Biodegradable plastic bags on the seafloor: a future threat for seagrass meadows?

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ABSTRACT: Marine plastic litter is a global concern. Carrier bags manufactured from non-biodegradable polymers constitute a large component of this litter. Because of their adverse impact on marine life, non-biodegradable bags have recently been replaced by biodegradable ones. However, growing evidence shows that these latter are not readily degradable in marine sediments and can alter benthic assemblages. The potential impact of biodegradable bags on seagrasses inhabiting sandy bottoms, which are the most widespread and productive ecosystems of the coastal zones, has been ignored. Mesocosm experiments were conducted to assess the effect of a commercialized biodegradable bag on a common seagrass species of the Mediterranean, *Cymodocea nodosa*, both at the level of individual plant (clonal growth) and of plant community (plant-plant relationships), under three culture regimes (plant alone, in combination with a neighbour of the same species or of the co-existing seagrass *Zostera noltei*) simulating different natural conditions (bare substrate, monospecific meadows or mixed meadows). The bag behaviour in marine sediment and sediment physical/chemical variables were also examined. After six months of sediment exposure, the bag retained considerable mass (85% initial weight) and reduced sediment pore-water oxygen concentration and pH. In the presence of bag, *C. nodosa* root spread and vegetative recruitment increased compared to controls, both intra- and interspecific interactions shifted from neutral to competitive, and the growth changed from guerrilla to phalanx but only with *Z. noltei*. These findings suggest that biodegradable bags altering sediment geochemistry could promote the spatial segregation of seagrass clones and influence species coexistence.

Keywords: biodegradable plastic, marine environment, plant interaction, seagrasses, sediments.

1.Introduction

Plastic pollution in the marine habitat is an environmental, growing problem at global scale (Derraik, 2002; Gross, 2013). Plastic carrier bags composed of polyethylene or polypropylene are a major component of the plastics accumulated in the marine environment (seawater and seafloor, Galgani et al., 1995; Thompson et al., 2004; Carson et al., 2011). Due to their extreme durability and adverse impact on marine organisms, these bags have been banned in many countries and replaced by biodegradable bags typically made from renewable raw materials such as starch or cellulose or bio-synthesized materials (Convery et al., 2007; UNEP, 2015). These materials are generally hygroscopic and/or have higher density than seawater thus the bags tend to settle onto the seafloor (Andrady, 2011) where they may be eventually entangled in marine vegetation and/or buried by sand. However, the expected benefits conferred by the increased use of biodegradable bags to marine organisms and ecosystems have been recently questioned (Accinelli et al., 2012; Tosin et al., 2012; Green et al., 2015). In fact, most polymers presently used for manufacturing biodegradable carrier bags (Accinelli and Abbas, 2011) are designed to breakdown into water, carbon dioxide/methane and biomass in a short time via microbial assimilation under standard conditions (i.e. soil, home or industrial compost facilities) that are generally not encountered in marine habitats. Very few companies worldwide claim to produce polymers designed to be biodegradable under marine environments (ASTM D7081-05), but the pass/fail criteria adopted for establishing the degradability are based on standard laboratory tests and their results cannot be extrapolated to real marine conditions. Indeed, the rate of degradation of bioplastics in marine environments strongly depends on local characteristics (including type of bacteria and organisms present, light, temperature and oxygen) and the compartment to which they are disposed, i.e. floating in seawater (pelagic zone) or in the seabed (Andrady, 2015). Studies have shown that some starch-based plastic bags degrade only partially after 236 days under sublittoral conditions (Tosin et al., 2012) remaining accessible for a given time to a suite of organisms living at or in the sediments.

The persistence of this material also inhibits gas changes between the overlying water and pore waters, and the resulting hypoxia or anoxia may alter macrobenthic community structure and interferes with the normal functioning of associated ecosystems (Green et al., 2015).

Surprisingly, no attention has been paid to assess the fate of plastics deposited on sandy bottoms colonized by marine vegetation (seagrasses) and their potential effect on plant growth. Seagrasses are clonal plants that colonize shallow coastal waters and estuaries in all continents except Antarctica (Short et al., 2007) and form both monospecific meadows dominated by a single foundation species and mixed meadows composed of species with different structural characteristics and functional traits (Duarte, 2000). Seagrasses depend on resources and conditions both above and within the sediments and are sensible to deterioration of sediment quality, although some species are able to cope with sediment alterations modifying biogeochemical conditions in their rhizosphere (Marbà and Duarte, 2001; Gacia et al., 2002; Borum et al., 2006). Seagrass meadows are vital to coastal ecosystems, provide numerous ecological services to human society (maintenance of marine biodiversity, regulation of the quality of coastal waters, protection of the coastline) and play a fundamental role in structuring communities (Costanza et al., 1997; Cullen-Unsworth and Unsworth, 2013). However, many species are presently under threat worldwide from localized (e.g., water pollution, eutrophication) and global stressors e.g., climate change), necessitating strategies to prevent further vegetation losses (Orth et al., 2000; Short et al., 2011). Currently, the amount of biodegradable plastics improperly discharged entering into the ocean it is unknown, but it is expected to become similar to the overall plastic input in future (UNEP, 2015). Therefore, understanding whether, and if so how, discarded biodegradable bags will influence the establishment, expansion and functioning of seagrass meadows in future is crucial.

In this study, we assessed in mesocosm the effect of a common type of bag manufactured with a starch derived polymer (Mater-Bi), which is available in the European market and certified as compostable and biodegradable, on the development of clones of a widely-distributed seagrass of the Mediterranean Sea, *Cymodocea nodosa* (Ucria) Ascherson. Specifically, we investigated the

response of the species to the bag, both at the level of individual plant (architecture and growth potential) and at plant community level (plant-plant interactions), over the first growing season (six-months) and under three culture regimes mimicking different naturally occurring situations: plant alone (i.e. when a clone colonizes novel substrate areas), in the presence of a neighbour of the same species (i.e., when a clone establishes into a monospecific stand) or of another seagrass, *Zostera noltei* Hornemann (i.e., when a clone establishes in a mixed stand). The behaviour of the bag in the marine sediment and its effect on sediment physical-chemical parameters, both in the absence of established vegetation and in the presence of *C. nodosa* or *Z. noltei*, were also examined in a parallel mesocosm experiment. The two species have contrasting clonal growth form and may coexist intermixed forming mixed beds (Kraemer and Mazzella, 1999). *C. nodosa* produces long internodes that ensure rapid and great occupation of new areas, and forms highly intermingled clumps of genets across the bed according to the guerilla-like growth form (Duarte et al., 2006). In contrast, *Z. noltei* produces short internodes, characteristics of the phalanx-like growth form (Ruggiero et al., 2005), and has a more compact structure that leads to uniform distribution of genets within the bed. We are particularly interested on the potential effect of the bag on the strength and direction of interactions both among clones of the same species and of different coexisting species, as plant-plant interactions and environmental stresses play a fundamental role in structuring plant communities and associated ecosystems (Tilman et al., 1981; Connell, 1983; Goldberg and Barton, 1992; Rose and Dawes, 1999).

2. Materials and methods

2.1. Experimental set-up and plant material

All the experiments were carried out in an aquaculture system (INVE Aquaculture Research Centre) located at Rosignano Solvay (Italy) that consisted of separate outdoor tanks (7000L) with

continuous flow of natural seawater equipped following a protocol previously established for successfully growing seagrasses (Balestri and Lardicci, 2012). The seawater level in the thanks was maintained at 0.5 m. Seawater temperature ranged from 16 to 25.8 °C, pH was 8-8.2, and salinity varied between 37.6 and 38.4 over the experimental period.

The type of biobag used in this study ("MB" hereafter) consists of Mater-Bi obtained from vegetable oils and corn starch (Novamont, http://www.novamont.com/) and it is certified as compostable under EN 13432 conditions and can be processed in home composting systems (Vincotte certification). Before the start of the experiment, MB bags were cut into equal pieces (14 cm x 14 cm, 0.48 ± 0.04 g dry weight, 20 µm of thicknesses). These pieces were placed in a tank at seawater-air surface and left to settle onto the bottom to simulate the natural entering in the marine environment from land sources. To establish plant cultures, plagiotropic rhizomes of *C. nodosa* and *Z. noltei* were collected in April 2016 in a shallow meadow (0.5 m depth) where the two species coexist (North western Mediterranean, Livorno, Italy). Collected plants were gently washed free of any adhering sediment particles and transported in seawater from the sampling site to the laboratory and planted within 2 h of sampling. Both the species exhibit typical unimodal growth at the collection site, reaching maximum development is summer and a cessation of growth in winter (Terrados and Ros, 1992; Marbà et al., 1996).

2.2. Behaviour of the bag buried in sediment and sediment variables

To examine the behaviour of the bag in marine sediments, 72 mesocosms (20 cm diameter x 19 cm depth) made in a non-biodegradable copolymer, polypropylene (Nuova Pasquini & Bini, Italy, www.pasquiniebini.it) were filled with natural sediment consisting of silica sand (<0.6-1 mm, <0.01% organic content) in March 2016. In each pot, a controlled-release fertiliser (Cifo Italy, N: P: K 20.10:10; six months) was applied at a rate of 1 g l^{-1} of sediment to assure the establishment of plants. The pots, hereinafter referred to as mesocosms, were placed in a tank and randomly assigned

to one replicate of two treatments, bag addition or positive control. In the bag addition treatment, one bag piece was individually inserted into the top 5 cm of sediment in the centre of the mesocosm while in the control a cellulose filter paper (15 cm diameter, Whathman n° 40) was inserted in the sediment. This latter treatment was used to assess whether the environment was sufficiently microbial active for biodegradation of starch/cellulose material. Each piece of bag or paper was individually inserted into a nylon mesh bag (1 mm mesh size) prior to be buried in the sediment to avoid the loss of particles due to fragmentation. The nets were retrieved from sediment every 15 days over six months (three replicates per treatment and harvest) and all material contained in each net was washed in distilled water, dried to constant weight (40 °C), weighed after sieving (2 mm sieve) with a digital microbalance (KERN ABT 220-50M, 0.1 mg accuracy). Weight loss of the bag as function of time was determined as difference between the final weight and the initial weight of bag and expressed in percentage. This test does not demonstrate biodegradability per se but gives data useful to predict the extent and rate of disintegration of the plastic material in real marine environments (EN 13432, 2000). At the end of the experiment, sub-samples (9 mm x 8 mm) of bag film were cut from freshly collected samples, washed with sterile water, dried using the critical point drying method, coated with gold-palladium (10 nm thickness) and then visualized under a scanning electron microscopy (SEM; JEOL USA JSM-5410) to examine changes in surface morphology and microbial growth. In addition, sub-samples of the same size were cut from untreated bag (pristine samples) at the beginning of the experiment and examined under SEM.

Additional mesocosms (18) were used to assess the individual and interactive effects of bag and seagrass vegetation on physical-chemical sediment parameters (temperature, pH and oxygen concentration). The experiment was a fully factorial design involving bag treatment (one bag piece was inserted directly into the sediment or no bag addition, control) and vegetation type (one rhizome of *C. nodosa* or *Z. noltei* was planted into the sediment or bare sediment) as fixed orthogonal factors (three replicates per each treatment combination). The temperature of sediment was measured every two weeks over the experimental period by carefully pushing the sensor of a

portable digital thermometer in the top 5 cm of sediment at the centre of each mesocosm. At the end of the study, pore-water samples were collected in the sediment by carefully inserting in the centre of each mesocosm (-5 cm) a 25-ml vacuumed sterile plastic syringe connected to a bottle. Immediately after collection, oxygen concentration in pore-water samples was measured using an electrode connected to a portable dissolved oxygen meter (OxyGuard Handy Polaris) while pH was measured using a portable pH/Redox meter (OxyGuard Handy Polaris). The electrodes were regularly calibrated with standard solutions and cleaned after each measurement.

2.3. Effect of bag on *Cymodocea nodosa* clonal growth and plant-plant interactions

In March 2016, 36 pots (25 cm diameter x 23 cm depth) filled with natural sediment as described above were equally distributed in two tanks and left undisturbed for 25 days to allow natural colonization. In April, collected plants were cut into apical rhizome fragments of similar size (7-8 cm long with a mean of 3.1 ± 0.3 SE shoots for *C. nodosa*, and 2-2.5 cm long with a mean of 2.6 ± 0.07 shoots for Z. *noltei*). Each fragment of *C. nodosa* was tagged in the youngest internode and randomly assigned to the treatments. The experiment had a mixed factorial design with tank as random factor (A or B), and bag (bag presence or no bag, control) and culture regime (plant alone, monoculture or mixed culture) as fixed orthogonal factors. In the bag treatment, one bag piece of the same size of that used for the experiment described above was previously horizontally inserted into the top 5 cm of sediment in the centre of the microcosm, while no bag piece was added in the control but the sediment was manipulated as that of treatment to avoid possible artefact effects. In the plant alone regime treatment, one individual of *C. nodosa* was planted into the sediment at the periphery of the mesocosm (i.e., in absence of interaction). In the monoculture treatment, two individuals of *C. nodosa* were planted in the mesocosm (i.e., in presence of intraspecific interaction) while in the mixed culture treatment one individual of *C. nodosa* was planted along with one of *Z. noltei* (i.e., in presence of interspecific interaction). There were three replicates for each treatment

combination in each tank. Plants were grown for their first growing season, and during this period the position of the mesocosms into each tank was randomly reassigned once a week to minimize position effects on plant performance. Plants were checked every week and new shoots recorded.

All plants were carefully harvested for measurements in September 2016, before leaves entered the senescence stage, and washed in seawater to remove the sediment. As the complete bag degradation was not achieved, plants were not destructively measured and then replanted into their original mesocosm for further study. Eight metrics of plant performance were used: total length of newly produced plagiotropic (horizontal) rhizomes, total number of new alive shoots and new rhizome branches, mean rhizome internode distance (spacer length), maximum leaf length, total number of new main roots and average total number of laterals on main roots. From these data, we calculated the following variables: shoot recruitment expressed as total number of newly produced shoots (both alive and dead) per plant relative to the initial number of shoots and shoot mortality estimated as number of dead shoots per plant relative to the total number of shoots (initial plus newly produced). The final number of alive shoots of *Z. noltei* was also determined. To examine how bag affects plant-plant interactions we calculated the index of relative interaction intensity (RII) (Armas et al., 2004) based on the total length of newly produced rhizome of the target plant (*C. nodosa*) for each treatment:

$$RII = (RL1 - RL0)/(RL1 + RL0) \quad \text{(eqn 1)}$$

where RL1 is the mean total length of the rhizome of *C. nodosa* at the same bag treatment level (bag addition or control) grown with a conspecific *i* or with a neighbour of *Z. noltei j* and RL0 is the is the total length of rhizome of *C. nodosa* grown alone. RII values indicate the outcome of interactions as negative (from −1 to 0, competition), neutral (equal to 0, no competition) and positive (from 0 to 1, facilitation).

2.4. Statistical analyses

One-way analysis of variance (ANOVA) was performed on the number of shoots of *C. nodosa* fragments randomly assigned to the different treatments at the start of the experiment to check for no difference in initial size. Three-way ANOVAs were separately performed to investigate the effect of tank, bag and culture regime, individually and in combination, on *C. nodosa* selected plant variables at the end of the experiment. Two-way ANOVAs were used to asses the effect of bag and vegetation type, individually and in combination, on sediment variables measured at the end of the study. Two-way ANOVAs were also individually performed to examine the effect of tank and bag treatment on RII values calculated for intra-specific and inter-specific interactions. To test for significant difference of RII values for intra and interspecific interaction from zero (no significant plant interaction) between treatments (presence or absence of the bag), *t* single mean tests were separately performed. Finally, two-way ANOVA was performed to test for differences in final number of shoots *Z. noltei* between tanks and treatments (bag presence and control). When the main test was significant, a post hoc pairwise comparison of the means (Student Newman SNK test, at $\alpha = 0.05$) was conducted to ascertain the a priori hypotheses. Before the analyses, data were checked for both normality (using Shapiro-Wilk test) and homogeneity of variance (using Cochran's test). Total length of newly produced rhizome and initial number of shoots of *C. nodosa* were log transformed while spacer length was square root (x +1) transformed before analysis. In all the figures the data are depicted in terms of the untransformed variable.

3.Results

3.1. Behaviour of bag buried in sediment and effect on sediment variables

Qualitative assessment of MB bag buoyancy showed that the bag remained at the seawater-air interface for 10-25 minutes before to sink and settle onto the bottom. Bag pieces and filter paper samples buried in bare sediment exhibited progressive changes in their coloration (from white to pink or yellow) over the experimental period due to initial surface biofilm formation. The deterioration process of filter paper started after two months of sediment incubation, and a considerable loss of mass (about 60% of initial weight) was recorded at the end of the experiment (Fig. 1). No macroscopic alteration of surface morphology was visible on the bag film during the first three months of sand burial. Initial signs of bag deterioration, such small perforations, appeared at the end of July (after 14 weeks of exposure), when sea water temperature reached values equal or higher than 23°C (Fig. 2). The integrity of the film slightly deteriorated thereafter and large bag pieces were still visible at the end of experiment (Fig. 1). The loss of mass was about 15% of initial weight (Fig. 2) and 25% relative to the cellulose reference sample. SEM images of pristine (untreated) bag film revealed uneven hilly surface with numerous bumps but no sign of alteration (Fig. 1a). After 24 weeks of burial, potential signs of initial degradation, such as cracks and holes, possibly due to dissolving or mineralisation of granules were observed on the surface of bag buried films (Fig. 1b).

At the beginning of the experiment, sediment pore-water oxygen, pH and temperature in the mesocosms assigned to the treatments were similar. At the end of the study, pH and oxygen concentration in sediment pore-water decreased in the presence of bag compared to controls (Fig. 3; $F_{1,12}$= 5.16, P = 0.04 for pH and $F_{1,12}$= 14.35, P = 0.002 for oxygen concentration). A significant interaction effect among treatments was found for sediment temperature (Fig. 2) which was lower in presence of bag than in controls but only in bare sediment ($F_{1,12}$ = 6.37, P = 0.01). Oxygen concentration in the presence of *Z. noltei* was about 2.5 mg/L higher compared to that in bare sediment ($F_{1,12}$= 15.88, P = 0.0004) while pH in the presence of *C. nodosa* decreased of approximately 0.2 pH units ($F_{1,12}$ = 14.86, P = 0.0006).

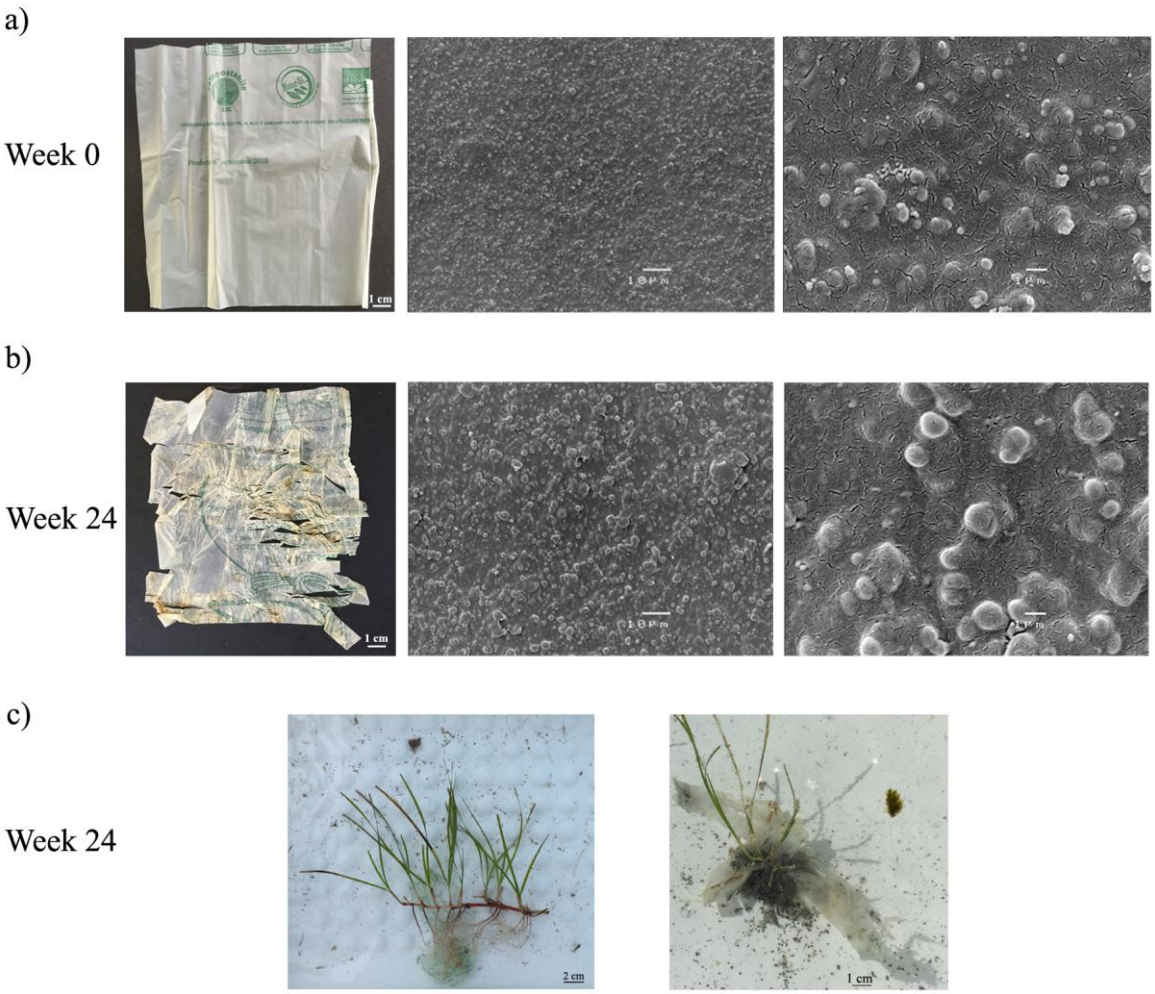


Figure 1. Photographs of MB bag samples and SEM images of the surface topography of bag films before (a) and after 24 weeks (b) of incubation in the marine sediment and photographs of MB bag fragments entangled into the root system of *C. nodosa* and *Z. noltei* clones after 24 weeks in mesocosm (c)

(Colour online only, 2-column fitting image)

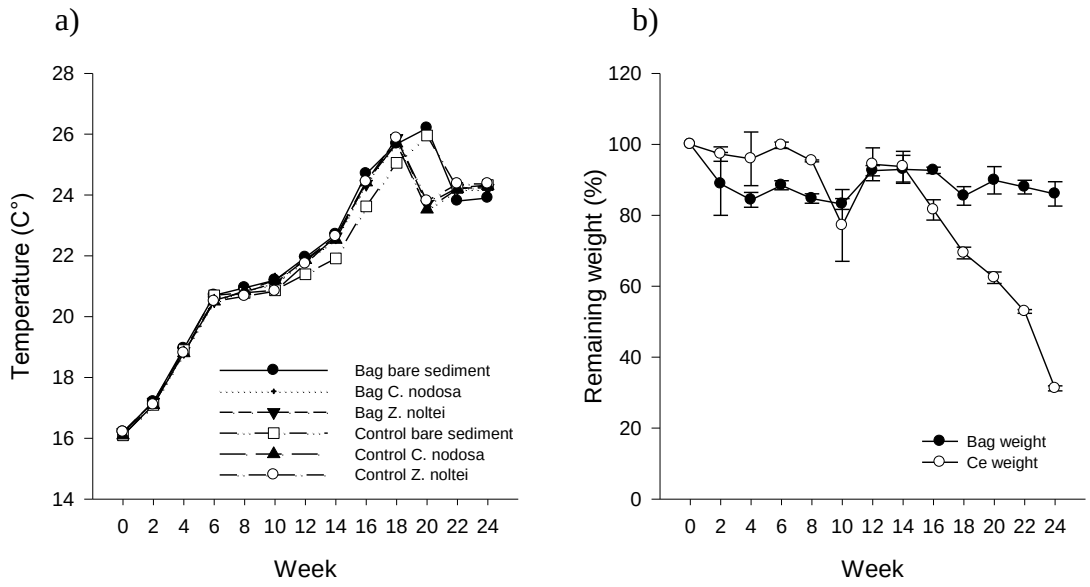


Figure 2. Temporal variation of sediment pore-water temperature recorded in the presence of bag or in the absence of bag (control) in bare substrate and in substrate colonized by *C. nodosa* and *Z. noltei* (a) and percent weight remaining of bag and cellulose samples respective to initial weight (b). Values are means ± SE, $n = 3$

(2-column fitting image)

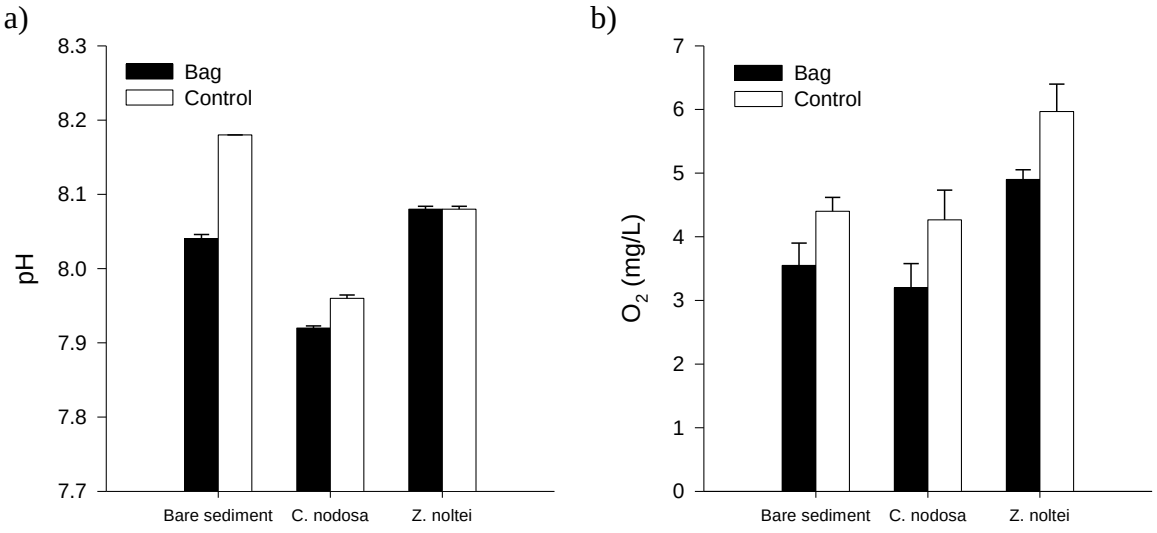


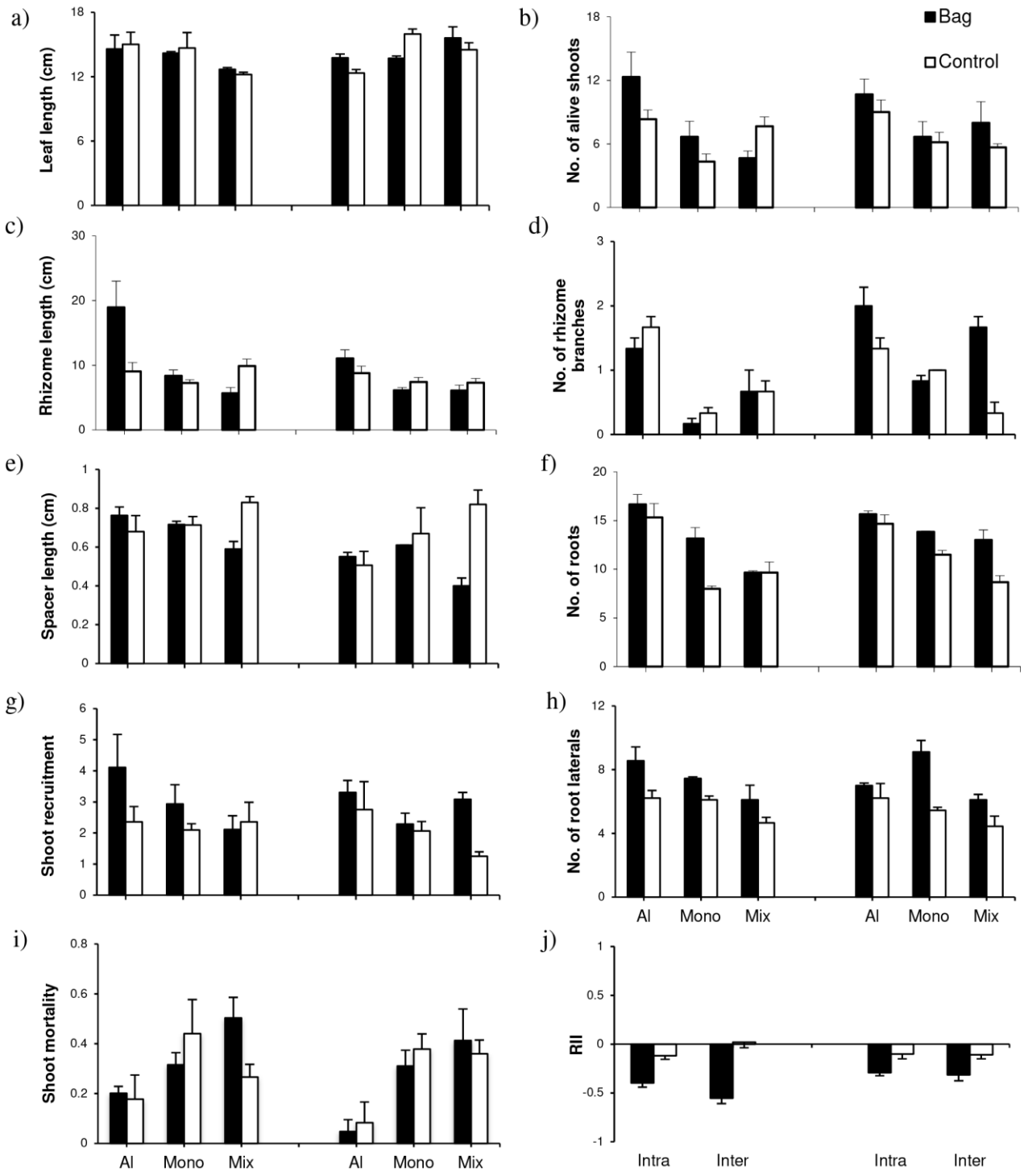
Figure 3. Sediment pore-water pH (a) and oxygen concentration (b) after 24 weeks of incubation in the presence of bag or in the absence of (control) in bare substrate and in substrate colonized by *C. nodosa* and *Z. noltei*. Values are means ± SE, $n = 3$

(2-column fitting image)

3.2. Effect of bag on *Cymodocea nodosa* clonal growth and plant-plant interactions

At the start of the experiment, the number of shoots present in *C. nodosa* fragments assigned to different treatments was similar ($F_{1,24}$ = 2.78, P = 0.34). All plants survived and propagated by clonal growth during the experiment. At the conclusion of the experiment, plants grown alone showed a higher number of alive shoots and rhizome branches, longer rhizome network and higher shoot recruitment but lower shoot mortality than those in monoculture or in mixed culture, regardless of bag treatment (Fig. 4; Table 1). Plants grown in the presence of bag exhibited higher shoot recruitment, total number of alive shoots and total number of roots and root laterals (Figure 4; Table 1), regardless of culture conditions. The formation of root laterals (9-16 per root) resulted in a tightly packed aggregation of roots near the bag (Fig. 1). There was a significant interaction between bag and culture regime for spacer length (Table 1); for plants grown in the presence of bag mean spacer length was shorter compared to that of control but only in mixed culture (Fig. 4). Relative interaction intensity index for total rhizome length ranged from 0.007 to - 0.58 (Fig. 4). There were significant differences between control and bag treatment for RII values of interspecific interaction ($F_{1,8}$ = 12.86, P = 0.007) and intraspecific interaction ($F_{1,8}$ = 8.43, P = 0.01). RII indices for plants grown in the absence of bag (Fig. 4) did not significantly differ from zero both for intraspecific interaction (t = -2.07, P = 0.09 data from two tanks pooled) and interspecific interaction (t = -0.61, P = 0.56 data from two tanks pooled), indicating no significant competitive effect from conspecifics as well as from the co-exiting species on *C. nodosa* growth In contrast, in the presence of the bag both the RII indices were negative (Fig. 4) and significantly different from zero (t = -6.5, P = 0.001 for intraspecific interaction and t = -4.7, P = 0.004 for interspecific interaction), indicating a competitive effect from conspecifics as well as from the co-existing species on *C. nodosa* growth. In the presence of bag, there was a reduction of more than 50% in rhizome length for a clone with a conspecific or with a clone of *Z. noltei* compared to control one grown in isolation. No significant effect of bag treatment or culture condition was detected for the

remaining *C. nodosa* variables (Fig. 4; Table 1) and no difference was found in the final number of new shoots (2-3 shoots per plant) present in *Z. noltei* plants grown in mixture both in the presence and in the absence of the bag ($F_{1,1} = 1.00$, $P = 0.50$).



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364 **Figure 4.** Plant-level effects of MB bag and culture regime on *C. nodosa* (a-i), and the effect of MB
365 bag on the relative index of interaction (RII) for intraspecific (Intra) and interspecific interactions
366 (Inter) (j). (Al: *C. nodosa* alone, Mono: monoculture, Mix: Mixed culture). Values are means ± SE,
367 $n = 3$ (2-column fitting image)

Table 1. Results of ANOVAs for morphological and growth variables, shoot recruitment and shoot mortality of *C. nodosa* plants subjected to different experimental treatments at the end of the experiment.. Results of SNK tests are reported. *Denotes post-hoc pooling. B^+ = bag added, Co = no bag added, Al = *C. nodosa* grown alone; Mon = *C. nodosa* grown in monoculture; Mix = *C. nodosa* grown in mixed culture.

		Leaf length (cm)		Rhizome length (cm)		Spacer length (cm)	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Tank = T	1	0.23	0.638	0.67	0.421	3.53	0.072
Bag = B	1	0.06	0.844	0.01	0.940	4.32	0.285
Culture = C	2	0.18	0.844	178.03	0.005	0.84	0.543
T x B	1	0.02	0.901	*		0.43	0.519
T x C	2	2.01	0.156	0.01	0.985	0.24	0.786
C x B	2	1.61	0.382	4.38	0.185	24.97	0.038
T x B x C	2	0.36	0.701	0.49	0.615	0.13	0.881
Residual	24						
SNK test				Al > Mon = Mix		Mix: B^+< Co	

		Shoot recruitment		Shoot mortality		No. alive shoots	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Tank = T	1	0.32	0.578	1.31	0.262	0.02	0.883
Bag = B	1	5.57	0.026	0.21	0.724	4.31	0.047
Culture = C	2	23.45	0.040	13.05	0.001	13.42	0.001
T x B	1	*		0.47	0.501	*	
T x C	2	0.14	0.867	*		*	
C x B	2	0.17	0.858	4.10	0.196	0.70	0.586
T x B x C	2	2.72	0.085	0.58	0.565	2.35	0.115
Residual	24						
SNK test		Al > Mon = Mix B^+>Co		Al < Mon = Mix		Al > Mon = Mix B^+>Co	

		No. rhizome branches		No. roots		No. root laterals	
	df	*F*	*P*	*F*	*P*	*F*	*P*
Tank = T	1	2.94	0.099	0.73	0.400	0.04	0.843
Bag = B	1	3.36	0.317	6.28	0.019	8.25	0.008
Culture = C	2	57.00	0.017	13.79	0.067	9.22	0.097
T x B	1	1.06	0.313	*		*	
T x C	2	0.01	0.992	0.83	0.445	0.32	0.728
C x B	2	6.33	0.136	0.51	0.661	0.31	0.761
T x B x C	2	0.15	0.857	1.24	0.306	0.74	0.485
Residual	24						
SNK test		Al > Mon = Mix		B^+>Co		B^+>Co	

4.Discussion

The present study is the first attempt to investigate the possible effect of biodegradable plastics on the development of natural marine plant communities, to our knowledge. This study also provides insights into the potential behaviour of biodegradable bags buried in marine sediments and in particular in shallow coastal bottoms colonized by seagrasses.

The negligible degradation of the biodegradable bag recorded after six months of exposure to marine sediments suggests that the soft bottom of shallow coastal areas of temperate seas could act as temporary sink for bioplastics (Nauendorf et al., 2016). Since the degradation of cellulose occurred at high rate, the poor bag deterioration might not be attributed to the lack of a favourable microbial community to biodegradation in the sediment. Instead, lack of UV-radiation and mechanical abrasion by wave in sediments might have played an important role. The extent of degradation observed here was, however, greater than that reported for other types of bags made from corn starch in previous field studies (1.5% of weight loss after three months of bag immersion in the sea, Accinelli et al., 2012) and in laboratory tests that simulated pelagic conditions (no fragmentation after 24 months of bag immersion in the sea, Tosin et al., 2012), but lower as compared to that recorded in other laboratory experiments that simulated the sublittoral zone (full degradation after about 9 months of bag exposure in seawater, Tosin et al., 2012) and at the seawater surface (full degradation after about 4 months of bag exposure, O'Brine and Thompson, 2010). The different degradation rates may clearly reflect differences in bag polymer composition, experimental or environmental conditions and duration of the experiment. Our findings support the hypothesis that degradation of biodegradable bags in marine sediments is a complex process and needs to be better investigated both under laboratory conditions and in the field.

In coastal sediments oxygen penetration depth is very limited varying from less than 1 millimetre in muddy sediments to few centimetres in sandy sediments (Glud, 2008). The sediment used in this study was initially normoxic (>7 mg/L oxygen), and in the presence of bag sediment pore-water

443 oxygen concentration declined to values near to that setting sediment hypoxic conditions (2 mg/L
444 oxygen, Diaz and Rosenberg, 1995). This decrease could be ascribed to the inhibition of gas change
445 from seawater and sediment and/or reduced diffusion of oxygen due to the physical presence of bag
446 pieces over the whole experimental period. A similar sealing sediment surface effect has been
447 hypothesized by Green et al. (2015) for other types of compostable bags manufactured from corn
448 starch. However, oxygen consumption by biofilm formation and increased production of CO_2 due to
449 initial degradation of starch in the film might not be excluded. The lower temperature recorded in
450 bare sediments in the presence of bag compared to controls also suggests that this material could act
451 as a thermal insulator, probably due to lower thermal conductivity than sediment as previously
452 reported for conventional plastics in beach sediments (Carson et al., 2011). Both *C. nodosa* and *Z.*
453 *noltei* influenced sediment variables. *C. nodosa* acidified the sediment, suggesting enhanced
454 microbial activity in sediments stimulated by decomposition of organic matter from detritus
455 produced by the plant itself and exudation of dissolved organic carbon from roots (Perry and
456 Dennison, 1999; Barròn et al., 2004; Fraser et al., 2016). Instead, *Z. noltei* ameliorated sediment
457 conditions, increasing oxygen concentration possibly due to the release of oxygen by its roots into
458 the sediment. The differential ability of these species to modify sediment conditions is in agreement
459 with previous studies which indicate that *C. nodosa* excretes more organic exudates from its roots
460 but oxidizes sediments much less than *Z. noltei* (Isaksen and Finster, 1996). For both the species,
461 plant metabolic activity appeared to counteract the negative effect of bag on sediment temperature.
462 Seagrasses generally grow in reduced sediments (Borum et al., 2006), and the release of oxygen by
463 roots is recognized to be an adaptive mechanism to enable plants to supply sufficient O_2 to their
464 belowground tissue to sustain aerobic metabolism, as well as to provide protection against invasion
465 of reduced phytotoxic compounds from the surrounding sediment (Armstrong, 1979; Borum et al.,
466 2005; Borum et al., 2006). Prolonged period of hypoxia or anoxia, however, may causes sudden
467 seagrass die-offs events (Terrados et al., 1999; Borum et al., 2005; Brodersen et al., 2014;
468 Brodersen et al., 2015). The root system of most seagrasses, including *C. nodosa*, is simple and

469 comprised of a root axis and 3-4 primary laterals (Marbà and Duarte, 2001), consistent with a
470 "herringbone" root architecture typical of species adapted to grow under low nutrient conditions
471 (Fitter et al., 1991). However, root morphology may vary in some species as a response to
472 environmental conditions, such as availability of oxygen and nutrients, and sediment nature and
473 texture (Kiswara et al., 2009; Hovey et al., 2012; Balestri et al., 2015). Since the amount of oxygen
474 transported and the potential for oxygen release to the sediment by roots are determined in part by
475 the total amount of active photosynthetic tissue (Smith et al., 1984), the proliferation of laterals on
476 roots along with increasing shoot production observed in *C. nodosa* in the presence of bag could be
477 a strategy to ensure enough flow of oxygen from the photosyntethic organs to the root so to
478 maintain a supply to roots and oxidise sulphide (or other phytotoxics) under declining oxygen
479 sediment concentrations. However, our results also showed that rhizome length did not consistently
480 increase in the presence of bag leading to more compact clones compared to controls grown in
481 isolation. In addition, both intra-specific and inter-specific interactions shifted from neutral to
482 competitive, and spacer elongation in clones grown with a neighbour of *Z. noltei* decreased
483 compared to control leading to a more phalanx growth form. These findings suggest that a *C.*
484 *nodosa* clone growing in the presence of neighbours may not readily escape from bag induced
485 deteriorated sediment conditions because of increased competition for available space and below-
486 ground resources. Modelling studies indicate that guerrilla species such as *C. nodosa* are specialized
487 in the occupation of free space and the long spacers between ramets they produce allow infiltration
488 in the surrounding vegetation (i.e. promote interspecific contacts as well as interaction with other
489 species allowing their coexistence). In contrast, phalanx species such as *Z. noltei* are specialized in
490 the consolidation of occupied spaces and the short spacers they produce impede the establishment
491 of other species (i.e. minimize interspecific contacts resisting competitors, Lovett Doust, 1981;
492 Murrell et al., 2001; Murrell et al., 2002; Benot et al., 2013). Alterations of competitive intensity
493 and clonal architecture in nature could translate to functional levels, affecting the spatial distribution
494 of ramets in the environment and relationships with other seagrass species. For example, the

presence of bag in monospecific stands could indirectly reduce the chance of interconnection among recruits of *C. nodosa* and hamper sexual reproduction promoting spatial segregation of male and female clones with possible local reduction of genetic diversity. Genetic diversity enhances the chance of long term persistence of seagrass populations to disturbances (Randall Hughes and Stachowicz, 2004). On the other hand, in mixed stands the bag could favour a more pronounced segregation of species in discrete competitively determined patches in which structural complexity is mediated locally by the dominant foundation species. Since the duration of the experiment was not long enough to achieve complete biodegradation of the bag, further longer term studies should be conducted to determine the time required for a bag to totally degrade in marine sediments and assess how the final products of the biodegradation process affect plant development and physiology.

5.Conclusions

The adoption of biodegradable bags is clearly a viable alternative solution to reduce the environmental impact plastics at global scale. However, the results of this study demonstrate that biodegradable MB bags degrade slowly when buried in marine sediments, and can be breakdown into fragments potentially harmful for a number of organisms living within in or above sediment similarly to conventional plastics. The deposition of bags on the sediment may also alter below and above seagrass compartments and more importantly increase the intensity of both plant intra- and interspecific competition. Given the augmented production of bioplastic bags, an increasing input of these bags into the marine environment is expected in next decades. Therefore, future studies should investigate more in detail the bag degradation process in sediments colonized by seagrasses in order to understand the possible consequences for associated ecosystems. and to develop new, standard tests for biodegradation of plastics in marine habitats.

Because of the current uncertainty concerning the final fate of biodegradable bags and its effect, not only on marine organisms but also on foundation marine plant species, we recommend extreme caution in the use of these bags to prevent dispersal and accumulation in the ocean. We also stress the need to quantify the presence of conventional plastics currently deposited within or near seagrasses beds as well as to assess their effect on seagrass ecosystems and functioning to better estimate the global environmental impact. of plastics.

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References

Accinelli, C., Abbas, H.K., 2011. New perspectives for the application of bioplastic materials in the biocontrol of *Aspergillus flavus* in corn. Toxin Rev. 30, 71–78, DOI:10.3109/15569543.2011.591517.

Accinelli, C., Saccà, M.L., Mencarelli, M., Vicari, A., 2012. Deterioration of bioplastic carrier bags in the environment and assessment of a new recycling alternative. Chemosphere 89, 136-143, DOI:10.1016/j.chemosphere.2012.05.028.

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62, 1596-1605, DOI: 10.1016/j.marpolbul.2011.05.030.

Andrady, A.L., 2015. Persistence of plastic litter in the oceans, in: Bergmann, M., Gutow, L., Klages, M., *Marine Anthropogenic Litter*. Eds., Springer International Publishing: pp. 57-72.

547 Armas, C., Ordiales, R., Pugnaire, F.I., 2004. Measuring plant interactions: a new comparative
548 index. Ecology, 85, 2682-2686, DOI: 10.1890/03-0650.
549 Armstrong, W.D., 1979. Aeration in higher plants, in: H.W. Woolhouse, *Advances in Botanical*
550 *Research*, Eds.; Academic Press: London, pp. 225-332.
551 ASTM D7081-05, 2005. Standard Specification for Non-Floating Biodegradable Plastics in the
552 Marine Environment (Withdrawn 2014), ASTM International, West Conshohocken, PA, 2,
553 www.astm.org.
554 Balestri, E., Lardicci, C., 2012. Nursery-propagated plants from seed: a tool to improve the
555 effectiveness and sustainability of seagrass restoration. J. Appl. Ecol. 49, 1426-1435,
556 DOI: 10.1111/j.1365-2664.2012.02197.x.
557 Balestri, E., de Battisti, D., Vallerini, F., Lardicci, C., 2015. First evidence of root morphological
558 and architectural variations in young *Posidonia oceanica* plants colonizing different
559 substrate typologies. Estuarine, Coastal Shelf Sci. 154, 205-213,
560 DOI:10.1016/j.ecss.2015.01.002.
561 Barròn, C., Marbà, N., Terrados, J., Kennedy, H., Duarte, C.M., 2004. Community metabolism and
562 carbon budget along a gradient of seagrass (*Cymodocea nodosa*) colonization. Limnol.
563 Oceanogr. 49, 1642–1651, DOI: 10.4319/lo.2004.49.5.1642.
564 Benot, M.L., Bittebiere, A.K., Ernoult, A., Clèment, B., Mony, C., 2013. Fine-scale spatial patterns
565 in grassland communities depend on species clonal dispersal ability and interactions with
566 neighbours. J. Ecol. 101, 626–636, DOI: 10.1111/1365-2745.12066.
567 Borum, J., Pedersen, O., Greve, T.M., Frankovich, T.A., Zieman, J.C., Fourqurean, J.W., Madden,
568 C.J., 2005. The potential role of plant oxygen and sulphide dynamics in die-off events of
569 the tropical seagrass, *Thalassia testudinum*. J. Ecol. 93, 148–158, DOI: 10.1111/j.1365-
570 2745.2004.00943.x.

571 Borum, J., Sand-Jensen, K., Binzer, T., Pedersen, O., Greve, T.M., 2006. Oxygen movement in
572 seagrasses, in: Larkum, A., Orth, R.J., Duarte, C.M., *Seagrasses: Biology, Ecology and*
573 *Conservation*. Eds.; Springer, pp 255-270.
574 Brodersen, K.E., Nielsen, D.A., Ralph, P.J., Kühl, M., 2014. A split flow chamber with artificial
575 sediment to examine the below-ground microenvironment of aquatic macrophytes. Mar.
576 Biol. 161, 2921-2930, DOI: 10.1007/s00227-014-2542-3.
577 Brodersen, K.E., Nielsen, D.A., Ralph, P.J., Kühl, M., 2015. Oxic microshield and local pH
578 enhancement protects *Zostera muelleri* from sediment derived hydrogen sulphide. New
579 Phytol. 205, 1264–1276, DOI: 10.1111/nph.13124.
580 Carson, H.S., Colbert, S.L., Kaylor, M.J., McDermid, K.J., 2011. Small plastic debris changes
581 water movement and heat transfer through beach sediments. Mar. Pollut. Bull. 62, 1708-
582 1713, DOI: 10.1016/j.marpolbul.2011.05.032.
583 Connell, J.H., 1983. On the prevalence and relative importance of interspecific competition:
584 Evidence from field experiments. The American Naturalist 122, 661, DOI:
585 10.1086/284165.
586 Convery, F., McDonnell, S., Ferreira, S., 2007. The most popular tax in Europe? Lessons from the
587 Irishplastic bags levy. Environ Resource Econ. 38, 1–11, DOI: 10.1007/s10640-006-9059-
588 2.
589 Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S.,
590 O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., van den Belt, M., 1997. The value of
591 the world's ecosystem services and natural capital. Nature 387, 253-260, DOI:
592 10.1038/387253a0.
593 Cullen-Unsworth, L., Unsworth, R., 2013. Seagrass meadows, ecosystem services, and
594 sustainability. Environ. Sci. Policy 55, 14-28, DOI: 10.1080/00139157.2013.785864.
595 Derraik, J.G.B., 2002. The pollution of the marine environment by plastic debris: a review. Mar.
596 Pollut. Bull. 44 , 842-852, DOI: 10.1016/S0025-326X(02)00220-5.

597 Diaz, R.J., Rosenberg, R., 1995. Marine benthic hypoxia: a review of its ecological effects and the
598 behavioral responses of benthic macrofauna. *Oceanogr.* Mar. Biol. 33, 245-303.
599 Duarte, C.M., 2000. Marine biodiversity and ecosystem services: an elusive link. J. Exp. Mar. Biol.
600 Ecol. 250, 117-131, DOI: 10.1016/S0022-0981(00)00194-5.
601 Duarte, C.M., Forqurean, J.W., Krause-Jensen, D., Olesen, B., 2006. Dynamics of seagrass stability
602 and change, in: Larkum, A., Orth, R.J., Duarte, C. M., *Seagrasses: Biology, Ecology and*
603 *Conservation.* Eds.; Springer, pp 271-294.
604 EN 13432: 2000. "Packaging - Requirements for packaging recoverable through composting and
605 biodegradation - Test scheme and evaluation criteria for the final acceptance of
606 packaging". European standard.
607 Fitter, A.H., Stickland, T.R., Harvey, M.L., Wilson, G.W., 1991. Architectural analysis of plant root
608 systems 1. Architectural correlates of exploitation efficiency. New Phytol. 118, 375-382,
609 DOI: 10.1111/j.1469-8137.1991.tb00018.x.
610 Fraser, M.W., Statton, J., Hovey, R.H., Laverock, B., Kendrick, G.A., 2016. Seagrass derived
611 organic matter influences biogeochemistry, microbial communities, and seedling biomass
612 partitioning in seagrass sediments. Plant Soil, 400, 133-146, DOI: 10.1007/s11104-015-
613 2721-0.
614 Gacia, E., Duarte, C.M., Middelburg, J.J., 2002. Carbon and nutrient deposition in a Mediterranean
615 seagrass (*Posidonia oceanica*) meadow. Limnol. Oceanogr. 47, 23-32, DOI:
616 10.4319/lo.2002.47.1.0023.
617 Galgani, F., Burgeot, T., Bocquènè, G., Vincent, F., Leautè, J.P., Labastie, J., Forest, A., Guichet,
618 R., 1995. Distribution and abundance of debris on the continental shelf of the bay of
619 Biscay and in Seine bay. Mar. Pollut. Bull. 30, 58-62, DOI: 10.1016/0025-326X(94)00101-
620 E.
621 Glud, R.N., 2008. Oxygen dynamics of marine sediments. Mar. Biol. Res. 4, 243-289, DOI:
622 10.1080/17451000801888726.

623 Goldberg, D.E., Barton, A.M., 1992. Patterns and consequences of interspecific competition in
624 natural communities: A review of field experiments with plants. The American Naturalist
625 139, 771-801, DOI: 10.1086/285357.
626 Green, S.D., Boots, B., Blockley, D.J., Rocha, C., Thompson, R., 2015. Impacts of discarded plastic
627 bags on marine assemblages and ecosystem functioning. Environ. Sci. Technol. 49,
628 5380−5389, DOI: 10.1021/acs.est.5b00277.
629 Gross, M., 2013. Plastic waste is all at sea. Curr. Biol. 23, 135-137, DOI:
630 10.1016/j.cub.2013.01.070.
631 Hovey, R.K., Cambridge, M.L., Kendrick, G.A., 2012. Season and sediment nutrient additions
632 affect root architecture in the temperate seagrasses *Posidonia australis* and *P. sinuosa.*
633 Mar. Ecol. Prog. Ser. 446, 23–30, DOI: 10.3354/meps09483.
634 Isaksen, M.F., Finster, K., 1996. Sulphate reduction in the root zone of the seagrass *Zostera noltii*
635 on the intertidal flats of a coastal lagoon (Arcachon, France). Mar. Ecol. Prog. Ser. 137,
636 187-194, DOI: 10.3354/meps137187.
637 Kiswara, W., Behnke, N., Van Avesaath, P., Huiskes, A.H.L., Erftemeijer, P.L.A., Bouma, T.J.,
638 2009. Root architecture of six tropical seagrass species, growing in three contrasting
639 habitats in Indonesian waters. Aquat. Bot. 90, 235–245, DOI:
640 10.1016/j.aquabot.2008.10.005.
641 Kraemer, G.P., Mazzella, L., 1999. Nitrogen acquisition, storage, and use by the co-occurring
642 Mediterranean seagrasses *Cymodocea nodosa* and *Zostera noltii*. Mar. Ecol. Prog. Ser.
643 183, 95-103, DOI:10.3354/meps183095.
644 Lovett Doust, L., 1981. Population dynamics and local specialization in a clonal perennial
645 (*Ranunculus Repens*): II. The Dynamics of leaves, and a reciprocal transplant-replant
646 experiment. J. Ecol. 69, 757-768, DOI: 10.2307/2259634.

Marbà, N., Cebrian, J., Enriquez, S., Duarte, C.M., 1996. Growth patterns of Western Mediterranean seagrasses: species-specific responses to seasonal forcing. Mar. Ecol. Prog. Ser. 133, 203-215, DOI: 10.3354/meps133203.

Marbà, N., Duarte, C.M., 2001. Growth and sediment space occupation by seagrass *Cymodocea nodosa* roots. Mar. Ecol. Prog. Ser. 224, 291-298, DOI: 10.3354/meps224291.

Murrell, D.J., Purves, D.W., Law, R., 2001. Uniting pattern and process in plant ecology. Trends Ecol. Evol. 16, 529-530, DOI: 10.1016/S0169-5347(01)02292-3.

Murrell, D.J., Travis, J.M.J., Dytham, C., 2002. The evolution of dispersal distance in spatially-structured populations. Oikos 97, 229–236, DOI:10.1034/j.1600-0706.2002.970209.x.

Nauendorf, A., Krause, S., Bigalke, N. K., Gorb, E.V., Gorb, S.N., Haeckel, M., Wahl, M., Treude, T., 2016. Microbial colonization and degradation of polyethylene and biodegradable plastic bags in temperate fine-grained organic-rich marine sediments. Mar. Pollut. Bull. 103, 168-178, DOI: 10.1016/j.marpolbul.2015.12.024.

O'Brine, T., Thompson, R.C., 2010. Degradation of plastic carrier bags in the marine environment. Mar. Pollut. Bull. 60, 2279-2283, DOI: 10.1016/j.marpolbul.2010.08.005.

Orth, R.J., Harwell, M.C., Bailey, E.M., Bartholomew, A., Jawad, J.T., Lombana, A.V., Moore, K.A., Rhode, J.M., Woods, H.E., 2000. A review of issues in seagrass seed dormancy and germination: implications for conservation and restoration. Mar. Ecol. Prog. Ser. 200, 277-288, DOI: 10.3354/meps200277.

Perry, C.J., Dennison, W.C., 1999. Microbial nutrient cycling in seagrass sediments. AGSO J. Aust. Geol. Geophys. 17, 227-231.

Randall Hughes, A., Stachowicz, J.J., 2004. Genetic diversity enhances the resistance of a seagrass ecosystem to disturbance. PNAS 101, DOI: 0.1073/pnas.0402642101.

Rose, C.D., Dawes, C.J., 1999. Effects of community structure on the seagrass *Thalassia testudinum*. Mar. Ecol. Prog. Ser. 184, 83-95, DOI: 10.3354/meps184083.

672 Ruggiero, M.V., Capone, S., Pirozzi, P., Reusch, T.B.H., Procaccini, G., 2005. Mating system and
673 clonal architecture: a comparative study in two marine angiosperms. Evol. Ecol. 19, 487-
674 499, DOI: 10.1007/s10682-005-0292-x.
675 Short, F.T., Carruthers, T., Dennison, W., Waycott, M., 2007. Global seagrass distribution and
676 diversity: A bioregional model. J. Exp. Mar. Biol. Ecol. 350, 3-20, DOI:
677 10.1016/j.jembe.2007.06.012.
678 Short, F.T., Polidoro, B., Livingstone, S.R., Carpenter, K.E., Bandeira, S., Bujang, J.S., Calumpong,
679 H.P., Carruthers, T.J.B., Coles, R.G., Dennison, W.C., Erftemeijer, P.L.A., Fortes, M.D.,
680 Freeman, A.S., Jagtap, T.G., Kamal, A.H.M., Kendrick, G.A., Kenworthy, W.J., La Nafie,
681 Y.A., Nasution, I.M., Orth, R.J., Prathep, A., Sanciangco, J.C., van Tussenbroek, B.,
682 Vergara, S.G., Waycott, M., Zieman, J.C., 2011. Extinction risk assessment of the world's
683 seagrass species. Biol. Conserv. 144, 1961–1971, DOI: 10.1016/j.biocon.2011.04.010.
684 Smith, R.D., Dennison, W.C., Alberte, R.S., 1984. Role of seagrass photosynthesis in root aerobic
685 processes. Plant Physiol. 74, 1055–1058.
686 Terrados, J., Ros, J.D., 1992. Growth and primary production of *Cymodocea nodosa* (Ucria)
687 Ascherson in a Mediterranean coastal lagoon: the Mar Menor (SE Spain). Aquat. Bot. 43,
688 63-74, DOI: 10.1016/0304-3770(92)90014-A.
689 Terrados, J., Duarte, C.M., Kamp-Nielsen, L., Agawin, N.S.R., Gacia, E., Lacap, D., Fortes, M.D.,
690 Borum, J., Lubanski, M., Greve, T., 1999. Are seagrass growth and survival constrained by
691 the reducing conditions of the sediment? Aquat. Bot. 65, 175-197, DOI: 10.1016S0304-
692 3770(99)00039-X.
693 Tilman, D., Mattson, M., Langer, S., 1981. Competition and nutrient kinetics along a temperature
694 gradient: An experimental test of a mechanistic approach to niche theory. Limnol.
695 Oceanogr. 26, 1020-1033, DOI: 10.4319/lo.1981.26.6.1020.

696 Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W.G., McGonigle,
697 D., Russell, A.E., 2004. Lost at sea: Where is all the plastic? Science 304, 838;
698 DOI:10.1126/science.1094559.
699 Tosin, M., Weber, M., Siotto, M., Lott, C., Degli Innocenti, F., 2012. Laboratory test methods to
700 determine the degradation of plastics in marine environmental conditions. Front.
701 Microbiol. 3, DOI:10.3389%2Ffmicb.2012.00225.
702 UNEP, 2015. Biodegradable Plastics and Marine Litter. Misconceptions, concerns and impacts
703 on marine environments. United Nations Environment Programme (UNEP), Nairobi.

Table

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Table 1. Results of ANOVAs for morphological and growth variables, shoot recruitment and shoot mortality of *C. nodosa* plants subjected to different experimental treatments at the end of the experiment. Results of SNK tests are reported. *Denotes post-hoc pooling. B^+ = bag added, Co = no bag added, Al = *C. nodosa* grown alone; Mon = *C. nodosa* grown in monoculture; Mix = *C. nodosa* grown in mixed culture.

		Leaf length (cm)		Rhizome length (cm)		Spacer length (cm)	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Tank = T	1	0.23	0.638	0.67	0.421	3.53	0.072
Bag = B	1	0.06	0.844	0.01	0.940	4.32	0.285
Culture = C	2	0.18	0.844	178.03	0.005	0.84	0.543
T x B	1	0.02	0.901	*		0.43	0.519
T x C	2	2.01	0.156	0.01	0.985	0.24	0.786
C x B	2	1.61	0.382	4.38	0.185	24.97	0.038
T x B x C	2	0.36	0.701	0.49	0.615	0.13	0.881
Residual	24						
SNK test				Al > Mon = Mix		Mix: B^+< Co	

		Shoot recruitment		Shoot mortality		No. alive shoots	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Tank = T	1	0.32	0.578	1.31	0.262	0.02	0.883
Bag = B	1	5.57	0.026	0.21	0.724	4.31	0.047
Culture = C	2	23.45	0.040	13.05	0.001	13.42	0.001
T x B	1	*		0.47	0.501	*	
T x C	2	0.14	0.867	*		*	
C x B	2	0.17	0.858	4.10	0.196	0.70	0.586
T x B x C	2	2.72	0.085	0.58	0.565	2.35	0.115
Residual	24						
SNK test		Al > Mon = Mix B^+>Co		Al < Mon = Mix		Al > Mon = Mix B^+>Co	

		No. rhizome branches		No. roots		No. root laterals	
	df	*F*	*P*	*F*	*P*	*F*	*P*
Tank = T	1	2.94	0.099	0.73	0.400	0.04	0.843
Bag = B	1	3.36	0.317	6.28	0.019	8.25	0.008
Culture = C	2	57.00	0.017	13.79	0.067	9.22	0.097
T x B	1	1.06	0.313	*		*	
T x C	2	0.01	0.992	0.83	0.445	0.32	0.728
C x B	2	6.33	0.136	0.51	0.661	0.31	0.761
T x B x C	2	0.15	0.857	1.24	0.306	0.74	0.485
Residual	24						
SNK test		Al > Mon = Mix		B^+>Co		B^+>Co	

Figure

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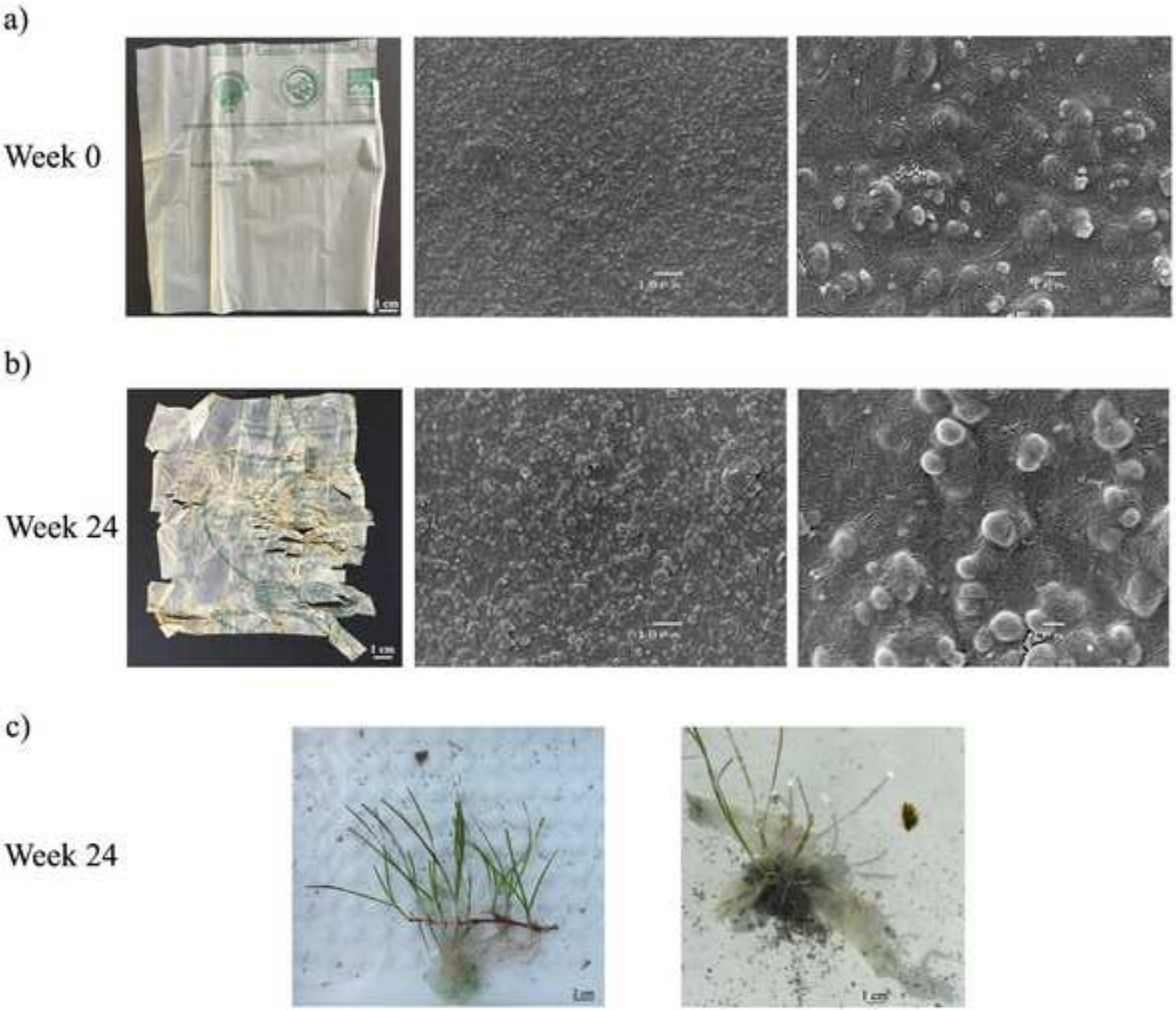

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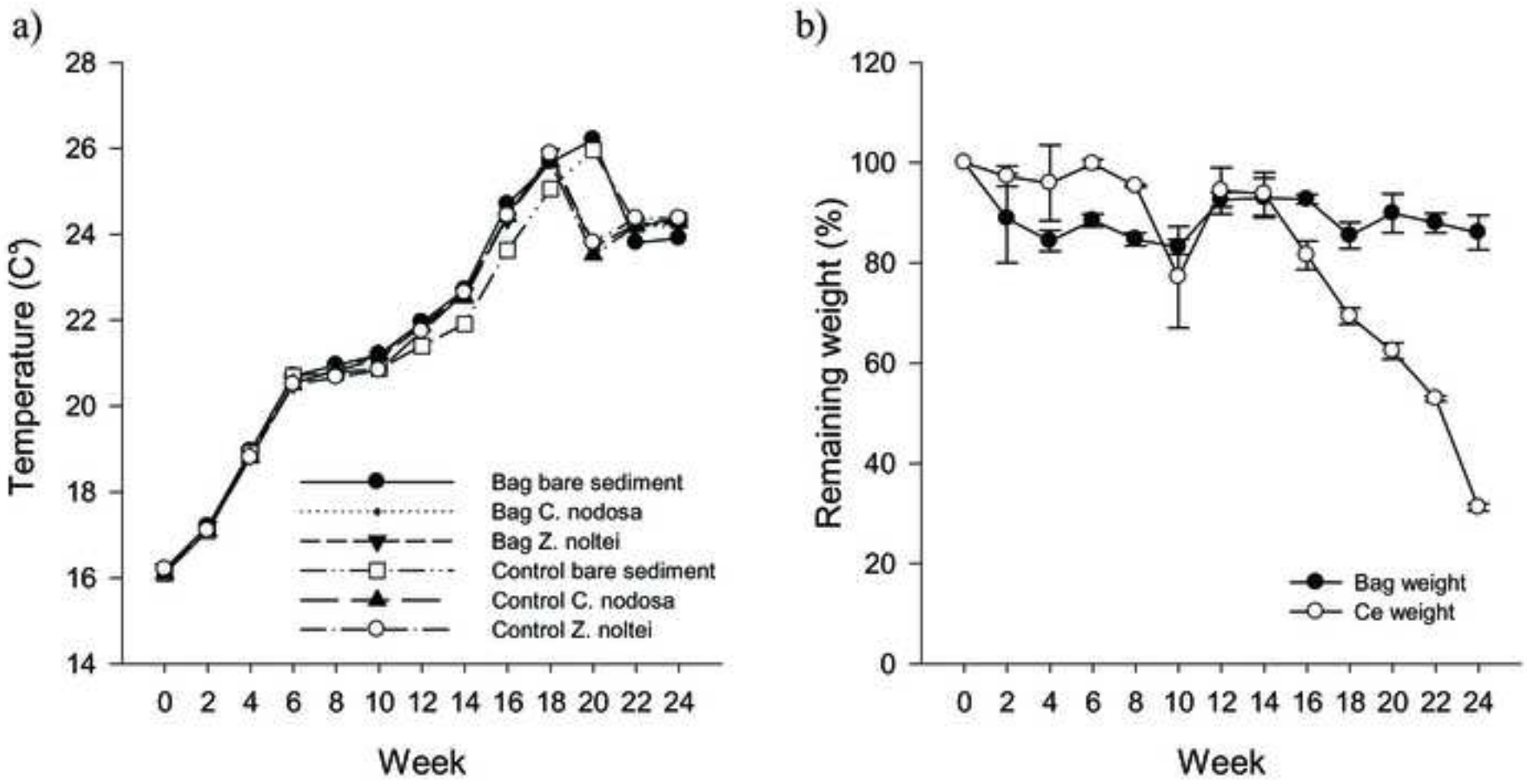

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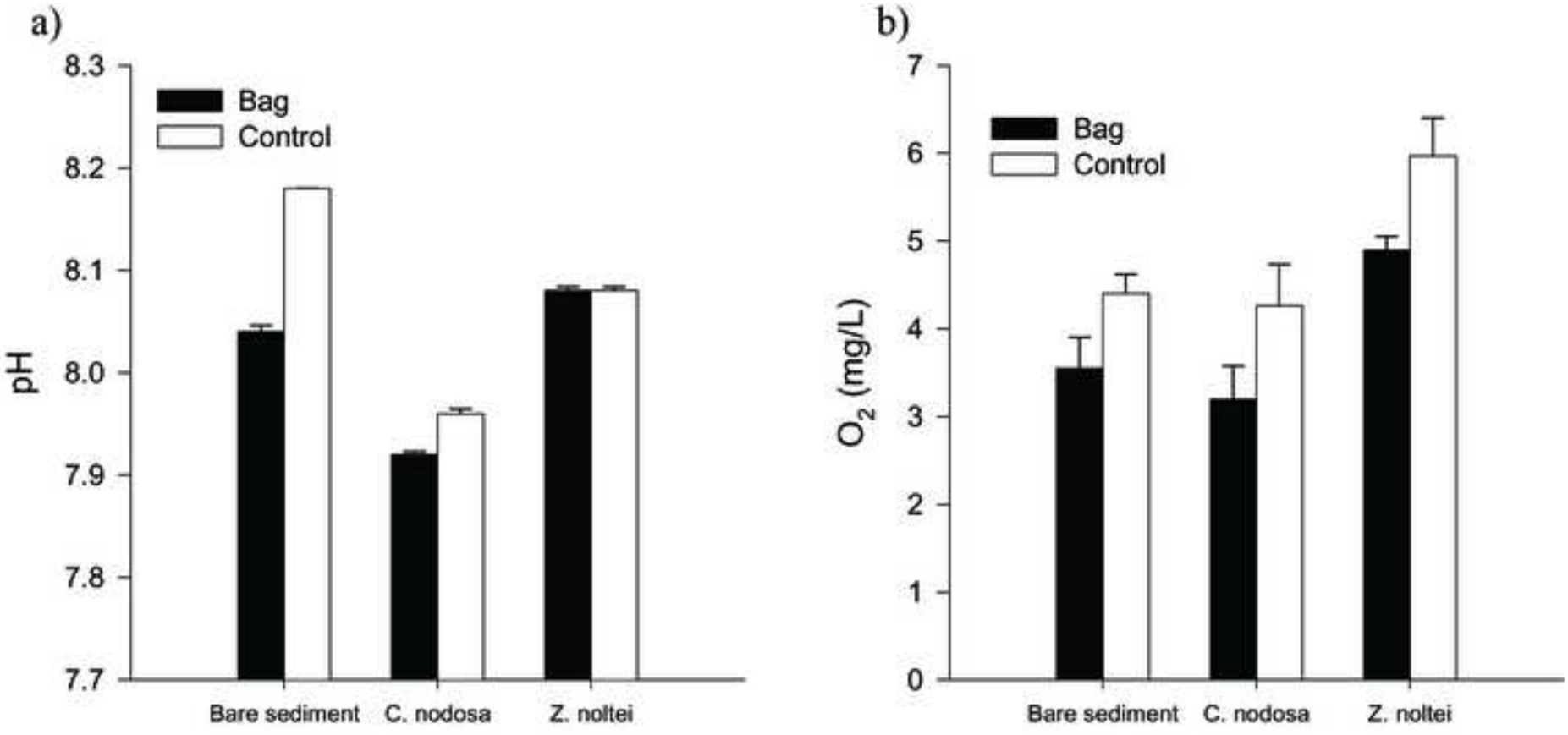

Figure

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