



## Effects of two submerged macrophyte species on microbes and metazoans in rooftop water-storage ponds with different labile carbon loadings

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### ABSTRACT

Nature-based solutions including rooftop-water storage ponds are increasingly adopted in cities as new eco-designs to address climate change issues, such as water scarcity and storm-water runoff. Macrophytes may be valuable additions for treating stored rooftop waters and provisioning other services, including aquaponics, esthetic and wildlife-conservation values. However, the efficacy of macrophyte treatments has not been tested with influxes of different labile carbon loadings such as those occurring in storms. Moreover, little is known about how macrophytes affect communities of metazoans and microbes, including protozoans, which are key players in the water-treatment process. Here, we experimentally investigated the effectiveness of two widely distributed macrophytes, *Ceratophyllum demersum* and *Egeria densa*, for treating drained rooftop water fed with two types of leaf litter, namely *Quercus robur* (high C lability) and *Quercus rubra* (low C lability). *C. demersum* was better than *E. densa* at reducing water conductivity (by 10–40  $\mu\text{S}/\text{cm}$ ), TDS (by 10–18 mg/L), DOC (by 4–5 mg/L) and at increasing water transparency (by 4–9%), water O<sub>2</sub> levels (by 19–27%) and daylight pH (by 0.9–1.3) compared to leaf-litter only microcosms after 30 days. Each treatment developed a different community of algae, protozoa and metazoa. Greater plant mass and epiphytic chlorophyll-a suggested that *C. demersum* was better at providing supporting habitat than *E. densa*. The two macrophytes did not differ in detritus accumulation, but *E. densa* was more prone to develop filamentous bacteria, which cause sludge bulking in water-treatment systems. Our study highlights the superior capacity of *C. demersum* and the usefulness of whole-ecosystem experiments in choosing the most adequate macrophyte species for nature-based engineered solutions.

### 1. Introduction

Rainwater harvesting from rooftops is becoming common in many parts of the world as aridity increases due to climate change (Campisano et al., 2017; Rodak et al., 2020). Stored rooftop water has many uses, including for drinking water (Lim and Jiang 2013), aquaponics (Million et al., 2016; Alsanus et al., 2017), and the creation of ponds with esthetic and wildlife conservation values (Hassall and Anderson 2015; Toboso-Chavero et al., 2019). Collected rooftop water can be treated

using technology for reducing nutrient content or microbial load (e.g., ultrafiltration, ozonation) (Rodak et al., 2020). However, these treatments may not always be cost-effective or do not have the additional values of 'green' technology, including providing raw materials for biofuel, food for livestock or habitat for biodiversity (Moore and Hunt 2012; Kurniawan et al., 2021).

Potential 'green' methods for treating rooftop water may be the use of submerged macrophytes, such as the hornwort *Ceratophyllum demersum* L and the Brazilian elodea *Egeria densa* Planch. 1849 (Brix

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1994; Feijóo et al., 2002). These fast-growing perennial macrophytes are recommended for wastewater treatment in constructed wetlands (Khan et al., 2009; Pietro et al., 2006; Zhang et al., 2020). However, as far as we are aware, there is no study comparing the performance of these macrophytes as water-treatment systems in poorly mineralized waters, such as storm-water runoff. Waters with low mineral content may alter the growth of these macrophytes directly (Walstad 1999) or by increasing the toxic effects of compounds, such as ammonia (Vines and Wedding 1960; Thurston et al., 1981). This is one of the toxins generated in waters after atmospheric nitrogen deposition, which increased globally from 86.6 to 96.6 TgN year<sup>-1</sup> between 1984 and 2016 (Decina et al., 2020).

The potential of *C. demersum* and *E. densa* as water-treatment systems can be from direct plant assimilation or to the microbes and metazoa that are associated with these macrophytes (Brix 1994; Pietro et al., 2006). These macrophytes may remove contaminants from water, including nutrients (Feijóo et al., 2002; Pietro et al., 2006; Zhang et al., 2020), and reduce harmful algal blooms by releasing algal growth inhibitors (Wium-Andersen et al., 1983; Pietro et al., 2006; Gross et al., 2003). Fewer algae may mean less food for their consumers, including protozoans (Carlough and Meyer 1990; Finlay and Esteban 1998), unless these taxa feed on bacteria and fungi, both of which participate in processing detritus from dead plants and consumers (Srivastava and Bell 2009; Gessner et al., 2010). The impacts of *C. demersum* and *E. densa* on microbial assemblages, including protozoa, have not been compared in detail because, to our knowledge, only *C. demersum* has protozoan inventories (Babko et al., 2010; Zhang et al., 2020).

It is also largely unknown how the potential of *C. demersum* and *E. densa* as water-treatment systems may be altered by changes in the biodegradability of organic matter in rooftop water, including leaf litter, which is a major source of organic carbon to freshwaters (Gessner et al., 2010; Stoler and Relyea 2016). Biodegradability is the rate of biological breakdown, which is influenced by the lability of organic carbon (e.g., C: N ratio, lignin content) and environmental factors (e.g., temperature, water-nutrient levels) (Stoler and Relyea 2016; Stanek and Stefanowicz 2019). Influxes of organic matter with very labile carbon may boost nutrient and microbe numbers in waters to levels that might lead to eutrophication (Mason 1996; Le Moal et al. 2019). However, if there is concurrent release of low-biodegradable substances, such as tannins, then nutrients may not boost ecosystem productivity. Tannins increase water turbidity (Julkunen-Tiitto and Haggman 2009), which decreases photosynthesis efficiency (e.g., Machado et al., 2020). Therefore, the potential of *C. demersum* and *E. densa* in 'green technology' applications will be better understood if their performance is tested experimentally with different labile organic carbon loadings.

The goal of this study was to investigate experimentally the effectiveness of *C. demersum* and *E. densa* in water-treatment systems and as supporting habitat for eukaryotic microbial and metazoan assemblages in drained, rooftop water provisioned with two types of leaf litter, namely *Quercus robur* (high biodegradability) and *Quercus rubra* (low biodegradability; Stanek and Stefanowicz 2019). Understanding the biological effects of *Q. rubra* is important because this American tree species is listed as naturalized in many countries within the distributional range of the European native *Q. robur* (Stanek and Stefanowicz 2019), including Spain, where our study was conducted. If *C. demersum* and *E. densa* are effective in water treatment, we expected an improvement in drained rooftop water with leaf litter shown by a reduction in nutrients, conductivity, and suspended solids. Moreover, if macrophytes are useful for widespread use in water treatment, we expected that the two macrophyte treatments would perform equally well and improve water quality and boost the density and biomass of high order consumers (e.g., rotifers, crustaceans), in particular with the more labile organic carbon source (*Q. robur* leaves). Last, detritus production is a major concern in water treatment, including those using nature-based solutions such as ours (Maceda-Veiga et al., 2015). Therefore, the better water treatment would be one that improves water

quality while also reducing sludge production, including aggregates of filamentous organisms (Martins et al., 2004; Perez-Uz et al., 2010).

## 2. Materials and methods

### 2.1. Overview of the experimental design

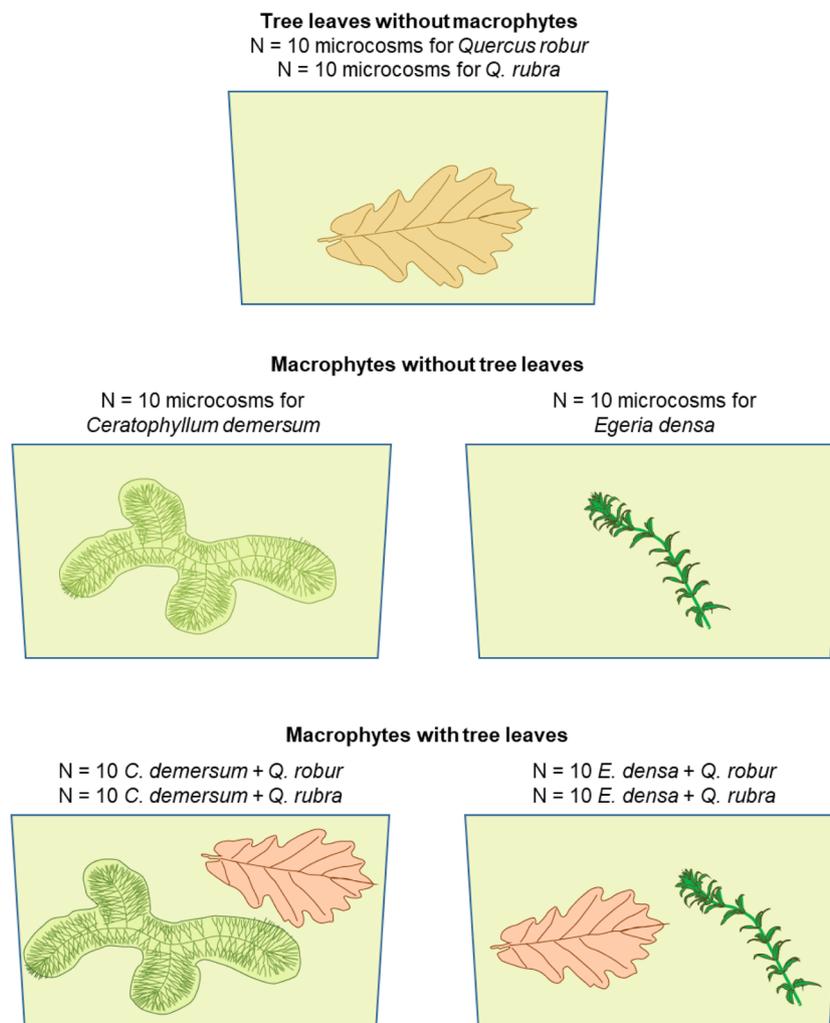
The factorial experiment was conducted over 30 days in August 2017 in cylindrical microcosms (12-cm height x 11-cm diameter, 1 L) in the O Incio Pilot Agro-Fluvial Observatory (42° 38' N – 7° 21' E, Lugo, Spain). The treatments (i.e., experimental factors) were the macrophyte species (*C. demersum* and *E. densa*) and the type of abscised tree leaves (*Quercus robur* and *Q. rubra*), which produced differences in labile organic carbon loadings (see details in section 2.2.2). Treatments were randomly allocated to 80 microcosms. There were 10 microcosms (replicates) each for tree leaves only, macrophytes only, and for tree leaves with macrophytes (Fig. 1). There were replicates for the two species of tree leaves and macrophytes (e.g., 10 microcosms with *Q. rubra* and *C. demersum* and 10 for *Q. rubra* and *E. densa*). We did not have rooftop-water only microcosms because this was the least realistic setting. All microcosms were placed on the ground under a balcony to protect microcosms from rain but to allow natural temperature and light fluctuations. Mean ( $\pm$  SD) daily air temperature was 20  $\pm$  7 °C. Mean light intensity at the top of microcosms was 6600  $\pm$  400 lux at 12 AM.

Microcosms were filled with 225 mL of water from a rainwater collector without macrophytes and with another 725 mL from a mixture of rooftop water from six houses in the municipality of O Incio, Lugo, northwestern Spain, after a storm. Two of the roofs were made of slate, two of asbestos sheeting and two of tiles, which were the three main roof types in the study area. The area is relatively far from industries (>30 km) but atmospheric pollution can arrive from further afield (Decina et al., 2020). We harvested the first 25 L of drained rooftop water from each roof and used the supernatant water of an homogenized sample to fill the microcosms within  $\leq$  24 h of harvesting. Water properties were: pH = 7.4, total nitrogen = 0.5 mg/L and dissolved organic carbon (DOC) = 5 mg/L. Macrophytes and tree leaves were added after 24 h (section 2.2.2) and we measured after 30 days: water properties, final plant size, remnant tree leaf biomass, microbes and metazoans (section 2.2.3). Thirty days is within the range of hydraulic retention times of constructed wetlands (Moore and Hunt 2012; Maceda-Veiga et al., 2015) and this duration is sufficient for biological assemblages, with fast generation times such as those of microbial eukaryotes, to reach mature developmental stages (e.g., Salvadó 1994).

### 2.2. Plant and labile organic carbon sources

Plant fragments of *C. demersum* ( $N = 30$ ) and *E. densa* ( $N = 30$ ) used in this study were apical shoots haphazardly selected from mother plants kept at the Pilot AgroFluvial Observatory O Incio, Spain (42°39'N– 7° 21'W). We introduced a fragment of 8-cm length from unbranched sections of a stem in microcosms with macrophytes. Plant fragments were gently brushed and rinsed in tap water to remove debris or epiphytes. All fragments of each plant species had similar morphologies (e.g., number and size internodes) and size expressed as biomass (Mean  $\pm$  SD; 0.52  $\pm$  0.05 g *C. demersum* and 0.45  $\pm$  0.03 g *E. densa*) or surface area (20  $\pm$  3 cm<sup>2</sup> and 16  $\pm$  4 cm<sup>2</sup>). Mother plants were brought in 2003 as four apical shoots of each species and have since grown in monocultures in two 200-L tanks fed with rainwater from rooftops (pH = 8.1, water conductivity = 116  $\mu$ S/cm, total nitrogen = 4.5 mg/L). The four shoots of each species were from four different aquarium plant importers to potentially increase the genetic pool.

Sources of labile organic carbon were freshly abscised leaves of two tree species, namely European oak *Quercus robur* L. ( $N = 30$  leaves) and American oak *Quercus rubra* L. ( $N = 30$  leaves), growing together in a forest patch from O Incio. We collected 30 leaves from five tree individuals and selected similarly sized tree leaves of the pool to be cut and



**Fig. 1.** Schematic view of the experimental design used to explore the effectiveness of the submerged macrophytes *Ceratophyllum demersum* and *Egeria densa* in treating drained rooftop water having two types of organic carbon loadings, namely abscised dead leaves of *Quercus robur* (high biodegradability) and *Q. rubra* (low biodegradability).

introduced part-by-part in microcosms until reaching 1 g. We chose 1 g of these tree leaves per microcosm because this dry mass of leaf litter typifies amounts in rooftops scaled by the volume of our microcosms. This was seen in our pilot study, which also found that differences in growth of *C. demersum* and *E. densa* depending on these leaf litter types, with the stronger negative effects for *E. densa* growth ( $N = 3$  microcosms each). Differences in tissue composition (mean  $\pm$  SE) among leaves mostly arise from elements related to biodegradability, namely C/N and lignin: *Q. robur* (C/N =  $37.7 \pm 5.1$ , lignin =  $234 \pm 15$ , Ca =  $12 \pm 0.5$ ; Mg =  $1.8 \pm 0.1$ ; P =  $1.7 \pm 0.1$  in  $\text{mg g}^{-1}$  litter) and *Q. rubra* (C/N =  $68.7 \pm 5.5$ , lignin =  $198 \pm 1$ , Ca =  $11.8 \pm 0.6$ ; Mg =  $1.4 \pm 0.1$ ; P =  $1.4 \pm 0.1$ ) (Hobbie et al., 2006). Given that optimal microbial metabolism is at C/N ratios from 20–30 (Puyuelo et al., 2011), *Q. robur* leaves should boost productivity, including macrophyte growth, more than *Q. rubra*.

### 2.3. Microcosm surveys

At the end of the experiment (30 days) we recorded: (1) ten water properties; (2) three macrophyte properties; (3) remnant tree leaf mass; (4) taxonomic composition of primary producers (algae) and consumers (protozoa and metazoa); and (5) three measures of sludge production. Samples from (1) to (3) were taken before microcosms contents were homogenized by 10 manual inversion shakes before taking samples for (4) and (5).

- $\text{O}_2$  saturation (%), dissolved  $\text{O}_2$  concentration (mg/L), pH, conductivity ( $\mu\text{S}/\text{cm}$ ), water turbidity (%) and total dissolved solids (TDS, mg/L) were measured *in-situ* using digital probes. General water hardness ( $^\circ\text{dGH}$ ) was measured using the colorimetric test Viscolor® (Macherey-Nagel, Germany). We also calculated the day-night water pH balance defined as the difference in pH between a period of high photosynthetic activity (14:00 h) and after the night (7:00 h). It is well-known that photosynthesis of submerged macrophytes requires  $\text{HCO}_3^-$  uptake that eventually increases water pH due to the release of  $\text{OH}^-$  from the plants (Pedersen et al., 2013). Another water property was the balance between  $\text{O}_2$  production and consumption for 30 min to assess the level of autotrophy, which relates to eutrophication (Staeher et al., 2012). Microcosms were topped with rainwater and sealed with plastic to act as metabolic chambers (see Staeher et al., 2012) by means of recording the difference in the initial and final  $\text{O}_2$  values at Day 21. Last, microcosms were emptied through Whatman GF/F glass fiber filters (U.K.). The filtrate was frozen at  $-20^\circ\text{C}$  until analyses of chlorophyll-a ( $\mu\text{g}/\text{L}$ ) were made (hereafter water chlorophyll-a). Water samples of 100 mL were frozen similarly to quantify total nitrogen (mg/L) and DOC at the University of Barcelona following well-established procedures outlined in Elosegui and Sabater (2009).
- Plant fragments were extracted from microcosms and brushed to detach epiphytes onto a tray filled with tap water (Elosegui and

Sabater 2009). The surface area of each plant ( $\text{mm}^2$ ) was calculated from scanned geometries and the final plant size was also expressed as mg WW. Epiphytic algal biomass was estimated by the concentration of chlorophyll-a in the rinsed water, which was measured as described in (1) and expressed as  $\mu\text{g}$  chlorophyll-a per g of macrophyte.

- Remnant dead tree-leaf mass after 30 days was calculated based on the difference in initial and final dry-weight mass values of leaves at  $70^\circ\text{C}$  for 72 h (Elosegui and Sabater 2009).
- The density of primary producers and consumers was determined in two steps: (a) the content of microcosm was sieved through a  $250\text{-}\mu\text{m}$  mesh to count large metazoa (crustacea); and (b) three 50-ml tubes of filtered water were collected per microcosm, two of which were fixed in 4% formaldehyde, for counting green algae, diatoms, amoeba, flagellates, ciliates and metazoans (crustaceans, rotifers, gastrotrichs and helminths). We examined  $75\text{-}\mu\text{l}$  of a homogenized water sample from each microcosm drop-by-drop with a microscope at  $\times 100$  and  $\times 400$  (Maceda-Veiga et al., 2015). The third tube was kept in the refrigerator at  $4^\circ\text{C}$  for fresh inspection at the field station to explore the taxa present prior to fixation and storage, which may cause protozoan shrinkage. From prior experience, the preservation of hypotrichs (e.g., *Aspidisca* and *Euplotes*) is poor, and so, these are excluded from analyses.

Taxonomic identifications were based on morphological traits following Foissner et al. (1991–1995) for ciliates, Krammer and Lange-Bertalot (1986–1991) for algae and Streble and Krauter (1987) for metazoans and algae. Density data ( $\text{ind}/\text{mL}$ ) were to genus or the lowest practical taxonomical category. Density data were converted into biomass following standard methods for each taxon (e.g., equations based on biovolume estimates; Dumont et al., 1975; Kremer et al., 2014). In the absence of published equations, we used carbon constants assumed for the group (e.g.,  $0.22\text{ pg C}/\mu\text{m}^3$  for nanoflagellates; Børshiem and Bratbak 1987). Protozoan assemblages were used to calculate the Saprobity index by Zelinka and Marvan (1961), which ranks organic-matter content in five ordinal categories (I, xenosaprobity; II, oligosaprobity; III,  $\beta$ -mesosaprobity; IV,  $\alpha$ -mesosaprobity and V, polisaprobity).

- The amounts of detritus, filamentous algae and filamentous bacteria, including cyanobacteria, were ranked from 0 to 5 using the samples processed for microscopy detailed above and their relative abundance in the  $75\text{-}\mu\text{l}$  of water examined (Eikelboom, 2000). We measured filamentous organisms because they prevent sedimentation and cause sludge bulking (Martins et al., 2004).

#### 2.4. Statistical analyses

All statistical analyses were conducted in R (R Core Team 2014). The distributions of all variables were inspected prior to modeling and variables other than pH were log-transformed to meet statistical requirements of models (e.g., reducing severe kurtosis).

To examine effects of the 30-day water treatment on macrophytes and dead tree leaves themselves, we compared the biomass and surface area of macrophytes and remnant dead tree litter mass among treatments using Gaussian error distribution in the R function *glm*.

To test how *C. demersum* and *E. densa* performed relative to one-another as water treatments, we used the R function *glm* to compare the eleven water properties between leaf-rooftop water filled microcosms with and without macrophytes. We also used taxon-rank abundance curves (the R function *rankabuncomp*; Kindt and Kindt 2008) to visually inspect the role of the two species of macrophytes as supporting habitat for autotrophs (algae) and heterotrophs (flagellates, amoeba, ciliates and metazoa). The rank-abundance diagrams do not rely on a single statistic, as diversity indices do, and inform richness and evenness by reporting the whole distribution of abundances (Magurran 2004).

The 'x-axis' is the observed richness and the overall slope is the evenness, with a steeper curve indicative of low evenness (Magurran 2004). We used the R function *glm* to statistically compare the total taxon richness and densities of autotrophs, heterotrophic microbial eukaryotes and metazoa among treatments. Differences in taxonomic composition were assessed using the R function *adonis* (PERMANOVA,  $n\text{perm} = 999$ , Oksanen et al., 2007) and the function *permutest.betadisper* was used to check whether differences in data dispersion affected PERMANOVA analyses (Oksanen et al., 2007). Last, we used the R function *IndVal* to identify the most characteristic taxa for each treatment groups based on permutation tests (De Cáceres et al. 2012). The indicator value of each taxon ranges from 0 to 1 and reaches its maximum value when all individuals of a taxon are in all microcosms of a group (e.g. *C. demersum* with *Q. robur* leaves).

To further study the performance of *C. demersum* and *E. densa* in microcosms, we used the R function *glm* to compare biomasses of autotrophs and heterotrophs and indicators of sludge production among experimental factors.

Statistical significance of experimental factors was tested with *F*-tests and pair-wise comparisons between levels of each treatment (e.g. macrophyte type) were examined using the R function *lsmeans* (Lenth and Hervé 2015). Statistical significance was at  $\alpha = 0.05$ .

### 3. Results

#### 3.1. Characteristics of macrophytes and dead-tree leaves at 30 days

The biomass and surface area of macrophyte fragments were greater in *C. demersum* than in *E. densa* (Table 1, Fig. 2AB). The concentration of epiphytic chlorophyll-a per gram of macrophyte was greater with *C. demersum* than with *E. densa* (Table 1, Fig. 2C). Drained rooftop water with different organic carbon loadings (i.e. types of dead tree leaves) did not have significant effects on the final biomasses and surface areas of the two macrophyte species (Table 1). All types of tree leaves significantly reduced epiphytic chlorophyll-a in *C. demersum* and *E. densa*, but the effects were more marked for *C. demersum* (by 47– 76%) (Table 1, Fig. 2C). The presence of dead tree leaves significantly affected the day-night pH balance, with greater values in microcosms with *C. demersum* and dead tree leaves (Table 1, Fig. 2D). *Q. robur* leaves had decomposed more (68%) than those of *Q. rubra* (75%), irrespective of the two macrophyte treatments (Fig. 2F).

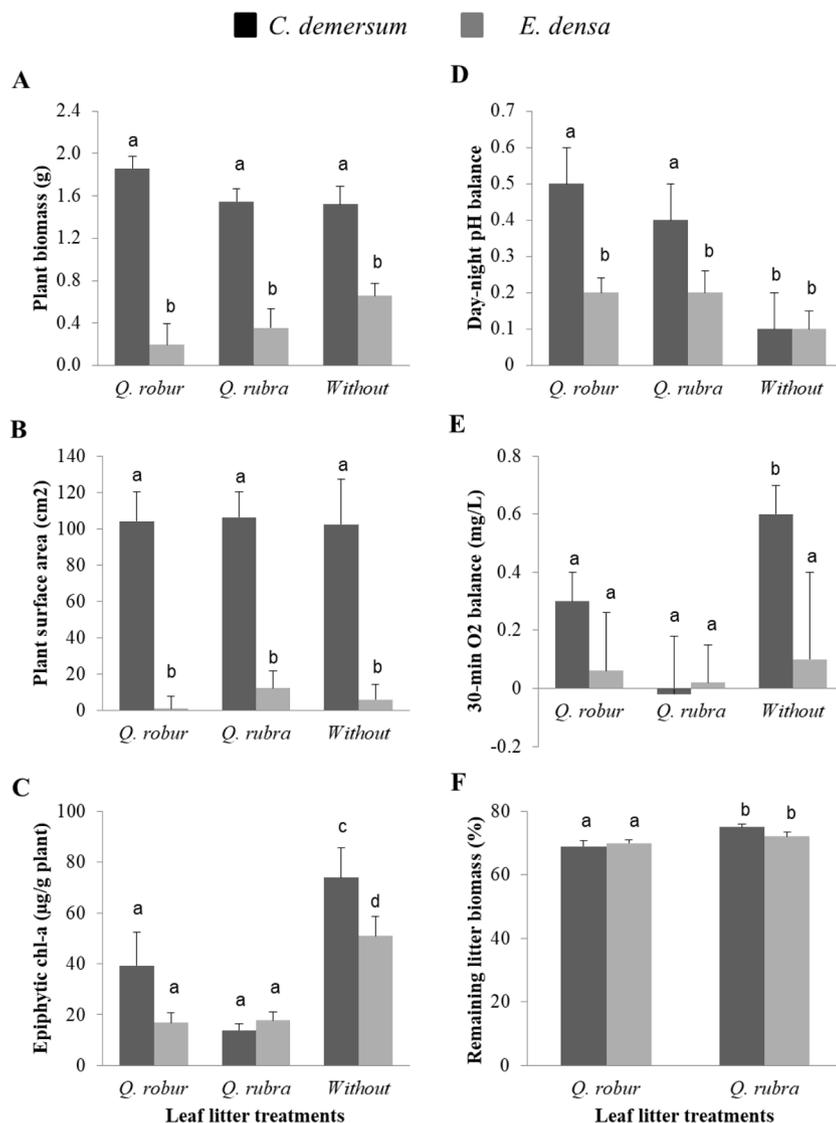
#### 3.2. Effects of the macrophyte treatments on water quality

Drained rooftop waters with *Q. robur* leaves had lower conductivity and less total dissolved solids (TDS) than microcosms with *Q. rubra* (Table 2, Appendix S1). Conversely, drained rooftop waters with

**Table 1**

Results for the comparison of macrophyte characteristics (*Ceratophyllum demersum* and *Egeria densa*) and their influence on the environment with different organic carbon loadings (leaves from *Quercus robur* and *Q. rubra*). Bold indicates treatments that were statistically significant at  $P \leq 0.05$ .

	Macrophyte species (M)	Terrestrial leaf litter (TL)	Interaction (M x TL)
Macrophyte biomass	<b><math>F_{1,54} = 52.4</math>; <math>P &lt; 0.01</math></b>	$F_{2,54} = 0.002$ ; $P = 0.99$	$F_{2,54} = 2.67$ ; $P = 0.07$
Macrophyte surface area	<b><math>F_{1,54} = 14.7</math>; <math>P &lt; 0.01</math></b>	$F_{2,54} = 0.88$ ; $P = 0.42$	$F_{2,54} = 0.43$ ; $P = 0.64$
Epiphytic algal biomass	<b><math>F_{1,54} = 27.9</math>; <math>P &lt; 0.01</math></b>	<b><math>F_{2,54} = 6.38</math>; <math>P &lt; 0.01</math></b>	<b><math>F_{2,54} = 42.51</math>; <math>P &lt; 0.01</math></b>
Day-night pH balance	<b><math>F_{1,54} = 10.8</math>; <math>P &lt; 0.01</math></b>	<b><math>F_{2,54} = 4.71</math>; <math>P = 0.01</math></b>	$F_{2,54} = 2.31$ ; $P = 0.10$
30-m $\text{O}_2$ balance	$F_{1,54} = 2.08$ ; $P = 0.15$	$F_{2,54} = 1.61$ ; $P = 0.21$	$F_{2,54} = 0.92$ ; $P = 0.40$
Litter decomposition	$F_{2,49} = 0.36$ ; $P = 0.70$	<b><math>F_{1,49} = 45.2</math>; <math>P &lt; 0.01</math></b>	$F_{2,49} = 0.19$ ; $P = 0.83$



**Fig. 2.** Effects of organic carbon loadings (i.e. drained rooftop water with and without leaves of *Quercus robur* and *Quercus rubra*) on stand characteristics of macrophytes *Ceratophyllum demersum* and *Egeria densa* (A, plant biomass; B, plant surface area; and C, epiphytic algal biomass) and the O<sub>2</sub> and CO<sub>2</sub> production and consumption, as estimated by: D) the 30-min O<sub>2</sub> balance and E) the day-night change in pH, respectively. 'F' shows the effects of the macrophytes on the remnant tree-litter biomass of 1 g of leaves introduced at the beginning of the experiment (30 days). The same letter (a or b) indicated homogeneous treatments at  $P \geq 0.05$  based on statistical outputs in Table 1. Mean  $\pm$  SE,  $N = 10$  microcosms.

*Q. robur* had the greatest values of water chlorophyll-a, water turbidity and DOC (Table 2, Appendix S1). O<sub>2</sub> production was greater than O<sub>2</sub> consumption when *Q. robur* microcosms were sealed for 30 min during daylight, while the net O<sub>2</sub> production was negative for *Q. rubra* microcosms (30-min O<sub>2</sub> balance in Table 2, Appendix S1). Leaf-litter type did not significantly affect the other six water-properties, including total N (Table 2, Appendix S1).

The *C. demersum* treatment was better than that of *E. densa* at reducing water conductivity (by 10–40 µS/cm), TDS (by 10–18 mg/L), DOC (by 4–5 mg/L) and at increasing water transparency (by 4–9%), water O<sub>2</sub> levels (by 19–27%) and daylight pH (by 0.9–1.3), all median values of the respective macrophyte treatments compared to microcosms with only leaf-litter at 30 days (Table 2, Appendix S1). However, despite its lower biomass, the *E. densa* treatment was better at reducing water chlorophyll-a (by 14–34 µg/L) (Table 2, Appendix S1). The day-night pH balance was significantly more positive for *E. densa* than for *C. demersum* in the presence of leaf litter (Table 2, Appendix S1). None of the two macrophytes had a significant effect on total N water concentrations (Table 2, Appendix S1).

### 3.3. Effects of the macrophyte treatments on the biological assemblages and sludge

The *C. demersum* and *E. densa* treatments did not significantly differ in the total density and taxon richness of autotrophic and heterotrophic organisms (Table 3, Fig. 3). The type of leaf-litter significantly affected the density and taxon richness of autotrophs (Table 3), with the greatest values for *E. densa* with *Q. robur* leaves (Fig. 3). The same relationship held for the density of metazoa but not for heterotrophic microbes because their density did not significantly differ among treatments (Table 3). The taxonomic composition of autotrophic and heterotrophic organisms was heterogeneous among the leaf litter-macrophyte treatments (Table 3, Fig. 3). There was greater taxon evenness for autotrophs than for heterotrophs (rank-abundance curves, Fig. 3). The most characteristic taxa for *E. densa* treatments were diatoms and bacteria, whereas *C. demersum* treatments included taxa for most of the studied groups (green algae, diatoms, amoeba, flagellates and ciliates) (Indicator Values in Table 4). The *C. demersum* treatment had less dead organic matter (mean  $\pm$  SD = 2.2  $\pm$  0.9) than one with *E. densa* (2.7  $\pm$  0.6) (Saprobity index,  $F_{1,51} = 5.40$ ;  $P = 0.02$ ) although it increased with dead tree leaves in both treatments ( $F_{2,51} = 25.41$ ;  $P < 0.001$ ).

The macrophyte treatments did not significantly differ in biomasses of autotrophs and heterotrophs, either for microbes or for metazoans

**Table 2**

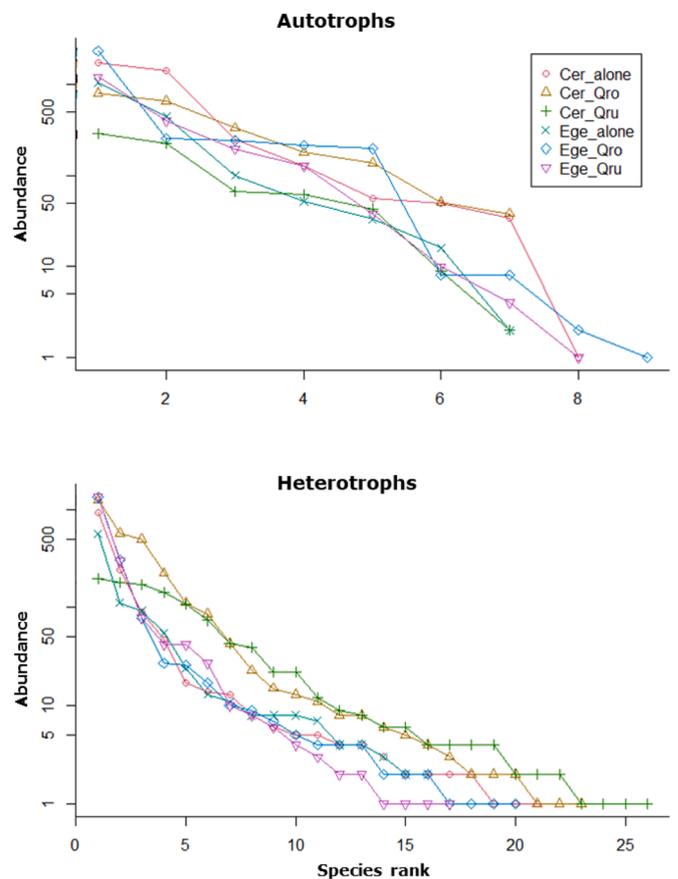
Results of the comparisons of water-quality properties of the drained rooftop water with two types of organic carbon loadings (leaves from *Quercus robur* and *Q. rubra*) and with and without two types of submerged macrophyte treatments (*Ceratophyllum demersum* and *Egeria densa*). Bold indicates statistically significant results at  $P \leq 0.05$ .

	Macrophyte species (M)	Terrestrial leaf litter (TL)	Interaction (M x TL)
O <sub>2</sub> saturation (%)	$F_{2,54} = 7.1; P = 0.01$	$F_{1,54} = 0.01; P = 0.92$	$F_{2,54} = 0.08; P = 0.91$
Dissolved O <sub>2</sub> (mg/L)	$F_{2,54} = 8.5; P < 0.01$	$F_{1,54} = 0.06; P = 0.80$	$F_{2,54} = 0.1; P = 0.87$
O <sub>2</sub> balance (mg/L)	$F_{2,54} = 0.6; P = 0.55$	$F_{1,54} = 5.4; P = 0.02$	$F_{2,54} = 0.7; P = 0.50$
pH	$F_{2,54} = 56.6; P < 0.01$	$F_{1,54} = 2.6; P = 0.11$	$F_{2,54} = 1.3; P = 0.28$
Day-night pH balance	$F_{2,54} = 14.3; P < 0.01$	$F_{1,54} = 3.1; P = 0.08$	$F_{2,54} = 0.7; P = 0.49$
Conductivity (µS/cm)	$F_{2,54} = 9.8; P < 0.01$	$F_{1,54} = 17.2; P < 0.01$	$F_{2,54} = 1.1; P = 0.34$
Total dissolved solids (mg/L)	$F_{2,54} = 9.9; P < 0.01$	$F_{1,54} = 16.6; P < 0.01$	$F_{2,54} = 1.3; P = 0.27$
Dissolved organic carbon (mg/L)	$F_{2,54} = 13.9; P < 0.01$	$F_{1,54} = 15.6; P < 0.01$	$F_{2,54} = 0.5; P = 0.66$
General water hardness (°dGH)	$F_{2,54} = 0.8; P = 0.46$	$F_{1,54} = 3.6; P = 0.06$	$F_{2,54} = 0.1; P = 0.87$
Total nitrogen (mg/L)	$F_{2,54} = 1.2; P = 0.31$	$F_{1,54} = 0.2; P = 0.65$	$F_{2,54} = 0.08; P = 0.93$
Chlorophyll-a (µg/L)	$F_{2,54} = 5.7; P < 0.01$	$F_{1,54} = 21.4; P < 0.01$	$F_{2,54} = 0.08; P = 0.92$
Water transparency (%)	$F_{2,54} = 60.7; P < 0.01$	$F_{1,54} = 12.3; P < 0.01$	$F_{2,54} = 7.8; P < 0.01$

**Table 3**

Results for comparisons of total density, total taxon richness, taxonomic composition and biomasses of autotrophic and heterotrophic organisms among treatment combinations. Changes in biomass of heterotrophic microbial eukaryotes and metazoa are provided given their potential importance as food for higher order consumers (e.g., fish). General linear models were used for density, richness and biomasses and Permutational Analyses of Variance (PERMANOVA) were used for the study of variation in taxonomic composition.

	Macrophyte species (M)	Terrestrial leaf litter (TL)	Interaction (M x TL)
<b>A) Autotrophs</b>			
Total taxon richness	$F_{1,53} = 0.05; P = 0.81$	$F_{2,53} = 3.4; P = 0.04$	$F_{2,53} = 0.07; P = 0.94$
Taxa composition	$F_{1,54} = 15.4; P < 0.01$	$F_{2,54} = 4.3; P < 0.01$	$F_{2,54} = 3.01; P < 0.01$
Total density	$F_{1,53} = 0.008; P = 0.93$	$F_{2,53} = 5.86; P = 0.01$	$F_{2,53} = 1.4; P = 0.25$
Total biomass	$F_{1,53} = 1.44; P = 0.23$	$F_{2,53} = 5.98; P = 0.01$	$F_{2,53} = 0.86; P = 0.43$
<b>B) Heterotrophs</b>			
Total taxon richness	$F_{1,53} = 0.22; P = 0.64$	$F_{2,53} = 0.59; P = 0.56$	$F_{2,53} = 0.31; P = 0.73$
Taxa composition	$F_{1,54} = 4.4; P < 0.01$	$F_{2,54} = 10.9; P < 0.01$	$F_{2,54} = 4.2; P < 0.01$
Total density	$F_{1,53} = 0.05; P = 0.82$	$F_{2,53} = 2.23; P = 0.12$	$F_{2,53} = 1.62; P = 0.21$
Microbes	$F_{1,53} = 0.001; P = 0.97$	$F_{2,53} = 1.56; P = 0.22$	$F_{2,53} = 1.46; P = 0.24$
Metazoa	$F_{1,53} = 0.04; P = 0.84$	$F_{2,53} = 27.72; P < 0.01$	$F_{2,53} = 0.12; P = 0.89$
Total biomass	$F_{1,53} = 3.00; P = 0.09$	$F_{2,53} = 7.38; P < 0.01$	$F_{2,53} = 9.06; P < 0.01$
Microbes	$F_{1,53} = 3.44; P = 0.07$	$F_{2,53} = 6.09; P < 0.01$	$F_{2,53} = 9.25; P < 0.01$
Metazoa	$F_{1,53} = 3.25; P = 0.08$	$F_{2,53} = 9.95; P < 0.01$	$F_{2,53} = 0.94; P = 0.39$



**Fig. 3.** Rank abundance diagrams of autotrophs (green algae, diatoms and euglenids) and heterotrophs (other flagellates, amoeba, ciliates and metazoan) in the macrophyte and tree-litter treatments. The y-axis is the abundance of organisms (ind/mL) in the treatments arranged by taxon richness (x-axis) and the overall slope is evenness. That is, the autotrophs had more similar taxonomic composition than did the heterotrophs, heterotrophs had greater taxon richness than did the autotrophs and the greatest taxon richness was for Cer\_rub. Acronyms: Cer\_alone, *Ceratophyllum demersum* without tree leaves; Ege\_alone, *Egeria densa* without tree leaves; Cer\_rob, *C. demersum* with *Quercus robur* leaves; Cer\_rub, *C. demersum* with *Quercus rubra*; Ege\_rob, *E. densa* with *Q. robur*; and Ege\_rub, *E. densa* with *Q. rubra*.

(Table 3; Fig. 3). However, microcosms with *Q. robur* had greater overall biomasses of autotrophs than those with *Q. rubra* (Fig. 4A). Biomasses of heterotrophic microbes were greater in microcosms with leaf litter compared to those with macrophytes alone, with the highest values being for *Q. robur* (Fig. 4B). The same relationship held for metazoans but the two types of leaf litter did not differ in metazoan biomass (Fig. 4C).

Detritus accumulation did not significantly differ between macrophyte species ( $F_{1,53} = 1.39; P = 0.24$ ) but was substantially greater in the presence of dead tree leaves ( $F_{1,53} = 8.19; P < 0.001$ ). Microcosms only with macrophytes had greater amounts of filamentous algae of genus *Cladophora* than when grown with dead tree leaves ( $F_{2,53} = 6.32; P = 0.003$ ), particularly *E. densa* ( $1.3 \pm 0.4$  vs  $0.3 \pm 0.1; P < 0.01$ ). The exception was *E. densa* with *Q. rubra*, which had similar amounts of filamentous algae to when growing alone (Macrophyte x Leaf litter:  $F_{2,53} = 8.77; P < 0.005$ ). *E. densa* had more filamentous bacteria ( $2.33 \pm 1.02$ ) than *C. demersum* ( $1.38 \pm 0.86$ ), either when growing without or with tree litter, particularly *Q. rubra* (Leaf litter:  $F_{2,53} = 3.94; P = 0.03$ ; Macrophyte x Leaf litter  $F_{2,53} = 1.22; P = 0.30$ ).

**Table 4**

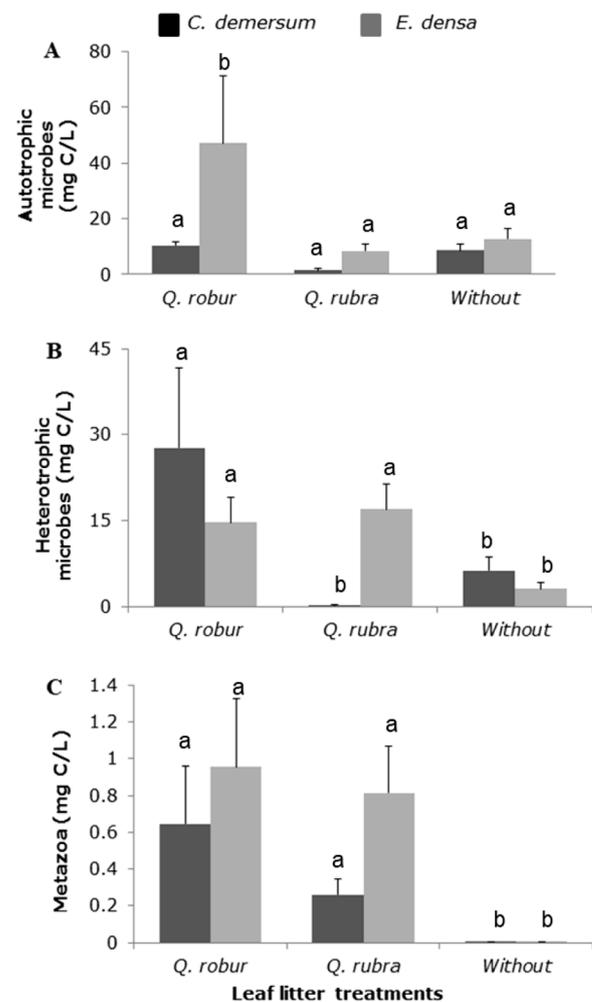
Eukaryotic microbes and metazoan indicator taxa for each treatment group with their indicator values and associated *P*-values computed using the R function *IndVal* (De Cáceres et al. 2012).

<i>C. demersum</i>	<i>E. densa</i>
<i>Q. robur</i> <i>Cyrtolophosis muscicola</i> (Ciliate) [IndVal = 0.91; <i>P</i> = 0.001]	<i>Q. robur</i> <i>Rhoicosphenia</i> sp. (Diatom) [IndVal = 0.40; <i>P</i> = 0.004]
	<i>Pinnularia</i> sp. (Diatom) [IndVal = 0.39; <i>P</i> = 0.012]
<i>Q. rubra</i> <i>Microthorax</i> sp. (Ciliate) [IndVal = 0.39; <i>P</i> = 0.008]	<i>Q. rubra</i> <i>Filamentous bacteria</i> [IndVal = 0.26; <i>P</i> = 0.001]
<i>Chrysophyceae</i> (Flagellate) [IndVal = 0.36; <i>P</i> = 0.008]	
<i>Pseudoconhilembus</i> sp. (Ciliate) [IndVal = 0.3; <i>P</i> = 0.02]	
Without leaves <i>Fragilaria</i> sp. (Diatom) [IndVal = 0.7; <i>P</i> = 0.001]	Without leaves <i>Cyclidium glaucoma</i> (Ciliate) [IndVal = 0.61; <i>P</i> = 0.001] <i>Nitzschia</i> sp. (Diatom) [IndVal = 0.47; <i>P</i> = 0.02]
<i>Cocconeis</i> sp. (Diatom) [IndVal = 0.5; <i>P</i> = 0.02]	
<i>Nucleariida</i> (Amoeba) [IndVal = 0.47; <i>P</i> = 0.003] <i>Ankistrodesmus</i> sp. (Green algae) [IndVal = 0.46; <i>P</i> = 0.005]	

#### 4. Discussion

Our study highlights the superior capacity of the European native macrophyte *C. demersum* for treating drained rooftop water when compared to the South American macrophyte *E. densa*. We proved the efficacy of the macrophytes with two types of organic carbon loadings (*Q. robur*: high biodegradability; and *Q. rubra*: low biodegradability) and found that *C. demersum* was better than *E. densa* at water oxygenation and at lessening water conductivity and turbidity arising, respectively, from the decomposition of leaves from the American oak (*Q. rubra*) and the native oak (*Q. robur*). Leaves of *Q. robur* decomposed faster than those of *Q. rubra*, which released more dissolved organic carbon, nitrogen and phosphorous to water and hence stimulated production of water chlorophyll-*a*. *Egeria densa* was better at reducing water chlorophyll-*a* after 30 days despite having less biomass than *C. demersum*. However, microcosms with *E. densa* developed more non-epiphytic organisms, which cause sludge bulking, which is a major issue in water-treatment systems (Madoni 2003; Martins et al., 2004).

The use of microcosms is common in aquatic ecology and to assess sewage treatments (e.g., Gause 1934; Srivastava et al., 2004; Benton et al., 2007; Altermatt et al., 2015; Maceda-Veiga et al., 2015), but microcosm-based inferences are inevitably limited by the spatial scale and duration of the experiment (Carpenter 1996; Englund and Cooper 2003). We dealt with potential temporal changes by measuring 27 biological and environmental properties with different potential response durations, ranging from hours (e.g., O<sub>2</sub> production and microbial densities) to weeks (e.g. plant growth) (Elosegui and Sabater 2009). Microcosms also were pertinent because we were interested in studying small organisms, metazoans (e.g., rotifers, gastrotrichs) and microbial eukaryotes (e.g., amoeba, ciliates), all of which have fast generation times (e.g., 13–33 generations of microbes in 4 weeks) and are the dominant biological communities in wastewater-treatment plants, alongside prokaryotes and viruses (Curds 1992; Madoni 2003; Perez-Uz et al., 2010). Although we did not measure non-filamentous bacteria, we examined ecological functions in which these bacteria participate (e.g., 30-min O<sub>2</sub> balance, leaf-litter decomposition) and we



**Fig. 4.** Biomasses of autotrophs (A, green algae, diatoms and euglenids) and heterotrophs, divided in: (B) microbial eukaryotes (other flagellates and ciliates) and (C) metazoa (crustaceans, rotifers, nematode and gastrotrichs), both of which may be food for adults and larvae of higher-order consumers (e.g. fish, newt larvae) and inform different stages of the dead organic matter processing. The letters (a or b) indicated homogeneous treatments at  $P \geq 0.05$  based on statistical outputs in Table 3. Mean  $\pm$  SE,  $N = 10$  microcosms.

studied microbial eukaryotes (e.g., flagellates and ciliates), whose densities typically depends on bacterial load (Curds 1992; Salvadó and Gracia 1993; Appendix S2). The expected temporal succession of these microbial eukaryotic assemblages also can be inferred from sewage treatment plant monitoring. Flagellates dominate in density in the initial, more disturbed assemblages, scuticociliates at intermediate levels and sessile peritric ciliates and testate amoeba dominate in the final, more stable eukaryotic microbial assemblages (Salvadó 1994). Last, rooftop water storage tanks are expected to differ from sewage-treatment plants in type, intensity, and frequency of sewage effluent volumes (Madoni 2003; Moore and Hunt 2012). Nevertheless, among the 50 taxa listed (Appendix S3), we recorded taxa common and abundant in sewage-treatment plants of different ages (e.g., heterotrophic nanoflagellates, the scuticociliate *Cyclidium glaucoma*, and the testate amoeba *Arcella* sp.; Salvadó 1994; Madoni 2003). There were also epibionts characteristic of *C. demersum* growing in natural conditions (e.g., *Cocconeis*, *Nitzschia*, Zhang et al., 2020). Therefore, we are confident that the manageable size of microcosms, allowing greater replication, is a good first step to get a general view of how these plant treatments may work before increasing experimental scales.

#### 4.1. *C. demersum* was superior to *E. densa* in treating poorly mineralized rooftop waters

Despite the two macrophytes being regarded as fast-growing plants with broad tolerances to a large range of conditions (Feijóo et al., 2002; Pietro et al., 2006), *C. demersum* had greater size than *E. densa* after 30 days in all experimental conditions. Mother plants of the two species had grown for 12 years as floating mats in rainwater collectors (see methods), so the plants should be acclimatized to the properties of rainwater. The lesser growth of *E. densa* probably was due to overgrowth of the filamentous non-epiphytic green algae *Cladophora*, which may have shaded the plant or have released plant growth inhibitors (Phillips et al., 2016; Zhang et al., 2019). Another factor, probably of lesser importance, might have been the shading from epiphytic algae (e.g., Tóth 2013) because *E. densa* had less epiphytic algae scaled by the macrophyte biomass than did *C. demersum*. The overgrowth of suspended algae also did not seem to affect *E. densa* performance because water chlorophyll-*a* from leaf-litter decomposition was reduced by the plant by up to 34  $\mu\text{L/g}$ , which is a value typical of highly eutrophic lakes (Nürnberg, 1996).

Besides direct plant effects, a decrease in algal biomass may be due to differences in the action of algal grazers, such as ostracods, which were in all microcosms fed with dead tree leaves at a mean density of  $28 \pm 36$  ind./L. However, ostracod grazing on filamentous algae is low compared to that of snails (Pinowska, 2002; Yan et al. 2020). There were no snails in microcosms because snail survival might be low given the low mineral content in rooftop water (Walstad 1999) but, if present, snails might have consumed more *E. densa* than *C. demersum* (Sheldon 1987; Pinowska, 2002). Another factor might have been that non-epiphytic algae had fewer competitors with *C. demersum*. Microcosms had c. 12 h of direct sunlight on a sunny day, whereas those of mother plants of each macrophyte species had c. 6 h (AMV observ.). However, the potential optimization of growth conditions for *E. densa* must be done in the native distributional range because the sale of *E. densa* is now prohibited in Europe, including Spain, because of its invasiveness (Curt et al., 2010).

#### 4.2. Tree-leaf runoff: from subsidies to contaminants

Although drained rooftop water may contain relatively high levels of nutrients (Lye 2009; Meera and Ahammed 2006), low total N values, such as in our microcosms ( $0.5 \text{ mg L}^{-1}$ ), are common in the highly variable contaminant loads in rooftop waters arising from time since rain or wind-dispersion (De Buyck et al. 2021). Despite this, microcosms with *Q. robur* and *Q. rubra* reached relatively high levels of eutrophication (e.g., chlorophyll-*a*  $\geq 30 \mu\text{L/g}$ ; Nürnberg, 1996) enabling us to test the capacity of *E. densa* and *C. demersum* to deal with eutrophic waters. Our experiment was realistic in that leaf-litter decomposition produces little biodegradable compounds, which stain water (Julkuunen-Tiitto and Haggman 2009), such as occurs in storm waters and sewage effluents (Madoni 2003; Shen et al., 2018). Therefore, *C. demersum* may have more potential than *E. densa* for treating other water types given that its growth was less affected by the runoff treatments.

The faster decomposition rate of *Q. robur* leaves than those of *Q. rubra* is consistent with work on the effects of *Q. rubra* on microbes in its invasive range in standardized, common-garden conditions (Hobbie et al., 2006; Stanek and Stefanowicz 2019). The slower leaf litter breakdown of *Q. rubra* leaves may be due to lower biomass of microbes arising from higher values of C/N ratio and phenols compared to *Q. robur*. Our results supported such effects by finding lesser biomass of heterotrophic eukaryotic microbes (flagellates and ciliates) in *Q. rubra* than in *Q. robur* microcosms. Although we did not study bacteria and fungi, both of which are involved in the leaf-litter decomposition (Szabó et al., 2000; Pascoal and Cásio et al. 2004), decreases in biomasses of these microbes probably would have decreased biomass of heterotrophic

eukaryotic microbes; the studied taxa (e.g., *Cyclidium glaucoma*, nano-flagellates) are bacterivores (Ježbera et al., 2005). However, the effects of changes in the quality of basal resources on upper trophic levels can be attenuated, at least partially, by food-web complexity (Srivastava and Bell 2009). The latter may explain why the biomass of metazoa did not show significant differences between *Q. rubra* and *Q. robur* leaves, although heterotrophic eukaryotic microbial biomass did so.

#### 4.3. Other advantages of macrophytes as water-treatment systems

Alongside improving water quality, an advantage of macrophyte-water treatments is that they have other environmental benefits compared to 'hard' engineered solutions (Moore and Hunt 2012; Kurniawan et al., 2021). One of these values is providing food for livestock, which may be useful for projects of circular economy, such as aquaponics or other multi-trophic systems (Million et al., 2016; Alsaniya et al., 2017; Stevčić et al., 2020), in which the water flows from fish tanks to plant tanks and return clean to the former. The 'green tank' may then provide both water and food for fish adults or larvae, such as ciliates, rotifers and *Daphnia* (Diver and Rinehart 2000; Stevčić et al., 2020) and raw material for biofuel (Kurniawan et al., 2021). The lack of significant differences in total metazoan biomass between *C. demersum* and *E. densa* might suggest a similar potential of the two plants in this regard. However, total biomass does not deal with food quality, which, when allied to the differences in the taxonomic composition of organisms between the two macrophyte species, suggests that *C. demersum* and *E. densa* are not equals in providing habitat for native biodiversity.

Winter water temperatures may reduce the usefulness of macrophyte-water treatments, but countries with Mediterranean climate probably have relatively long growing periods for the two plants. The temperature tolerance range of *C. demersum* is 5–35 °C (Ma et al., 2009) and that of *E. densa* is 10–35 °C (Haramoto and Ikusima, 1988) and mean monthly air temperature of Barcelona city ( $\pm\text{SD}$ ) was  $16 \pm 5$  °C (mean monthly minimum = 8 and maximum = 24) between 1981–2010 (www.aemet.es). Nevertheless, mats of *C. demersum* and *E. densa* in the study area survive winters with air temperatures  $<1$  °C and this does not seem to affect much the summer performance of *C. demersum*. Four rooftop water storage ponds (400 L) were built after this study in the O Incio Observatory, two with *C. demersum* and two without plants, and were visually inspected from 2018 to 2021. Waters were clearer for *C. demersum* than for ponds without the plant (farmers' personal observations). Given that runoff water quantity and quality probably varied substantially these years, the benefits of *C. demersum* seen in microcosms might well apply to broader spatial and temporal scales, at least in man-made ecosystems for water-treatment purposes.

## 5. Conclusions

Our study suggests that *C. demersum* is superior to *E. densa* for treating rooftop water and for providing food for consumers. Water transparency, conductivity, water chlorophyll-*a* and oxygenation were the water properties most affected by the type of macrophyte, probably due to a combination of nutrient competition and the release of algal growth inhibitors (Pietro et al., 2006; Gross et al., 2003). Influxes of two types of carbon loading affected the taxonomic composition and biomass of various groups of algae and eukaryotic microbes in both *C. demersum* and *E. densa* microcosms, as expected from the importance of leaf litter in fuelling food-webs (Srivastava and Bell 2009; Gessner et al., 2010; Stoler and Relyea 2016). Given food-web complexity, our study suggests the usefulness of whole-ecosystem experiments in selecting the most suitable macrophyte species for nature based engineered solutions.

#### Declaration of competing interest

Authors declared no conflict of interest

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117999.

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