be sensitive to other modified conditions such as litter mixture with invading species.

Peatlands contain up to a third of the world soil C (Gorham 1991). Their accumulation capacity originates from the particularly low decomposition rates of *Sphagnum* species (Clymo 1983). Any modification in their decomposition rate, through the litter mixing effect caused by vegetation change, would thus undermine the regulatory role of peatlands on the global C cycle (Limpens et al. 2008). Hoorens et al. (2002) showed that an increased atmospheric CO_2 concentration can lead to an excess microbial respiration in *Sphagnum recurvum* and *Carex rostrata* mixtures. In spite of the importance of such a result and the unfolding vegetation change in peatlands, very few studies have actually addressed the issue of a litter mixing effect in such ecosystems.

The two aims of this work are (i) to determine the occurrence of a mixture effect of *Sphagnum* and vascular plant litters and (ii) to identify the mechanisms behind such an effect. For this, two experiments were undertaken: a field experiment to test the effect of mixing *Sphagnum rubellum* and *cuspidatum* with *Betula* spp and *Molinia caerulea* on litter remaining mass, and a laboratory incubation experiment testing the effect of mixing *Sphagnum rubellum* and *Molinia caerulea* on solid, water extractable and gaseous C from these litters. In the latter experiment, the model developed in Gogo et al. (2014) was implemented to estimate overall exo-enzyme activity and respiration rate. Litter mixture effect can result from nutrient transfer between litters, influences caused by specific compounds released by the litters, changes in microclimatic conditions and modification of trophic levels interactions (Gartner and Cardon 2004; Hättenschwiler et al. 2005). Nutrient content of *Sphagnum rubellum* and *Molinia caerulea* is very low (Gogo et al. 2011). Thus, nutrient transfer may not be a pertinent cause of possible litter mixture effect. Instead, physical changes may occur. *Sphagnum* can affect the pH significantly and these species can retain up to 20 times its weight in water (Rydin and Jeglum 2013). Both

parameters are known to have an effect on OM decomposition (Bergman et al. 1999; Prescott 2010). To explain potential litter mixture effects in the laboratory experiment, the following two hypotheses were tested: compared to the theoretical expected values, mixing the two litters increases (a) litter water content or (b) pH of the water extract. To test these hypotheses, the water content of all litters was assessed as well as the pH of the water extract. To analyse in greater detail the water extract characteristics, the UV absorption (aromaticity) and the fluorescence properties (differentiation of pools of OM) of the soluble OM were investigated. Fluorescence analyses of DOM gave excitation-emission matrices (EEMs) that were treated using PARAFAC modelling.

2 Materials and methods

2.1 Sampling site

All litters for *in situ* and lab experiments were collected in La Guette peatland (Sologne, France), a site of the French Peatland Monitoring Service: Service National d'Observation Tourbières. It is located in Neuvy-sur-Barangeon (Cher) in the south-eastern part of the French Centre Region (altitude: 154m, N: 47°19', E: 2°16', Gogo et al. 2 011). La Guette peatland is composed of vegetation patches ranging from a transitional fen to wet heathland dominated by *Molinia caerulea*, *Erica teralix* and *Calluna vulgaris*. The dominant *Sphagnum* species are *Sphagnum cuspidatum* and *Sphagnum rubellum*. In recent decades, the abundance of vascular species already present in the ecosystem, *Molinia caerulea* and *Betula* spp., increased dramatically. Trees covered 15% of the peatland surface in 1980, 50% in 2000 and 69% in 2009. The reasons for such an abrupt increase may be hydraulic works and pine plantation in the surroundings of the site, as well as nutrient inputs.

2.2 Coupling field and laboratory experiments

Studying the effect of mixing litters with *Sphagnum* species is not as straightforward as with vascular plants. In the field, the incubation environment and the *Sphagnum* litter to decompose in the bag are the same material: *Sphagnum* litter. Whether in mixture with *Sphagnum* or in monoculture, the other litter composing the mixture experiences the same incubation conditions. To avoid this issue, Hoorens et al. (2003) and Hoorens et al. (2010) decided to incubate the litter bags in another environment than the natural one, with similar abiotic conditions. This

allows a fair contribution of both litters, but as the incubation conditions are not strictly the same as the natural ones, the results are difficult to transpose to the true environment.

Gartner and Cardon (2004) suggested that the remaining mass of each litter incubated in mixture should be weighed. Thus, following this recommendation, it was initially planned to separate the litter in mixture samples. However, preliminary tests showed that separating the *Sphagnum* litter which can be composed of very small leaves is a difficult and time consuming task that can lead to biased results as it is difficult to be sure that all the *Sphagnum* leaves have been separated,

In this study, it was decided to implement the field experiment in *Sphagnum* hummocks and lawns to test only the effect of the vascular plant litter on *Sphagnum* litter decomposition. Vascular plant litters were also incubated in their native environment. The field experiments were further complemented with a laboratory incubation experiment, where all the litters were incubated in the very same conditions. It has been shown that the initial laboratory respiration rate could give a fair representation of long-term litter decomposability (Aerts and de Caluwe et al. 1997). In addition to respiration, the litter remaining mass and WEOC quantity and characteristics were also measured.

2.3 Field experiment

Litter bags (0.5 mm mesh) were filled with air-dried litter (about 750 mg) of different species: *Sphagnum cuspidatum*, *Sphagnum rubellum*, and leaves of *Betula* spp and *Molinia caerulea*. These samples are referred to as “monoculture”. The mixture samples were composed of 50% of *Sphagnum* litter (either *S. cuspidatum* or *S. rubellum*) with 50% of a vascular plant litter (either *M. caerulea* or *Betula* spp). The litter bags (n=4) were placed in situ in March 2009. In *S. rubellum* hummocks were placed: *S. rubellum*, *M. caerulea* and *Betula* spp litters in monoculture and in mixture (3 monocultures and 2 mixtures: *S. rub* + *M. cae* and *S. rub* + *Bet. spp*). In S. cuspidatum lawns were placed: *S. cuspidatum*, *M. caerulea* and *Betula* spp in monoculture and in mixture (3 monocultures and 2 mixtures: *S. cus* + *M. cae* and *S. cus* + *Bet. spp*).

In addition, the same protocol was followed to prepare *M. caerulea* and *Betula* spp. litter bags in monoculture that were placed in their native decomposition environment. Litter bags of *M. caerulea* were placed in the litter of *M. caerulea* in tussocks. The litter bags of *Betula spp* were

placed in the litter of *Betula* spp. Both experiments were located in the same area and undertaken at the same time.

The litters were collected in March 2010 after one year of incubation. In the laboratory, the roots were retrieved from the litter bags with tweezers and the litters were gently washed with deionised water. The litter bags were dried at 50℃ for 2 days and weighed and the proportion of remaining mass was calculated (100 x remaining dry mass / initial dry mass).

There was an attempt to separate the litters, but this raised some issues. First, as the samples were extracted with water, some materials were found on the filter and some of these materials were so small that it was impossible to pool them with one or another litter. Furthermore, most of the vascular plant litter remained in one piece, which was absolutely not the case for *Sphagnum* litter, which scattered. This made the recovering of *Sphagnum* litter difficult to undertake without introducing an error in the estimation of the mass remaining, and thus a possible error in the detection of a mixture effect. It was preferred not to separate the litter to have a better guarantee on the whole mixture effect.

2.4 Laboratory experiment

As the field experiment showed significant differences between measured and expected values in the *S. rubellum* and *M. caerulea* mixture, the laboratory experiment focused on these 2 litters. The litters were collected in April 2011 and air dried before incubation, until they reached constant weight Surface peat water was collected in the same site. There was enough free water to collect it with a beaker and poor it into a flask. Then the water was filtered at 100 µm to get rid of the coarsest particles, without excluding microbial communities.

2.4.1 Sample preparation and incubation

The day after the water was collected from the peatland, air dried *S. rubellum* and *M. caerulea* litter samples were weighed (about 250 mg) and inoculated with 20 ml peat water and left overnight. Then, the excess water of the litters was removed with a tissue and the litters were placed in a 50 ml tube, which contained 5 ml of a potassium sulfate saturated solution (120 mg l^{-1}) to maintain moist conditions (Aerts and de Caluwe 1997). Glass marbles were also placed at the bottom of the tube and covered with a PVC filter (0.5 mm mesh) to avoid contact of the litter with the potassium sulfate solution. A total of 126 samples were prepared: 3 litter types (*S.*

rubellum, *M. caerulea* and the mixture of the two species, 50% each), 42 samples per litter type, and incubated in controlled conditions. An insulated container (0.3 m x 1 m x 1 m) was built with pieces of PVC covered with aluminum foil to ensure constant environmental conditions during incubation. To obtain a water-saturated air, the atmosphere of the container was filled with air that was pumped through warm water. Sets of samples (*S. rubellum*, *M. caerulea* and the mixture) were randomly put in racks that were placed in the container. The position of the racks within the container was changed every week. Temperature and humidity were monitored and showed that the experimental setup ensured a constant water-saturated atmosphere and an air temperature of 22.5 ± 0.3 °C (n = 32).

A total of 126 samples allowed a kinetic study of 7 dates with 6 samples of each litter type at each date. To calculate expected mixture results from additive effect (50% of each litter type), groups were composed of one sample of each type of litter (total of 3 samples per group: 1 *M. caerulea* alone, 1 *S. rubellum* alone, 1 mixture of both). At each date, 6 groups were randomly retrieved after 2, 6, 12, 20, 33, 47 and 64 days of incubation. Immediately after the sample had been taken out, the CO_2 production was measured. Then the water extraction of litters was undertaken and finally the sample was dried at 50°C during 2 days before weighing.

2.4.2 CO_2 production

The tubes were placed in a chamber during 20 to 30 minutes. The cover of the chamber was fitted with a mounting flange, which could receive a Vaisala CO_2 infra-red probe (GMP343). The CO_2 concentrations were monitored and recorded directly on a computer. The CO_2 production was calculated from the slope of the increasing concentration with time.

2.4.3 Water Extractable Organic Carbon (WEOC)

After the CO_2 production measurement, the litter was taken out of the tube and placed in an aluminum cup at room temperature. The litter was rinsed twice with 15 ml and once with 10 ml deionised water and the extract was filtered (0.45 µm). The filter was weighed before filtering and was dried 48 h at 50 °C after filtering. The difference in filter mass was added to the mass of litter left in the aluminum cup (litter also dried at 50 °C during 48 h and weighed) to obtain the whole remaining mass. A filtrate subsample was dedicated to dissolved organic carbon (DOC) analysis (Shimadzu TOC 5000), which allowed the calculation of the WEOC (in g C g^{-1} initial

litter dry weight). Another filtrate subsample was dedicated to UV absorption and fluorescence spectroscopy analyses. Before injection into the Shimadzu TOC 5000, the samples were acidified and bubbled to eliminate dissolved inorganic C, leaving only the DOC to be analyzed. Phtalate was used for calibration and each sample was replicated until the coefficient of variation was less than 10% (maximum replication = 5). This procedure was undertaken with each sample (n = 6) from each treatment. Just after extraction, the pH of the extracts was measured on subsamples.

2.4.4 Calculation of remaining mass

For both field and laboratory experiments, i.e. laboratory and field incubations, the litters were air-dried until reaching a constant weight. Then, a set of air-dried samples of each litter was dried at 50 ℃ during 48 h and weighed. This allows the determination of the air to oven dried ratio. This ratio was used to calculate the initial oven dried mass of all incubated air-dried litters. The sum of the litter left after extraction and the litter on the filter gave the remaining mass. This percentage was calculated by dividing the remaining mass at any time by the initial mass of the litter and multiplied by 100. The mass of wet samples was also measured and the water content in g of water per g of remaining mass was calculated.

2.4.5 $SUVA_{280}$

The water samples were neutralized with NaOH before measurement as recommended by Weishaar et al 2003). Most of the samples were in the range of 10 to 20 mg L^{-1}, with no samples higher than 31 mg L^{-1} or lower than 7 mg L^{-1}. UV absorption at 280 nm of all water samples was acquired with a Hitachi U-1900 absorbance spectrophometer using a 1 cm path-length quartz cell (6030-UV Hellma Analytics). The zero absorbance adjustment was carried out with deionised water before each batch of measurements. Absorbance spectra was measured between 1100 nm and 200 nm. The absorbance at 280 nm was divided by the DOC content of the sample to give the Specific UV Absorption at 280 nm ($SUVA_{280}$ in L cm^{-1} mg^{-1}). The $SUVA_{280}$ informs on the aromaticity of the DOC (Chin et al 1994).

2.4.6 Natural dissolved organic matter fluorescence (FDOM) measurement and signal processing

Fluorescence properties of DOM have been used to study the mixing of fresh and salt waters in estuaries (Coble 1996; Parlanti et al. 2000, Huguet et al. 2009), as well as humic substances in soil and peat water (Zsolnay et al. 1999; Alberts and Takács 2004; Zaccone et al. 2009). In this study, FDOM was measured to assess whether this technique could differentiate between sources of DOM in decomposition experiments.

After UV-vis analysis, the same sample was used to characterize FDOM. Excitation–Emission Matrices (EEMs) were acquired with a Hitachi F-7000 fluorescence spectrophotometer equipped with FL solutions software, using 10 x 10 mm quartz cell (1000-OS Hellma Analytics). For excitation and emission monochromators, slit widths were set at 2.5 nm. EEMs were recorded using a scan speed of 1200 nm/min, at 950 V, over ranges of excitation and emission wavelengths of 250–500 and 300–600 nm, respectively. Excitation and emission wavelengths were stepped by 10 and 1 nm respectively and no inner effect correction was necessary. After subtraction of the blank (deionised water), the EEMs were treated by CP/PARAFAC algorithm to extract independent fluorescence contribution.

PARAFAC analysis identified four groups of components in the water extracted organic matter (WEOM) from incubated litter (Table 1) with a CORCONDIA of 68.9%. Three ratios were calculated using the C2 fluorophores corresponding to α or C (Table 1) as the denominator. The ranges of excitation-emission of the four groups were compared to published data (Table 1). The C2 and C3 components were related to humic substances (Table 1). It is assumed that C3/C2 ratio corresponds to α'/α ratio. As both humic substances have α' and α components, it is not possible to use this ratio to distinguish humic substances. The component α is composed of more humified and older compounds than those found in the component α'. An increase in C3/C2 ratio could indicate an increasing proportion of recent material. C1 Em/Ex couples were similar to those found in extracts of IHSS standard humic substances (Alberts and Takács 2004) and marine samples corresponding to marine humic-like substances (Coble 1996; Parlanti et al. 2000). The C1/C2 ratio was also calculated and the correlation between the C1/C2 and C3/C2 ratios was significant ($R = 0.87$, $P<0.0001$). This suggests that, like the C3/C2 ratio, the C1/C2 ratio cannot be used to distinguish humic substances.

The C4 component is related to protein-like compounds (Table 1). It is assumed that the C4/C2 ratio corresponds to the γ/α ratio used to assess the relative variations of protein-like substances compared to humic substances (Sierra et al. 2005). Zsolnay et al. (1999) observed

302 a relative increase in the γ fluorophores (C4 component in this study) compared to the α
303 fluorophores (C2 component in this study) in soil water extract after fumigation. In the microbial
304 biomass estimation, fumigation is used to achieve lysis of microbial cells, so cellular
305 constituents are released into the soil solution and can be readily extracted with water and
306 measured (Brookes et al. 1985). Low values of the C4/C2 ratio could indicate that protein-like
307 substances are in living microbial cells and not extractable, whereas high values could indicate
308 that microbial cell death has released protein-like compounds in the soil that are extractable. To
309 corhoborate this assumption, C4/C2 was related to proxies of microbial activities (catalysis
310 activities and respiration).
311
312 2.5. Model description and parameters estimation
313 As the experimental design was similar to that in Gogo et al. (2014), the data were calculated to
314 allow their use as inputs to the Gogo et al. (2014) model. This model is based on Schimel and
315 Weintraub (2003). It assumes that solid organic matter is catalysed by exo-enzymes into soluble
316 organic matter. Then the soluble matter is absorbed by the microbial and used as an energy
317 source for different microbial function (enzymes production, maintenance, growth) and released
318 into the environment in the form of CO_2 (Schimel and Weintraud 2003). Gogo et al. (2014)
319 simplified this model to make it experimentally testable. It consist of a three compartment: (i) the
320 “L” compartment corresponding to the fraction of C contained in the litter (solid fraction), (ii) the
321 “W” compartment corresponding to fraction of C contained in the WEOC (dissolved fraction) and
322 (iii) the “G” compartment corresponding to fraction of C contained in the cumulative CO_2
323 released by microbial respiration (gaseous fraction). The flux between the L and W
324 compartment corresponds to the exo-enzymes catalysis rate. The flux between the W and G
325 compartment correspond to the respiration. Equations were written to give account of the
326 simultaneous change in time of the state variable (L, W, and G) and the reaction rates. At any
327 time of the course of the experiment the sum of all these three fractions is equal to 1. The three
328 fractions corresponding to the three compartments were experimentally measured. The two
329 reaction rates (catalysis or “c” and respiration or “r”) were tuned simultaneously to fit the model
330 to the three experimentally assessed compartments.
331 Gogo et al (2014) showed that allowing the reaction rate to change in the course of the
332 experiment improved the goodness of fit. The reaction rates were allowed to follow a negative

exponential decrease with time with the following parameters to describe the shape of the curve: “a + b” is the initial reaction rate, “a” is the final rate, “m” rate of decay with time of the reaction rate (Rovira and Rovira 2010).

2.6 Statistical analyses

Paired *t*-tests and repeated measures ANOVA (with litter type or mixture type as within effect) were conducted with Statistica (2008) to test for differences in response variables between *Sphagnum* spp and vascular plant litters and for differences between measured and calculated values of the mixture. Correlations were calculated on log transformed data when necessary to assess the relationship between two response variables, using Statistica (2008). Model II regression (ranged major axis; Legendre and Legendre 1998) was performed on log transformed data when necessary to calculate equation coefficients.

3 Results

3.1 Field experiment

S. rubellum litter decomposed significantly faster in the presence of *Molinia caerulea* than without ($P < 0.02$; Table 2). This was the only significant non-additive litter mixture effect observed. On the whole, in the field conditions and over a year, *Molinia caerulea* and *Betula* spp decomposed faster than *Sphagnum* species (Table 2). *M. caerulea*, but not *Betula spp*., decomposed faster in the *Sphagnum* environments than in its native one (Table 3). As a mixture effect was found only with *S. rubellum* and *M. caerulea and as Sphagnum environments affected only Molinia caerulea*, the laboratory experiment focused on this species.

3.2 Laboratory experiment

3.2.1 C dynamics

S. rubellum litter mass decreased faster, contained more WEOC and tended to produce more CO_2 than *M. caerulea* litter (Fig. 1a, c, e; Table 4). The measured remaining mass of the *S. rubellum* and *M. caerulea* mixture was significantly lower than expected from calculations ($P < 0.002$; Fig. 1b; Table 4). The non-additive effect of the mixture was also observed on the WEOC and the CO_2 production, with higher measured values than expected (Fig. 1d, f; Table 4).

Applying the model of Gogo et al. (2014) gave a good account of the observations with RMSE in the same range as Gogo et al. (2014): between 0.16 and 17.8 in this study (Table 5) and between 0.23 and 14.5 in (Gogo et al. 2014) for *M. caerulea*. Initial exo-enzyme activity and respiration rate in the measured mixture were in the expected range (no more than 10% of difference; Table 5; Fig. 2b, d). However, the rates at which the activities decreased afterward (the "m" parameters in Table 6) were much lower than expected. This implies that both activities in the measured mixture were higher than expected (Fig. 2). The difference was more pronounced for exo-enzyme activities (75% difference) than for respiration (46% difference; Table 6).

Contrary to the stated hypothesis, the water content in the measured mixture was not different than the calculated one (Table 4; Fig. 3 a and b). The pH was different and significantly lower in the measured mixture than in the calculated one (Table 4; Fig. 3 c and d).

3.2.3 Specific Ultraviolet Absorption at 280 nm

In all litters, $SUVA_{280}$ decreased with time (Fig. 4). On the whole WEOC from *Molinia caerulea* litter was more aromatic than the *Sphagnum rubellum* WEOC ($P < 0.0001$; Fig. 4ab). Also, the WEOC measured in the mixture was found to be more aromatic than expected (Fig. 4c).

3.2.4 Fluorescent dissolved organic matter

Both C1/C2 and C3/C2 ratios increased with time (Fig. 5a, b, c, d), showing an increased contribution of more recent humic substances. The C1/C2 ratio in *M. caerulea* WEOM was significantly higher than in *S. rubellum* ($t = 6.86$, $P < 0.0001$, $n = 42$; Fig. 5a), but there was no difference between measured and calculated mixtures ($t = 0.035$, $P = 0.97$, $n = 42$; Fig. 5b). In contrast, the C3/C2 ratio was significantly lower in the measured than in the calculated mixture ($t = 3$, $P < 0.005$, $n = 42$; Fig. 5d). However, this ratio in the single litter WEOM was not different ($t = 0.82$, $P = 0.42$, $n = 42$: Fig. 5c).

The C4/C2 ratio in all litter types increased with time, showing an increased contribution of protein-like compounds compared to humic substances (Fig. 5e, f; the same pattern is observed when C3 or C1 substitute C2). This increase was particularly high for the *M. caerulea* litter compared to the *S. rubellum* litter ($t = 8.93$, $P < 0.0001$, $n = 42$; Fig. 5e). However, as well as C1/C2, there was no significant difference between measured and calculated mixtures (t =

0.055, P = 0.96, n = 42; Fig. 5f). In our study, the C4/C2 ratios were always negatively correlated to the modelled exo-enzyme activity and respiration rates (all R < -0.89, all P < 0.01; Fig 6). Also, the relationship was in a chronological order: as time passes, the microbial activity decreased, whereas the relative fluorescence of protein-like compounds increased.

4 Discussion

4.1 Laboratory synergistic effect of litter mixture on OM dynamics

In the laboratory experiment, mixing *M. caerulea* and *S. rubellum* litters increased litter mass loss (decomposition) and CO_2 production (microbial respiration) compared to what can be expected in an additive scenario (Fig. 1; Table 4). Krab et al. (2013) already demonstrated a *Sphagnum*-vascular plant mixture effect on mass loss in the field, but with *S. fuscum* and *Betula pubescens*. To our knowledge, such a non-additive effect of mixing *M. caerulea* and *S.rubellum* litters on microbial activity has never been shown before. The mixture effect between *M. caerulea* and *S.rubellum* litters could result from a higher exo-enzyme activity and respiration rate in measured than in calculated mixtures during all or most of the incubation time respectively (Fig. 2b, c).

WEOC content was also increased by the mixture. The WEOC compartment receives input from litter exo-enzyme catalysis (provoking litter mass loss), and is consumed by the microbial biomass (resulting in output by respiration in the form of CO_2; Schimel and Weintraub 2003; Gogo et al. 2014). As such, WEOC is sensitive to any change in input and output rates (Gogo et al. 2014). The build-up of WEOC built up in the measured mixture, compared to the calculated mixture, can only result from a relatively higher input than output rate. This suggests that in the first stage of decomposition, mixing *S. rubellum* and *M. caerulea* increased exo-enzyme activity more than respiration, compared to what could be expected. The model showed that both rates decreased with time in all types of litter. Thus, the higher WEOC content in the measured mixture could only be caused by an output rate that decreased faster than the input rate (Table 6).

4.2. Increase of aromatic compounds in the WEOC

The higher WEOC in the measured mixture was simultaneous to a significant increase of WEOC aromaticity (Fig. 4). Aromaticity variation can be due to (i) input/output of aromatic

compounds in the WEOC compartment and/or (ii) input/output of non-aromatic compounds (e.g. carbohydrates), mechanically decreasing/increasing the $SUVA_{280}$.

Degradation of lignin, tannin and/or polyphenols of the litters can be a significant source of soluble aromatic compounds into the WEOC. Dignac et al. (2005) showed in a cropland soil that lignin turnover could be faster than the whole soil organic carbon turnover. In the present study, it was shown that decomposition rate of the solid phase in the measured mixture was faster than expected, with thus the potential to draw more aromatic compound into the WEOC than expected. Furthermore, aromatic compounds such as tannin are more difficult to metabolise by the microorganisms, than carbohydrates monomers (Davidson and Janssens 2006). In the present study, it was shown that respiration rate was faster in the measured mixture than in the expected one, leaving into the WEOC probably more aromatic compounds than carbohydrates. The increased of WEOC aromaticity in the measured mixture is thus coherent with the combine effect of increased rate of solid matter decomposition and WEOC respiration.

4.3 Contribution of fluorescence analysis to the study of litter C dynamics

Among the fluorescent components highlighted by the PARAFAC analysis, C4 is the only one that is not attributed to humic substances, but to tyrosine and tryptophane containing materials (i.e. proteins; Coble 1996; Parlanti et al. 2000). These compounds contain nitrogen, which is one of the most limiting nutrients in soils. The occurrence of such fluorophores may be related to a release of exo-cellular enzymes. However, if a high contribution of protein-like fluorophores were to be related to an increased release of exo-enzymes, an increased decomposition rate would be expected when the C4/C2 ratio is high. This was not the case. First, the *S. rubellum* litter decomposed faster than the *M. caerulea* litter, whereas its C4/C2 was much lower (Figs. 1a and e and 5e). Second, as time passes, the microbial activity decreased in all samples, whereas the γ fluorophores contribution increased. The occurrence of such fluorophores may also be related to the release into the solution of proteins associated to the decomposing tissues. If this were the case, increased contribution of the γ fluorophores would be associated to an increased mass loss. However, the exact opposite was observed: *M. caerulea*, which decomposition was slow, had a WEOC with the most important γ fluorophore contribution. Furthermore, because of the translocation process (Taylor et al. 2001) *M. caerulea* litter has a very low N content: more than 2 times less than *S. rubellum*, with 1.0 mg g^{-1} and 2.2 mg g^{-1}

456 respectively (Gogo et al 2010). Such a high input of protein-like compounds from such a slow
457 decomposing N poor litter may not be realistic.
458 In peat, the microbial biomass concentrates a significant fraction of the soil total N (peat C/N
459 from 16 to 36, microbial biomass C/N from 6 to 14; Francez et al. 2000). Parlanti et al. (2000)
460 showed in a macro-algae degradation experiment that the γ/α ratio increased in the first 15 days
461 of the incubation corresponding to degradation products of the organisms, probably originating
462 from cell membranes. Zsolnay et al. (1999) also showed that such fluorophores occurred when
463 the microbial cell were lysed, leaving in solution the protein rich cytoplasmic content. As
464 suggested above, protein amount in soil can increase because of exo-enzymes release.
465 However, the expected increase of OM degradation in such case was not observed: microbial
466 activity decreased as the protein-like fluorophores contribution increased. What could happen is
467 that as the microbial biomass dies off, the cell contents are released in the media without being
468 taken up again, provoking a build-up of proteinaceous compounds in the WEOM. Thus, it
469 suggests that the relative increase in the C4/C2 ratio in time could reflect an increasing
470 solubilisation of microbial cell products after their death. The C4/C2 ratio in the context of litter
471 laboratory incubation can be used as an index of the microbial dead/living biomass ratio.
472 As solid OM composition differs between *Sphagnum* species and *M. caerulea* litter (Gogo et al.
473 2011; Gogo et al. 2014), it was expected that the fluorescence of the WEOM produced by solid
474 OM degradation would differ as well. Such a difference would allow identification of the relative
475 contribution of each litter in the mixture, making it possible to identify which litter contributes
476 more to the input into the WEOM. However, the results show that when a difference was found
477 between single litters, there was no non-additive effect in the mixture (C1/C2 and C4/C2, Fig.
478 5a, b, e and f). In contrast, a non-additive effect was detected in the mixture where no difference
479 was found between single litters, (C3/C2, Fig. 5c, d). Thus, in the present case, fluorescence
480 analysis is not a pertinent tool to study chemical changes linked to mixture.
481 Most fluorescent materials (C1, C2, C3) are humic substances (Coble 1996; Parlanti et al. 2000;
482 Alberts and Takács 2004). They are not the direct product of litter exo-enzymatic attack. They
483 were certainly present in the water used to inoculate the litter. Humic substances are known to
484 be recalcitrant to decay (Jenkinson and Rayner 1977). This may explain why the non-additive
485 effect observed on mass loss, WEOC content and respiration was not reflected in these
486 compounds.

487

488 4.4 Mechanisms of in situ and laboratory litter mixture effect

489 As in the laboratory, there was an in situ synergistic effect of mixing *S. rubellum* and *M.*
490 *caerulea* litters on OM decomposition after one year of incubation (Table 2). This effect of
491 mixing *M. caerulea* with a *Sphagnum* moss on OM decomposition has never been evidenced
492 before. Because the *M. caerulea* decomposing environment is composed of *Sphagnum* litter, it
493 was not possible to test the effect of *Sphagnum* litter on *M. caerulea* litter decomposition. It can
494 however be concluded that the in situ mixture effect observed originated from an effect of *M.*
495 *caerulea* on the rate of decay of *S. rubellum*, which is an additional information compared to the
496 laboratory incubation.

497 The effect of the *Sphagnum* environment on *M. caerulea* can be approached by comparing the
498 decomposition rate obtained in *Sphagnum* and in the native tussock environment. *M. caerulea*
499 litter in its in situ native environment decomposed at a much slower rate than in both *S.*
500 *rubellum* and *S. cuspidatum* environments (Table 3). Van Vuuren and van der Eerden (1993)
501 also found low decomposition rate of *M. carulea* in its native environment in a heathland (80%
502 of remaining mass after 11months of incubation). The decomposition of *M. caerulea* litter
503 increased by 17 - 21% in a *Sphagnum* environment compared to their native environment
504 (Table 3). The non-additive litter mixture effect may result from changes in physical properties
505 caused by one litter (Gartner and Cardon 2004). *M. caerulea* is a tufted plant forming tussocks
506 and because of this microtopography most of the litter experiences dry conditions in the first
507 year that limit decomposition. Moisture in the range of 60-75% (wet weight basis) does not limit
508 decay, whereas its variations could influence mass loss in the range of 75-80% and more
509 extensively in the range of 30-60% (Prescott 2010). Microbial activity decreases as the
510 conditions become drier and wetter. A change toward an increase of moisture compared to its
511 native environment would stimulate *M. caerulea* litter decomposition. Through their
512 morphological characteristics, *Sphagnum* mosses are able to retain up to 15 to 20 times their
513 dry weight in water (Rydin and Jeglum 2013, in accordance with Fig. 3), which maintains moist
514 conditions over dry periods. Thus, in field conditions, when *M. caerulea* litter decomposes in a
515 *Sphagnum* environment, it spends more time in favourable conditions for decomposition than in
516 its native environment.

Water content seems to explain the differences observed. Although *M. caerulea* did decompose faster than in its native environment, the decomposition rate in *Sphagnum* environment was low (about 70% of mass remaining after one year; Table 3). Hoorens et al. (2003) reported a faster decomposition rate of *M. caerulea* litter in a *Polytrichum commune* decomposing environment (59%). Once a limiting factor was alleviated, other factor influencing OM decomposition may take place. *Sphagnan* a carbohydrate polymer specific to *Sphagnum* environment is thought to preserve OM through different mechanisms (Hájek et al. 2011). A longer experiment and/or comparison of *M. caerulea* litter decomposition in other environments (*M. caerulea* can grow in a wide array of environments) would help to assess the effect of *Sphagum* environment on *M. caerulea* decomposition.

As mentioned above, *M. caerulea* is a tufted plant forming tussocks, which implies dry conditions in the first year that limit decomposition. From the in situ experiment, it was suggested that litter moisture is an important factor controlling the effect of the *Sphagnum* environment on the decomposition of *M. caerulea*. In the laboratory conditions, all the litters were subjected to the same conditions. The *M. caerulea* litters in both treatments (in monoculture and in mixture) were moist (unlike native field conditions). A significant correlation was found between microbial activity and water content for all litters (Fig. 6), which supports the role of water content on microbial activity. However, no significant difference in litter water content was found between measured and calculated mixtures (Table 4; Fig. 3a and b). This suggests that additional mechanisms are at work to explain the observed non-additive effect of *S. rubellum* on *M. caerulea* in the laboratory and of *M. caerulea* on *S. rubellum* in the field.

Microbial activity can be very sensitive to changes in pH. The optimum pH for CO_2 production in peat is above the pH usually measured in peatlands (Bergman et al. 1999). In our study, although the pH in the mixture extract decreased compared to an additive scenario, microbial activity was enhanced by mixing the two litters. This rules out any direct role of this factor on the mechanism that could explain the synergistic effects observed. However, increase of acidity could have an indirect effect on labile carbon supply to microorganisms. Low pH could accelerate litter OM hydrolysis, releasing both lignin (as suggested above) and carbohydrate monomers. The latter could have stimulated microbial activity, which in turn could have increased litter degradation. Such an hypothesis has to be further explored.

4.5 Implications and perspectives

Hoorens et al. (2010) showed that the litter mixture effect may be additive at the plant functional level, implying that the decomposition rate at the ecosystem level could be calculated from the abundant literature on individual litter decomposition rates. While this may be true for ecosystems supporting enough biodiversity, it is to stress that biodiversity is dramatically decreased in peatlands invaded by *Molinia caerulea*. In the study site, with the exception of some wetter areas dominated by *Rynchospora alba*, *M. caerulea* together with very sparse *Eriophorum angustifolium* dominates the herbaceous strata and covers almost all the site. In some wetter places, where *S. rubellum* can develop, *M. caerulea* is the only herbaceous plant. *M. caerulea* cover increases because of disturbances. In such a situation, the negative and positive non-additive interaction may not cancel out as suggested by Hoorens et al. (2010), leaving only a positive non-additive effect. The ecosystem level decomposition rate estimation using individual litter data in ecosystems where *M. caerulea* dominates should therefore not be used in modelling. Furthermore, the occurrence of a mixture effect between different *Sphagnum* species and different invading vascular plants (Krab et al. 2013; this study) clearly highlights the need to take peatland vegetation change into account when simulating future C stock and fluxes.

One must be careful when transposing results obtained in the laboratory to the field. That is why here laboratory experiment was coupled to in situ incubation. Both experiment showed an acceleration of decomposition in litter in mixture. However, the laboratory experiment was much shorter than the field experiment and mixture effect observed in both experiment may not originate from the same process.

One aim of this study was to elucidate the mechanisms behind the mixture effect. However, direct effect of the variations of the factors studied (water content and pH) could not explain the observation. As an alternative, the results of the present study suggest that the interaction between labile OM and microbial biomass activity may be the key interaction to focus on in further research. This is crucial because such a synergistic effect can jeopardize the C storing capacity of *Sphagnum* peatlands in the context of vegetation change. Considering the vast areas covered by *Sphagnum* species, especially in Siberia and Canada and the increasing abundance in Northern peatlands of *M. caerulea* and other colonising vascular plant species (e.g. *Betula spp*, *Pinus spp*, *Phragmites australis*), the litter mixture effect of invading species

litter with *Sphagnum* spp litters definitely requires more attention. For example, other mixing experiment should be undertaken with *Sphagnum* species and other vascular plant to assess to which extent the mixture effect is occurring in these ecosystems.

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692

693 **Tables**

694

695 Table 1. Major components found by a PARAFAC analysis of the fluorescence excitation-
696 emission matrices of the water extractable samples, and the assumed corresponding origin of
697 the substances of each component from different literature sources. M and β were usually
698 attributed to marine humic substances, but were also found in extract of humic substances
699 standard (Alberts and Takács, 2004).

PARAFAC Components	Coble et al. (1996)	Parlanti et al. (2001)	Excitation max. (mm)	Emission max. (mm)	Compound type
C1	M	β	300-340	400-440	Humic substances
C2	C	α	330-370	420-480	humic substances
C3	A	α'	250-270	380-480	humic substances + recent materials
C4	B-T	γ	270-280	300-340	Protein-like material

700

701

Table 2. Mean (± 1 s.e., n = 4) remaining mass (%) after one year *in situ* incubation of *Sphagnum* and vascular plant litters, in monoculture and in mixture (Mix). Significant difference between *Sphagnum* and the vascular plant litters decomposition are in italics and significant difference between measured and calculated decomposition in mixture are in bold italics.

Incubation site	Litter	Mass remaining ± s.e.	*P*
S. rubellum hummocks	*S. rubellum*	86.9 ± 0.95	*0.007*
	M. caerulea	68.5 ± 2.56	
	Mix measured	71.1 ± 1.74	***0.02***
	Mix calculated	77.6 ± 1.39	
	S. rubellum	86.9 ± 0.95	*0.01*
	Betula spp.	59.6 ± 5.36	
	Mix measured	74.0 ± 1.45	0.88
	Mix calculated	72.9 ± 2.19	
S. cuspidatum lawns	*S. cuspidatum*	83.1 ± 1.66	0.05
	M. caerulea	71.6 ± 2.74	
	Mix measured	71.5 ± 4.09	0.16
	Mix calculated	77.2 ± 1.41	
	S. cuspidatum	83.1 ± 1.66	*0.004*
	Betula spp.	63.7 ± 2.27	
	Mix measured	71.3 ± 1.46	0.64
	Mix calculated	72.9 ± 1.96	

708 Table 3. Mean (± 1 s.e., n = 4) remaining mass (%) after one year *in situ* incubation of *Molinia*
709 *caerulea* and *Betula spp*. in their native decomposition environment, in S. rubellum hummock
710 and in S. cuspidatum lawn environments. *P* values correspond to the comparison of remaining
711 mass of a litter type in *Sphagnum* environment to their native decomposition environment.
712 Significant are in italics.

Incubation site	Litter	Mass remaining ± s.e.	*P*
M. caerulea		86.4 ± 2.84	
S. rubellum hummocks	*M. caerulea*	68.5 ± 2.56	*0.0025*
S. cuspidatum lawns		71.6 ± 2.74	*0.008*
Betula spp.		67.4 ± 1.61	
S. rubellum hummocks	*Betula spp.*	59.6 ± 5.36	0.12
S. cuspidatum lawns		63.7 ± 2.27	0.57

713

714

715 Table 4. Mean remaining mass after 64 days of incubation (± 1 s.e., n = 6, %), and mean water
716 extractable organic carbon (WEOC) concentration (± 1 s.e., n = 42, mg g^{-1} dry weight), CO_2
717 production (± 1 s.e., n = 42, µg C-CO_2 g^{-1} dry weight h^{-1}), litter water content (± 1 s.e., n = 42, g
718 H_2O g^{-1} dry weight) and WEOC pH (± 1 s.e., n = 42) over 64 days of the laboratory experiment
719 of *Sphagnum* spp. and vascular plant litters, in monoculture and in mixture (Mix). Significant
720 differences are in italics.

	M. caerulea	*S. rubellum*	P	Mix measured	Mix calculated	P
Remaining mass	96.1 ± 0.38	92.6 ± 0.52	*<0.0001*	91.8 ± 0.53	93.8 ± 0.38	*<0.0001*
WEOC	1.77 ± 0.12	3.40 ± 0.09	*<0.0001*	3.51 ± 0.16	2.53 ± 0.09	*<0.0001*
CO_2 production	19.7 ± 2.61	29.1 ± 4.58	0.06	35.9 ± 3.89	24.2 ± 2.78	*0.01*
Water content	1.28 ± 0.21	17.2 ± 0.66	*<0.0001*	8.08 ± 0.66	8.96 ± 0.37	0.09
pH	5.09 ± 0.04	4.52 ±0.08	*<0.0001*	4.62 ± 0.06	4.80 ±0.04	*0.01*

721

722

723 Table 5. Relative mean square error (RMSE) achieved when the Gogo et al. (2014) model was
724 fitted to the data of this study.

	Remaining mass	WEOC	Cumulative CO_2	Sum
S. rubellum	0.76	17.8	6.72	25.27
M. caerulea	0.16	11.5	5.89	17.58
Mixture measured	0.20	13.6	3.91	17.74
Mixture calculated	0.14	12.75	4.37	17.27

725

726

727 Table 6. Model parameters and percentage of change between measured mixture and expected

728 mixture: (measured – expected) / expected x 100.

Parameters		*S. rubellum*	*M. caerulea*	Mix. Measured	Mix. Calculated	% of difference
Exo-enzyme catalysis	m_c	0.063	0.081	0.016	0.065	-75.5
	a_c	0.0011	0.0004	8.27E-07	0.0007	
	b_c	0.0015	0.0019	0.0026	0.0018	
	initial rate (a_c + b_c)	0.0026	0.0023	0.0026	0.0024	8.1
Respiration	m_r	0.012	0.027	0.010	0.019	-46.1
	a_r	5.52E-10	0	6.68E-10	1.38E-10	
	b_r	0.36	0.51	0.38	0.42	
	initial rate (a_r + b_r)	0.0122	0.0271	0.3793	0.4215	-10.0

729

730

Figure captions

Fig. 1 Measured (mean, n = 6, dots) and modelled (lines, modelling based on Gogo et al., 2014) mass loss (a, b), WEOC (c, d) and cumulative CO_2 (e, f) in *Sphagnum rubellum* (black circles), *Molinia caerulea* (white triangles), and litters in mixture (measured – dark grey diamonds, and expected – light grey squares)

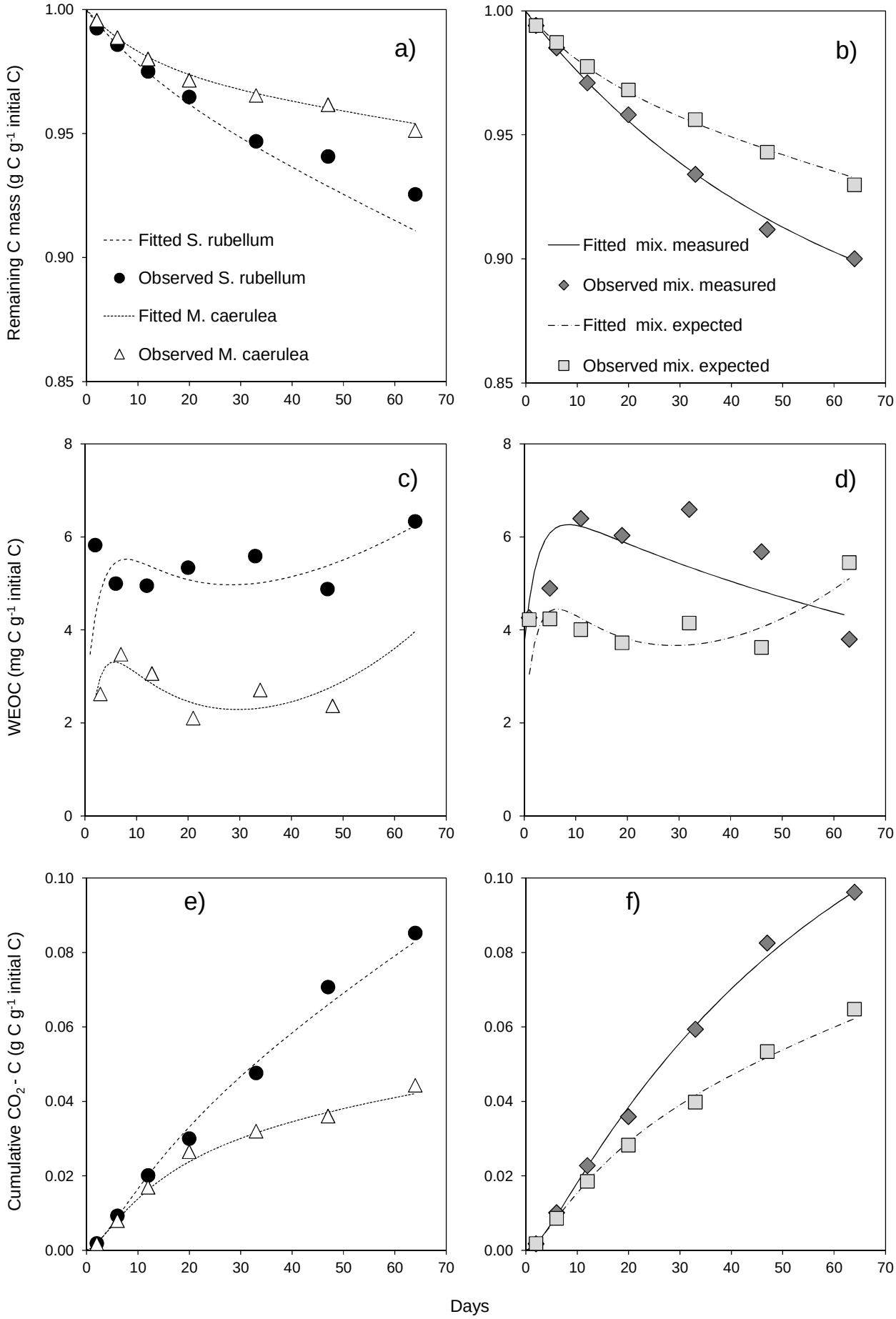
Fig. 2 Kinetics of the exo-enzyme catalysis rate (a, b) and respiration rate (c, d) in *Sphagnum rubellum* (a) and *Molinia caerulea* (b) litters in monoculture and in measured (c) and expected mixture (d)

Fig. 3 Relationship between $SUVA_{280}$ (L cm^{-1} mg^{-1}, ± 1s.e., n = 6) and dissolved organic carbon (DOC, mg L^{-1}) of the WEOC extracted from *Sphagnum rubellum* (a) and *Molinia caerulea* (b) litters in monoculture and in measured (c) and expected mixture (d). The black line corresponds to the "conservation" line, where aromatic content experienced no variation (either input = output ≠ 0 or input = output = 0)

Fig. 4 Kinetics of the C1/C2 (a, b), C3/C2 (c, d) and C4/C2 (e, f) ratios obtained after the PARAFAC analysis of the excitation-emission matrices of the WEOM extracted from *Sphagnum rubellum* (black circles) and *Molinia caerulea* (white triangles) litters in monoculture and in measured and expected mixture (measured – dark grey diamonds, and expected – light grey squares)

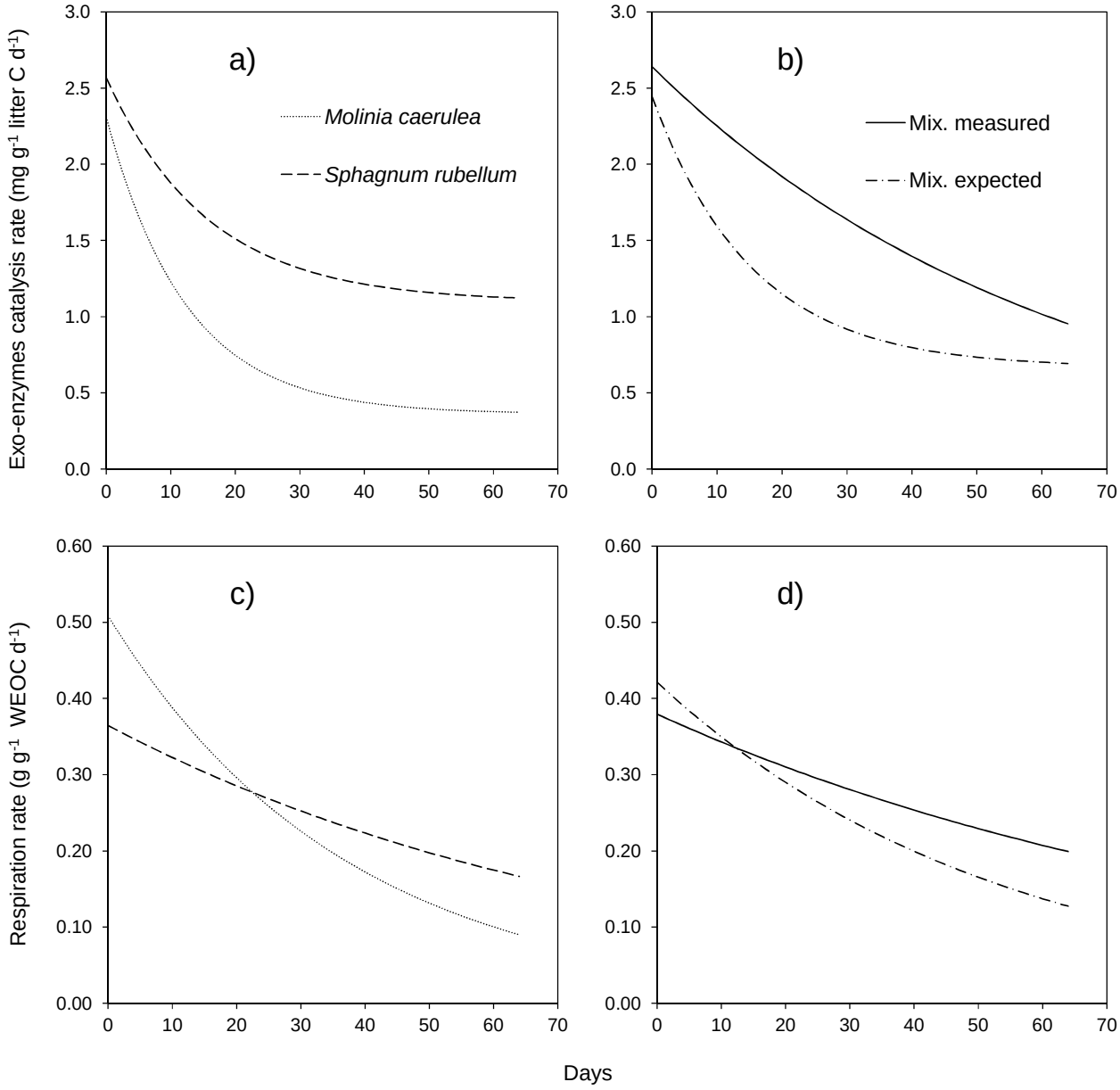
Fig. 5 Relationship between modelled enzyme activity (a, b) and respiration (c, d) and the C4/C2 ratio in *Sphagnum rubellum* (black circles) and *Molinia caerulea* (white triangles) litters in monoculture and in measured and expected mixture (measured – dark grey diamonds, and expected – light grey squares). All correlations are significant ($P < 0.05$)

Fig. 6 Relationship between modelled enzyme activity (a, b) and respiration (c, d) and the litter water content in *Sphagnum rubellum* (black circles) and *Molinia caerulea* (white triangles) litters in monoculture and in measured and expected mixture (measured – dark grey diamonds, and expected - light grey squares). All correlations are significant ($P < 0.05$).



763

764 Figure 1.



765

766 Figure 2.

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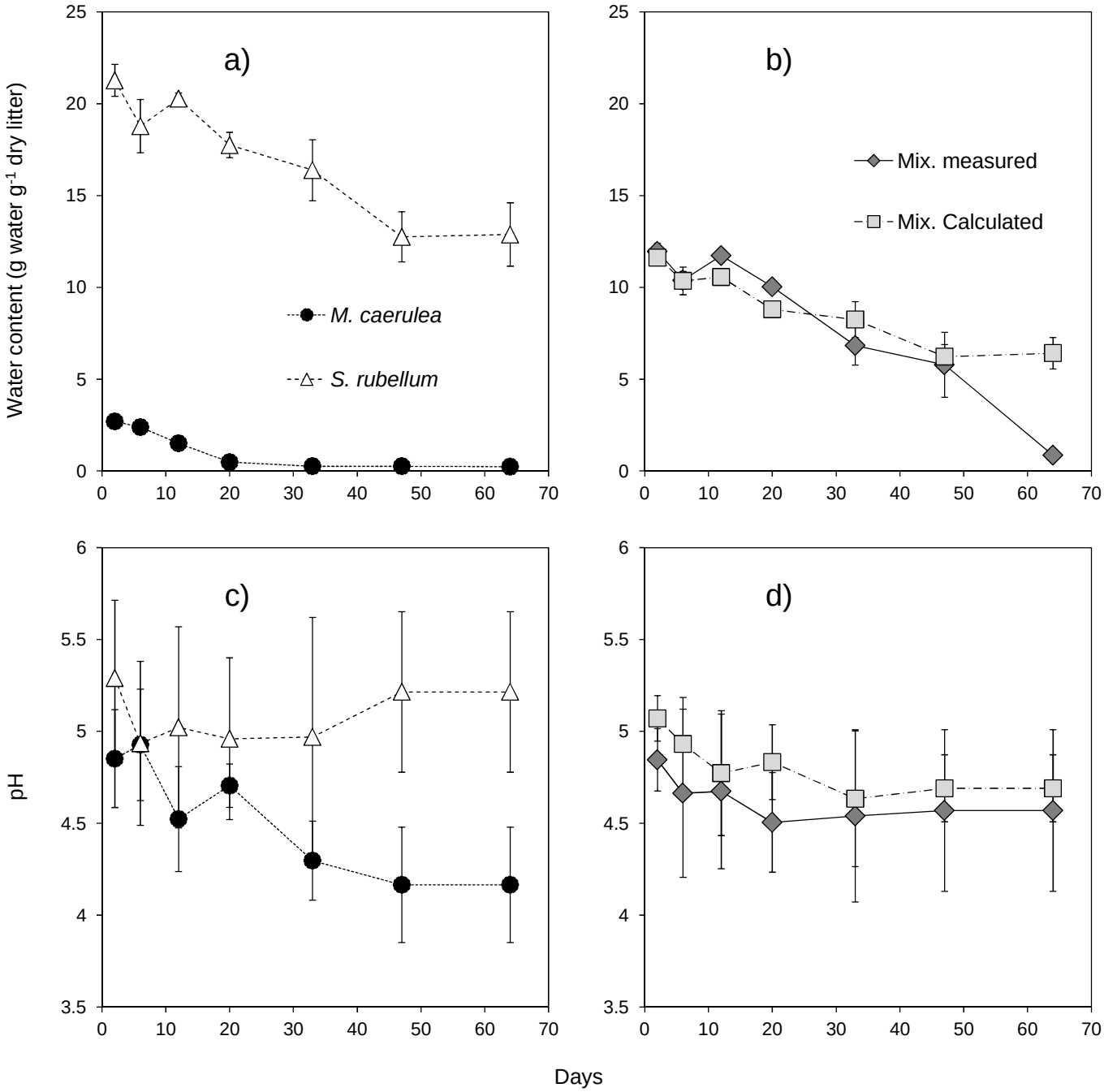

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769 Figure 3.

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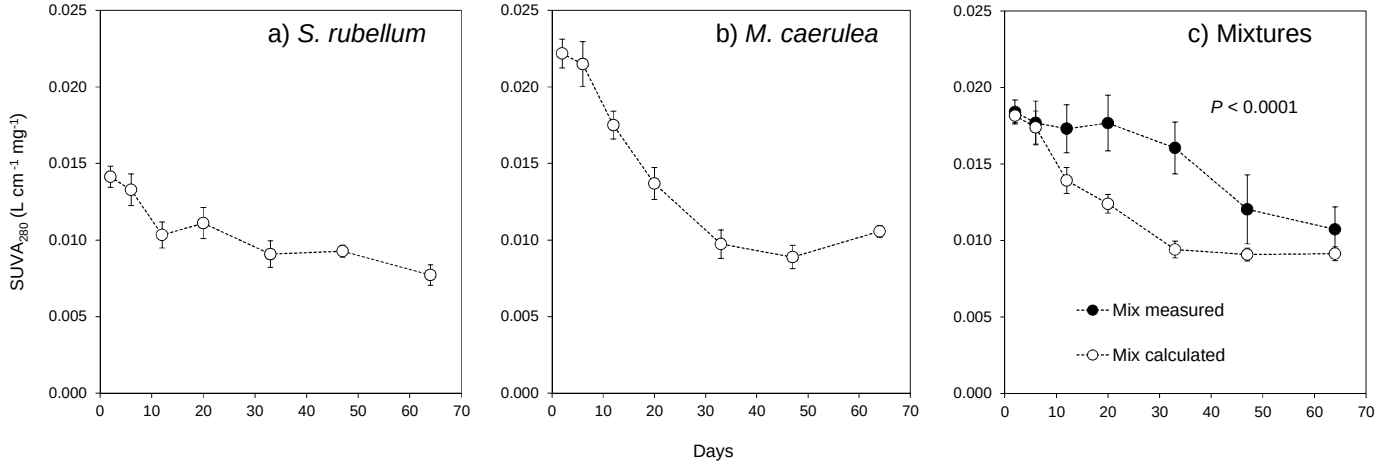

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772 Figure 4.

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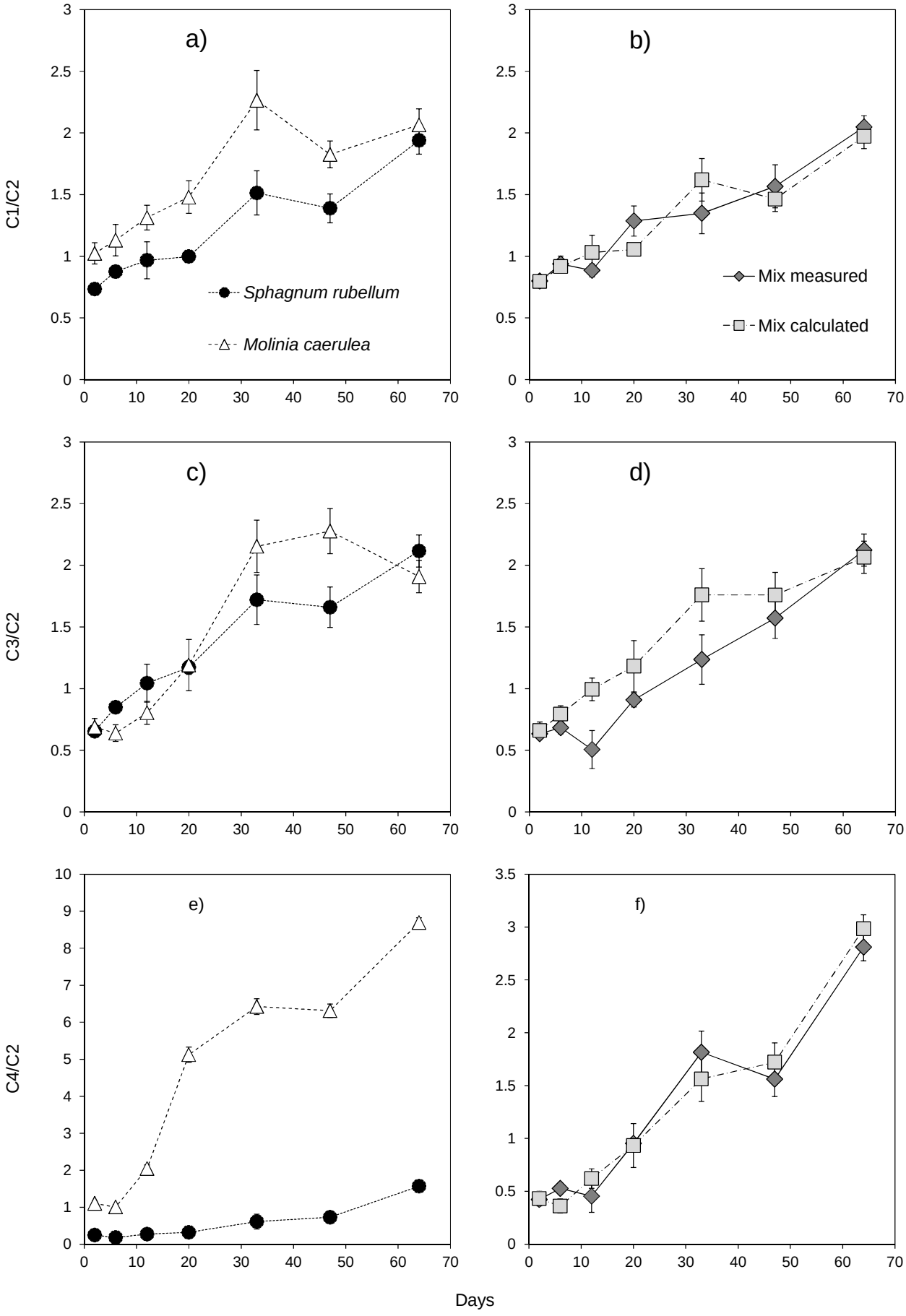

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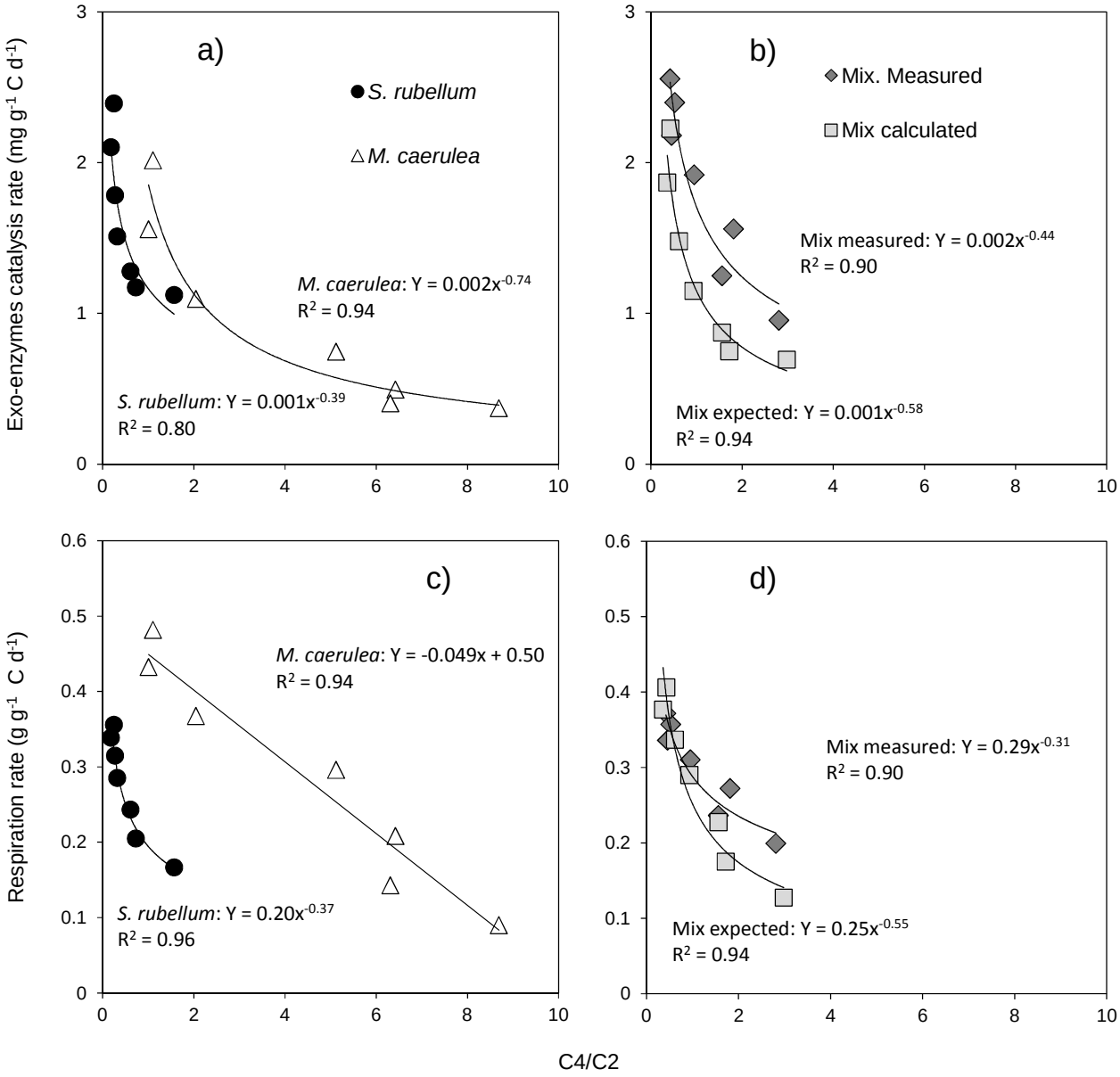
775 Figure 5.



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777 Figure 6.