OCEAN ACIDIFICATION AND HYPOXIA ALTER ORGANIC CARBON FLUXES IN MARINE SOFT SEDIMENTS

Running title: Effects of multiple stressors on carbon fate

Chiara Ravaglioli[1*], Fabio Bulleri[1], Saskia Rühl[2,3], Sophie J. McCoy[4], Helen Findlay[2], Steven Widdicombe[2] and Ana M. Queirós[2]

[1]Dipartimento di Biologia, Università di Pisa, CoNISMa, Via Derna, 1, 56126 Pisa, Italia

chiara.ravaglioli@for.unipi.it; fabio.bulleri@unipi.it

[2]Plymouth Marine Laboratory, PL1 3DH Plymouth, UK

hefi@pml.ac.uk; swi@pml.ac.uk; anqu@pml.ac.uk

[3]Southampton University, University Road, Southampton, SO17 1BJ

sru@pml.ac.uk;

[4]Dept. of Biological Sciences, Florida State University, Tallahassee, FL 32306-4295 USA

sophiemccoy@gmail.com

*Corresponding author

Tel: +390502211415

Fax +390502211410

Email: chiara.ravaglioli@for.unipi.it

Abstract

Anthropogenic stressors can alter the structure and functioning of infaunal communities, which are key drivers of the carbon cycle in marine soft sediments. Nonetheless, the compounded effects of anthropogenic stressors on carbon fluxes in soft benthic systems remain largely unknown. Here, we investigated the cumulative effects of ocean acidification and hypoxia on the organic carbon fate in marine sediments, through a mesocosm experiment. Isotopically-labelled macroalgal detritus (^{13}C) was used as a tracer to assess carbon incorporation in faunal tissue and in sediments under different experimental conditions. In addition, labelled macroalgae (^{13}C), previously exposed to elevated CO_2, were also used to assess the organic carbon uptake by fauna and sediments, when both sources and consumers were exposed to elevated CO_2. At elevated CO_2, infauna increased the uptake of carbon, likely as compensatory response to the higher energetic costs faced under adverse environmental conditions. By contrast, there was no increase in carbon uptake by fauna exposed to both stressors in combination, indicating that even a short-term hypoxic event may weaken the ability of marine invertebrates to withstand elevated CO_2 conditions. In addition, both hypoxia and elevated CO_2 increased organic carbon burial in the sediment, potentially affecting sediment biogeochemical processes. Since hypoxia and ocean acidification are predicted to increase in the face of climate change, our results suggest that local reduction of hypoxic events may mitigate the impacts of global climate change on marine soft-sediment systems.

Keywords: carbon sequestration, climate change, enhanced CO_2, hypoxia, infauna, macroalgal detritus, multiple stressors, stable isotope

1. Introduction

Marine sediments are key for the accumulation and burial of organic matter (Berner, 1982, Smith *et al.*, 2015). The input of organic detritus from the water column is one of the main sources of carbon to resident burrowing fauna, mediators of benthic pelagic exchange processes that in turn determine the fate of organic matter at the global scale (Middelburg, 2018, Snelgrove *et al.*, 2018). These processes include direct metabolic carbon uptake and mineralisation (Woulds *et al.*, 2016), particle reworking and burrowing ventilation, which affect detritus availability to other biota (Kristensen *et al.*, 2012, Snelgrove *et al.*, 2018). Anthropogenic stressors, including global warming, ocean acidification and oxygen depletion, impact the structure and functioning of these communities, having thus the power to influence benthic-pelagic carbon fluxes (Godbold & Solan, 2013, Laverock *et al.*, 2013, Widdicombe *et al.*, 2009). However, the influence of anthropogenic stressors on marine sediment carbon cycling remains largely unquantified (Keil, 2017).

Hypoxia (defined as oxygen concentration $\leq$ 2 ml of O_2/L; Diaz & Rosenberg, 2008) has increased in many coastal areas worldwide, as a consequence of both natural and anthropogenic influences (Breitburg *et al.*, 2018, Levin, 2018, Schmidtko *et al.*, 2017, Vaquer-Sunyer & Duarte, 2008). Among anthropogenic stressors, eutrophication is one of the main drivers of coastal hypoxia. Enhanced nutrient loading to seawater stimulates algal biomass accumulation and the subsequent microbial degradation of organic matter to the seabed lowers oxygen levels (Reed & Harrison, 2016, Steckbauer *et al.*, 2011). The intensity, duration and frequency of hypoxic events are expected to increase because of global warming, which is reducing O_2 solubility, whilst increasing primary production, thermally induced stratification and biotic respiration (Keeling *et al.*, 2009, Schmidtko *et al.*, 2017). In addition to this chronic reduction in oxygen availability, acute sporadic oxygen depletion events can occurs near the coastal seabed, following periods of intense autotrophic growth in surface waters, which are followed by fast, intense deposition of decaying phyto- and zooplankton on the sediment surface (Tait *et al.*, 2015, Zhang *et al.*, 2015). The effects of hypoxia on benthic community structure and functioning are well known (Levin *et al.*, 2009,

75 Middelburg & Levin, 2009, Zhang *et al.*, 2010). For instance, hypoxia can result in shallower
76 infaunal activity within the sediment (Riedel *et al.*, 2014), metabolic depression and, over time,
77 decreased body size (Diaz & Rosenberg, 1995), ultimately altering sediment biogeochemistry
78 (Middelburg & Levin, 2009). Hypoxia can also restrict macrofaunal burrowing activity to
79 superficial sediment layers, thus reducing the vertical, downward transport of material and
80 increasing the proportion of organic matter degradation that occurs near the sediment surface
81 (Middelburg & Levin, 2009). Finally, lower levels of aerobic respiration slow down carbon
82 mineralization (Jessen *et al.*, 2017, Woulds *et al.*, 2009, Woulds *et al.*, 2007).
83 In addition to hypoxia, increased anthropogenic CO_2 emissions are driving up levels of
84 atmospheric CO_2, which in turn increases the rate of oceanic CO_2 uptake. Once dissolved in the
85 surface ocean, this CO_2 drives a series of changes and reactions in the marine carbonate system and
86 these chemical changes are collectively known as ocean acidification (OA) (Doney *et al.*, 2009).
87 Under current rate of CO_2 emission, seawater CO_2 concentrations are expected to increase from
88 ~385 ppm to ~700-1000 ppm by the end of the century, based on the 5th IPCC Assessment Report's
89 Representative Concentration Pathway (RCP) 8.5 (Riahi *et al.*, 2011, Stocker *et al.*, 2013). In
90 addition, in coastal hypoxic regions, with a strong vertical stratification and high nutrient loadings,
91 levels of seawater CO_2 already exceed those predicted by the end of the century (pCO_2 > 1000
92 µatm), as heterotrophic degradation of organic matter increases metabolic CO_2 release because of
93 respiratory processes (Cai *et al.*, 2011, Melzner *et al.*, 2012). Thus, much higher CO_2 values are
94 expected to occur concomitantly with hypoxia in many shelf and estuarine regions worldwide, as a
95 consequence of climate change (e.g. warming and ocean acidification) (Breitburg *et al.*, 2018,
96 Carstensen & Duarte, 2019).
97 The potential for elevated CO_2 to negatively impact a wide variety of marine organisms and
98 biological processes is well documented (Gaylord *et al.*, 2015, Kroeker *et al.*, 2013, Sunday *et al.*,
99 2016, Vargas *et al.*, 2017). However, the impacts of elevated CO_2 on the structure and functioning
100 of soft-sediment ecosystems remain less understood (Godbold & Solan, 2013, Keil, 2017,

101 Laverock *et al.*, 2013). Although elevated CO_2 does not always cause mortality to infaunal species,
102 a trade-off between the maintenance of core activities (*e.g.* respiration and growth) and locomotion,
103 tightly linked to fauna particle transport (Queirós *et al.*, 2013), might be expected, resulting from
104 the allocation of additional energy (*i.e.* ATP) to physiological stress response pathways (Pan *et al.*,
105 2015, Widdicombe & Spicer, 2008, Wood *et al.*, 2008). In addition, elevated CO_2 can indirectly
106 alter the relationship between consumers and organic matter sources, modifying the nutritional
107 quality of food (*i.e.* higher C:N ratio), thus affecting carbon uptake (Duarte *et al.*, 2016, Kamya *et*
108 *al.*, 2017, Poore *et al.*, 2013, Rossoll *et al.*, 2012).
109 Enhanced CO_2 concentration in seawater can further alter sedimentary carbon cycling in
110 marine sediments through changes in primary production and respiration (Engel *et al.*, 2013, Molari
111 *et al.*, 2018, Piontek *et al.*, 2013, Riebesell *et al.*, 2007). Elevated CO_2 may stimulate primary
112 production (Engel *et al.*, 2013), but reduce organic carbon remineralisation due to changes in C:N
113 ratio (Riebesell *et al.*, 2007), potentially enhancing organic carbon sequestration in sediments. On
114 the other hand, elevated CO_2 may reduce carbon burial through the stimulation of organic matter
115 microbial degradation (Grossart *et al.*, 2006, Piontek *et al.*, 2013) and faunal respiration (Molari *et*
116 *al.*, 2018). These contrasting effects of elevated CO_2 on bulk organic carbon may be the result of
117 complex pathways of impacts on benthic communities and carbon sediment stores, potentially
118 resulting in cumulative neutral impacts and challenging predictive frameworks (Zark *et al.*, 2015).
119 Although coastal areas with low O_2 and elevated CO_2 have been largely documented
120 worldwide and will continue to increase under future climate conditions (Melzner *et al.*, 2012), the
121 vast majority of studies have focused on the effects of these stressors in isolation. A few recent
122 studies that have examined hypoxia and elevated CO_2 together have reported either additive or
123 synergistic effects of hypoxia and elevated CO_2 on the survivorship, development and growth of
124 different species of marine invertebrates (Gobler & Baumann, 2016, Gobler *et al.*, 2014,
125 Steckbauer *et al.*, 2015). However, to date, no study has investigated the effects of both stressors
126 simultaneously on infaunal communities and the carbon fluxes they mediate.

Here, using a four-week mesocosm study, we investigated the compound effects of hypoxia and elevated CO_2 on the fluxes of organic carbon in soft sediments, considering faunal-driven benthic-pelagic processes. Using isotopically-labelled macroalgal detritus (^{13}C), a common source of organic matter supplied to the coastal ocean (Krause-Jensen & Duarte, 2016, Queirós *et al.*, 2019), we traced organic carbon uptake of a pulsed supply into sedimentary faunal tissues and organic carbon stores under different oxygen availability and CO_2 levels. We predicted that the combined effects of elevated CO_2 and hypoxia could significantly reduce the faunal uptake of algal detritus, by causing metabolic depression in marine invertebrates (Levin *et al.*, 2009, Widdicombe *et al.*, 2009). Alternatively, elevated CO_2 could increase resource uptake by fauna (Queirós *et al.*, 2015, Thomsen *et al.*, 2013), due to increasing energetic demands associated to physiological responses under OA (*e.g.* protein synthesis, pH homeostasis, calcification) (Pan *et al.*, 2015, Ramajo *et al.*, 2016b, Stumpp *et al.*, 2012), thus counteracting the negative effects of hypoxia on feeding activities. In addition, the increase in sediment carbon incorporation expected under hypoxic conditions (Jessen *et al.*, 2017) could be dampened by elevated CO_2, possibly stimulating microbial degradation of algal detritus (Grossart *et al.*, 2006, Piontek *et al.*, 2013).

Moreover, in order to assess whether OA could alter organic carbon cycling directly (*e.g.* metabolic processes) or indirectly (*e.g.* modification of food quality), we carried out an independent experiment, where isotopically-labelled macroalgae (^{13}C), pre-exposed to elevated CO_2 for ten days, were used as a tracer to assess the organic carbon uptake by fauna and sediments, when both sources and consumers were exposed to OA. Elevated CO_2 could increase the C:N ratio of algal detritus (Mercado *et al.*, 1999, Stiling & Cornelissen, 2007), possibly resulting either in a decrease of carbon uptake by fauna, due to lower organic matter palatability (Duarte *et al.*, 2010, Kamya *et al.*, 2017), or in increased consumption of less nutritional food (Cruz-Rivera & Hay, 2001, Duarte *et al.*, 2011). An increase in C:N ratio of algal detritus under elevated CO_2 condition could also increase the organic carbon burial in the sediment, possibly due to lower organic matter remineralisation (Riebesell *et al.*, 2007).

2. Materials and methods

2.1 Sediment and macroalgal collection and preparation

Sediments were collected on board of the Plymouth Marine Laboratory's RV Quest, at Station L4 (50° 13' 22.7" N, 4° 11' 23" W, also known as Hilmar's Box), located about 13 km southwest of Plymouth, in the Western English Channel. L4 is one of the most comprehensively studied coastal systems in the world, having been monitored routinely for over 100 years, generating a wide range of environmental and biological benthic-pelagic observations which are used, e.g. by the European Union's Water Framework Directive (Smyth *et al.*, 2015). The site is representative of the vast majority of shelf environments around the world and, at present, neither hypoxia nor acidification are a regular occurrence in this system (publicly available data at http://www.westernchannelobservatory.org.uk, not shown). Phytoplankton blooms at L4 are generally observed in spring and autumn, representing the main source of organic supply, together with macroalgal detritus, at the seabed (Queirós *et al.*, 2019, Smyth *et al.*, 2015, Widdicombe *et al.*, 2010). During summer months, this site is generally characterized by thermal stratification and inorganic nutrient depletion in the surface water, suggesting N-limitation of primary production (Smyth *et al.*, 2015). The seawater pCO_2 at the seabed has been shown to vary between 351-432 µatm, with a pH value always above 8.0 throughout the year (Kitidis *et al.*, 2012). This site is generally not exposed to seasonal hypoxic events. A significant reduction of oxygen levels below the thermocline has been recorded during the summer of 2012, probably due to the largest and long lasting phytoplankton bloom recorded locally over the past 20 years (Smyth *et al.*, 2015, Tait *et al.*, 2015, Zhang *et al.*, 2015).

On the 16th March 2016, 22 cores were collected from the soft-sediment bed of the benthic monitoring site of the L4 station, using six separate deployments of a 0.1 m^2 box-core, at about 50 m depth. Seawater temperature (~10.5°C) and dissolved oxygen (~270 µM) at the seabed, during sampling day, are reported in Queirós *et al.* (2019). On retrieval to the deck, sediment with resident

179 fauna and overlying water were immediately sub-sampled from each box-core by pushing a
180 maximum of four acrylic core tubes (10 cm diameter x 30 cm high) to a depth of approximately 12
181 cm. This method allows the preservation of the structural integrity of sediment in each core tube
182 (Evrard *et al.*, 2012, Queirós *et al.*, 2019, Woulds *et al.*, 2016), which is essential to maintain as
183 much as possible on going sedimentary gradients and ecosystem processes (Stocum & Plante,
184 2006). The core tubes were equipped with oxygen sensor spots (PreSens), previously attached to the
185 inner wall of the cores with silicone glue, just above the sediment surface (see below). Each core
186 was, then, gently removed from the box-core and capped at the bottom with a PVC lid fitted with an
187 O-ring, further sealed by a plastic cap, which was glued to the core with biological grade silicon
188 (Gold Label, Huttons Aquatic Products). The top of each core was sealed with an acrylic lid, onto
189 which the tubing for an airstone sitting near the surface of the water in the core had been fitted. All
190 cores were placed in two water baths containing seawater from the collected site and covered with
191 black plastic sheets during transport to Plymouth Marine Laboratory to reduce temperature changes.
192 Once in the mesocosm laboratory at Plymouth Marine Laboratory, the core tubes were randomly
193 allocated to two 1-m^3 mesocosm tanks. The laboratory is a temperature controlled room where air
194 temperature is maintained such that aquarium water in the room follows the seasonal cycle of
195 bottom water at the L4 station (Findlay *et al.*, 2008, Queirós *et al.*, 2015). The 1-m^3 mesocosm
196 tanks were used as water baths to ensure that base temperature and light (absence of) conditions
197 experienced by each core tube were as similar as possible during laboratory exposures, and water
198 was not circulated between individual (microcosm) sediment cores. Water in each core was aerated
199 for 24 h prior to start the experiment by use of the fitted airstones, which promoted a gentle flow
200 inside the core without causing resuspension.
201 The macroalga *Laminaria digitata* was used as a labelled food source in our experiment.
202 *Laminaria* spp., together with other macroalgal species, have been shown to occur as organic
203 detritus within the sediment at L4, and they are one of the organic matter sources preferentially
204 assimilated by infaunal assemblages at the site (Queirós *et al.*, 2019), as indeed potentially in much

of the coastal ocean (Krause-Jensen & Duarte, 2016). In February 2016, individuals of *L. digitata* were collected by hand from the low intertidal rocky shore at Rame Head (50°18'41.11"N, 4°13'14.89"O; England). All individuals were immediately transported to the mesocosm facility at the Plymouth Marine Laboratory, where they were placed in a recirculating water system tank and kept at ambient CO_2 for approximately ten days. The tank was lit by two LED strip lights, positioned at a distance of about 40 cm from the water surface. Algae were maintained under constant light to maximize growth. Seawater was collected from the Western Channel Observatory during the previous week to each of the exposures (pH: mean ± SE = 8.09 ± 0.01; salinity: mean ± SE = 36.25 ± 0.75). Ten days later, some individuals of *L. digitata* were transferred to a separate tank in which conditions were otherwise the same, except for elevated seawater CO_2 level, and held there for two weeks. The CO_2 level in this tank was used to create a low pH treatment (pH mean ± SE = 7.75 ± 0.07) and was in line with the Intergovernmental Panel on Climate Change 5^{th} Assessment Report's Representative Concentration Pathway (RCP) 8.5 atmospheric CO_2 for the year 2100, the scenario in which emissions are highest, and which does not include specific climate mitigation targets (Riahi *et al.*, 2011, Stocker *et al.*, 2013). The elevated seawater CO_2, and the resultant lower pH, in this tank was achieved by using a premixed gas system modified from Findlay *et al.* (2008). Briefly, the enrichment was achieved by mixing pure CO_2 gas with CO_2-free air using flow meters and mixing vessels, monitored with a CO_2 analyser (820, Li-Cor). The water bath with the low pH water was covered with sealed plastic sheets in order to insulate the tank's atmosphere from the laboratory atmosphere, allowing CO_2 in seawater and the air above it to equilibrate.

Individuals of *L. digitata* from the two treatments were then transferred to two clear acrylic aquaria, filled with seawater at either ambient or elevated CO_2 levels. The seawater in these aquaria contained 200% ^{13}C-enriched bicarbonate (98% ^{13}C, Sigma Aldrich) to label algae, and allow its subsequent tracing within the sediment cores. The aquaria were sealed with clear acrylic lids and maintained under constant light and ambient temperature for 72 hours. Labelled algae were then

231 rinsed with unlabelled seawater to remove adhering ^{13}C-bicarbonate and stored at -78° C before
232 freeze-drying. Algal detritus marked with ^{13}C (~ 13.23 % and 66.7 %, respectively for macroalgae
233 labelled at ambient and elevated CO_2 levels) were then ground to a fine powder using pre-acid
234 washed and muffle-furnaced agate pestle and mortars before being added to the experimental cores
235 (Evrard *et al.*, 2012, Hunter *et al.*, 2019). ^{13}C labelling was used to enable tracing of carbon
236 between source and sedimentary consumers, and the use of the same population of macroalgae is
237 also necessary because of strong variations that occur within and across individuals, as well as
238 different populations (Phillips *et al.*, 2014). Carbon and nitrogen content in macroalgal tissue was
239 analysed using an elemental analyser. C:N ratio in macroalgae maintained at ambient CO_2 seawater
240 was significantly lower than those at elevated CO_2 (20.103 ±0.37 % and 22.73 ±0.45 %,
241 respectively; t= -4.50; *P*= 0.024, n=2).
242
243 *2.2. Macrofauna and sediment organic carbon uptake experiment*
244 The sedimentary core experiment was set up for 4 weeks to examine the separate and
245 cumulative effects of CO_2 concentration [CO_2] (ambient versus elevated CO_2) and oxygen
246 concentration [O_2] (normoxia versus hypoxia) on faunal and sediment incorporation of labelled
247 algae, which was previously maintained at ambient CO_2. Four replicate cores were then randomly
248 allocated to each experimental treatment. Treatments were achieved by: selecting the air-CO_2 mix
249 bubbled in each sediment core (manipulated as before); whether or not a hypoxia event was
250 simulated; which macroalagal detritus was added to which core. Seawater was not circulated
251 between individual (microcosm) cores. Only three replicate cores were used to simulate control
252 conditions (ambient CO_2, normoxia), due to loss of one core during field sampling. Four cores were
253 also used to test the effects of elevated CO_2 on faunal carbon uptake using labelled algae that were
254 pre-exposed to elevated CO_2. Three additional control cores were maintained at ambient seawater
255 CO_2, oxygen concentration and without labelled algae, and used to determine the ^{13}C background
256 content in faunal tissue and sediment (see below). Two CO_2 treatments were established, as used

with the macroalgae, to compare present day (ambient) values with those expected by the end of the century under RCP 8.5. pH_{NBS} was measured every two days and the average value (± SE) for the ambient and elevated CO_2 treatments were 8.17 ±0.01 and 7.65 ±0.02, respectively. Seawater temperature and salinity were measured every two days, while alkalinity samples were collected weekly from each core and measured using an automated titrator (Apollo SciTech Alkalinity Titrator Model AS-ALK2). Carbonate system parameters were calculated from measured pH, alkalinity, temperature and salinity using CO2SYS program for Excel with constant from Mehrbach *et al.* (1973) and adjusted by Dickson and Millero (1987) (See Table S1 in Supplementary information).

After two weeks from the start of the experiment, water mixing was interrupted and 0.115 ± 0.0002 g of ^{13}C-labelled *L. digitata* (equivalent to a C addition of ~ 1g C m^{-2}; Woulds *et al.* 2016) was added to the overlying water of each core and allowed to settle to the sediment surface. Correspondingly, 0.113 ± 0.0003 g of ^{13}C-labelled algae pre-exposed to elevated CO_2 were added in four cores exposed to elevated CO_2. Airflow was re-instated one hour later in all but the hypoxia treatment cores. In these, airflow was interrupted for 46 hours by sealing the lids (and their openings) to cores with silicone grease (biological grade, Gold Label). Oxygen concentration in the water column was measured using the oxygen sensor spots (PreSens) and a fibre-optic oxygen transmitter equipped with a computer to collect the data. The oxygen sensors consisted of an oxygen-permeable foil, in which a chemical luminescence reaction takes place. The photoluminescence lifetime of the luminophore within the sensor was measured by pointing the fibre optic towards the outside of the wall in within which the sensor was glued. Before each measurement, a two-point calibration was performed in all spot sensors, following manufacturer recommendation (0 and 100%). The 0% oxygen saturation was calibrated by adding sodium sulphide to distilled water. Seawater was, then, aerated with ambient air and stirred for 20 min to avoid oversaturation. At this point, it was used in the calibration as the 100% dissolved oxygen solution. We used the definition of hypoxia as oxygen levels of ≤ 2 mg L^{-1} (Diaz & Rosenberg,

2008), which hypoxia treatment cores reached after 46 hours. The cores were monitored using optodes, so that oxygen depletion was not extreme for too long. The average oxygen saturation of each treatment was 103.12 ± 0.545 % and 18.74 ± 3.08 %, which correspond to $[O_2]$= 10.20 ± 0.446 mg L^{-1} and $[O_2]$= 1.794 ± 0.294 mg L^{-1}, respectively for normoxia and hypoxia treatments. All experiments were carried out in the dark and the incubations were terminated after four weeks from the start of the experiment. The duration of the experiment was appropriate to ensure that isotopic signal in traced carbon could be detected in primary consumers, whilst reducing the changes of complexity in measured response variables as the labelled detritus is cycled by subsequent consumers within the sedimentary food-web (Middelburg, 2014, Queirós *et al.*, 2019)

2.3. Sample collection and analysis

At the end of the experiment (13 April 2016), the cores were processed for stable isotope analyses of organic carbon content in faunal tissue and sediment. For each core, sediment was sectioned into 0-2, 2-6 and 6-10 cm depth layers using a custom built sediment slicer. Each layer was subsampled for the analysis of $^{13}C_{org}$ content in sediment, using a syringe that fitted tightly into a 50 mL falcon tube, and immediately frozen at -20°C until processing. The sediment remaining from each layer was used for the determination of $^{13}C_{org}$ incorporation into faunal tissue. Each sediment layer was sieved over a 0.5 mm sieve, and specimens were identified to the lowest taxonomical level possible using pre-combusted sorting equipment and then frozen in pre-weighed and pre-combusted petri dishes at -80 $^{\circ}$C until processing (within two weeks). Sediment and fauna samples were oven dried at 60°C for 48 h. Each sample was then ground to a fine powder using agate pestle and mortars and, then, they were acidified by adding drops of 10% HCl, until all carbonates had been dissolved. All samples were oven dried at 60 °C for 48 h. Elemental and isotopic analyses of sediment and fauna samples were measured on constant flow isotope ratio mass spectrometers (Sercon model 20-20's, dual turbo pumped, CF/IRMS) connected to a Thermo EA1110 elemental analyser at OEA Labs (UK).

308 $^{13}C_{org}$ incorporation into fauna (% ^{13}C mg^{-1} m^{-2}) and sediment (% ^{13}C) was then calculated as
309 the product of the excess ^{13}C (*E*) and C_{org} content in the fauna /sediment (expressed as percentage).
310 *E* is the difference between the labelled fraction (*F*) of fauna/sediment sample and background
311 fauna/sediment sample: $E = F_{sample} - F_{background}$, where $F = {}^{13}C/({}^{13}C+{}^{12}C) = R/(R+1)$, where $R = (d^{13}C/1000 +1) \times R_{VPDB}$, and $R_{VPDB}=0.0112372$ (Sweetman *et al.*, 2016). The carbon uptake by
313 fauna was standardized for faunal biomass (mg DW) for each layer. Data from layers were summed
314 to produce C uptake by fauna for each core. Background isotope information for sediment was
315 taken from control cores (without labelled algae). Isotope signature for faunal invertebrates was
316 unavailable from control cores, probably due to the low sample weight, so *E* was calculated using
317 background F values from samples collected from the field at the same site in March 2016 (Queirós
318 *et al.*, 2019). $^{13}C_{org}$ content in faunal tissue and sediment samples were corrected for the fact that the
319 added macroalgal detritus is not the 100% ^{13}C labelled: C-uptake= ^{13}C incorporated
320 (%^{13}C)/fractional abundance of ^{13}C in algal detritus.
321
322 *2.4 Statistical analysis*
323 Effects of $[CO_2]$ and $[O_2]$ on infaunal assemblages, within each sediment layer, were tested
324 by means of a permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001),
325 based on Bray-Curtis dissimilarity matrix of untransformed data. The model included two fixed
326 factors: $[CO_2]$ (ambient versus elevated CO_2) and $[O_2]$ (normoxia versus hypoxia). In a separate
327 analysis, the effects of $[CO_2]$ on the infaunal assemblages fed with algae previously exposed to
328 enhanced CO_2, were tested using one-way PERMANOVA comparing the following treatments:
329 control (ambient CO_2/control algal detritus), elevated CO_2/control algal detritus, elevated CO_2/ algal
330 detritus exposed to enhanced CO_2. Two-way analyses of variance (ANOVA), with $[CO_2]$ and $[O_2]$
331 as fixed orthogonal factors, were carried out on univariate data (total infaunal density, species
332 diversity, fauna and sediment incorporation of labelled algae, previously maintained ambient CO_2).
333 In a separate analysis, the effects of $[CO_2]$ on infaunal density, species diversity and faunal and

sediment incorporation of algae, previously exposed to enhanced CO_2, were tested using one-way ANOVA, comparing the same treatments described for one-way PERMANOVA analysis. Cochran's C-test was used to check for homogeneity of variances and, when necessary, data were log- or square root transformed. Student-Newman-Keuls (SNK) tests were used for comparison of the means.

3. Results

3.1 Infaunal assemblage analyses

Animals (ind. m^{-2}) were mainly found in the uppermost 2 cm of sediment (0-2 cm: 563.48 ± 92.41; 2-6 cm: 58.33 ± 12.88; 6-10 cm: 8.88 ± 4.79; data are mean ± SE value averaged across experimental treatments; n=15). A taxonomic list of infauna found within each sediment layer is reported in Appendix 1 (Supplementary information). There were no effects of [CO_2] and [O_2] on the structure of infaunal assemblages, within each sediment layer (Table S2). In addition, there were no differences in the infaunal assemblage composition between different sources of algal detritus or under ambient and elevated CO_2 conditions, within each sediment layer (Table S3). In the upper 2 cm, echinoderms were the most abundant group with 56.18%, followed by polychaetes (27.29%), bivalves (5.79%), nematodes (4.96%), crustaceans (4.13%) and chelicerates (1.65%). In the 2-6 cm, infaunal assemblage was dominated by polychaetes (85.72%), and the rest of the assemblage included Sipuncula (7.14%), cnidarians (3.57%) and bivalves (3.57%). Only one species of bivalve (*Lucinoma borealis*) was found in the deeper layer of sediment. Furthermore, there were no significant effects of [CO_2] and [O_2] on the total infaunal density and species diversity, within each sediment layer (Table S4a and Table S5a). Finally, no differences were found in the total infaunal density and species diversity between different sources of algal detritus or under ambient and elevated CO_2 conditions, within each sediment layer (Table S4b and Table S5b). The number of species and biomass per feeding modes within each sediment layer is reported in Appendix II.

360 *3.1. Organic carbon assimilation in faunal tissue and sediment*

361 There was a significant interaction between [CO_2] and [O_2] on the organic carbon uptake by
362 fauna (Table 1a). At ambient CO_2, there were no differences in the organic carbon uptake by fauna
363 between oxygen treatments, while, under elevated CO_2 level, the faunal carbon uptake was higher at
364 normoxic than hypoxic conditions (Fig. 1).

365 Sediment organic carbon enrichment was detected only in the 2-6 cm sediment layer (Fig.
366 2), while there was no increase in the organic carbon compared to the background in the 0-2 cm and
367 6-10 cm sediment layers (Fig. S1 in supplementary information). ANOVA on the 2-6 cm sediment
368 layer showed no significant effect of [CO_2] and [O_2] on the organic carbon incorporation in the
369 sediment (Table 1a); however, there was a tendency ($F= 3.767$, $P= 0.08$) for the organic carbon
370 burial to increase under hypoxia compared to normoxia, regardless of CO_2 treatments (Fig. 2).
371

372 **Table 1.** ANOVAs on the effects of **a)** [CO_2] (ambient, elevated CO_2) and [O_2] (normoxia,
373 hypoxia) and **b)** food quality (ambient CO_2/control algal detritus, elevated CO_2/control algal
374 detritus, elevated CO_2/algal detritus exposed to enhanced CO_2) on the organic carbon incorporation
375 in faunal tissue and in sediments.

a)		Fauna C-incorporation			Sediment C-incorporation		
Source of variation	df	MS	*F*	P	MS	*F*	P
[CO_2]	1	0.0002	0.081	0.783	0.0080	0.032	0.863
[O_2]	1	0.0017	0.788	0.401	0.9533	3.767	0.088
[CO_2] x [O_2]	1	0.0127	5.943	**0.041**	0.0191	0.075	0.791
Residual	8	0.0021			0.2531		
Transformation		log (x+1)			log (x+1)		
Cochran's test		*P< 0.05*			ns		

b)		Fauna C-incorporation			Sediment C-incorporation		
Source of variation	df	MS	*F*	P	MS	*F*	P
Food quality	2	0.0040	1.214	0.361	2.0836	5.455	**0.045**
Residual	6	0.0033			0.3820		
Transformation		log (x+1)			log (x+1)		
Cochran's test		*P< 0.05*			*P< 0.05*		

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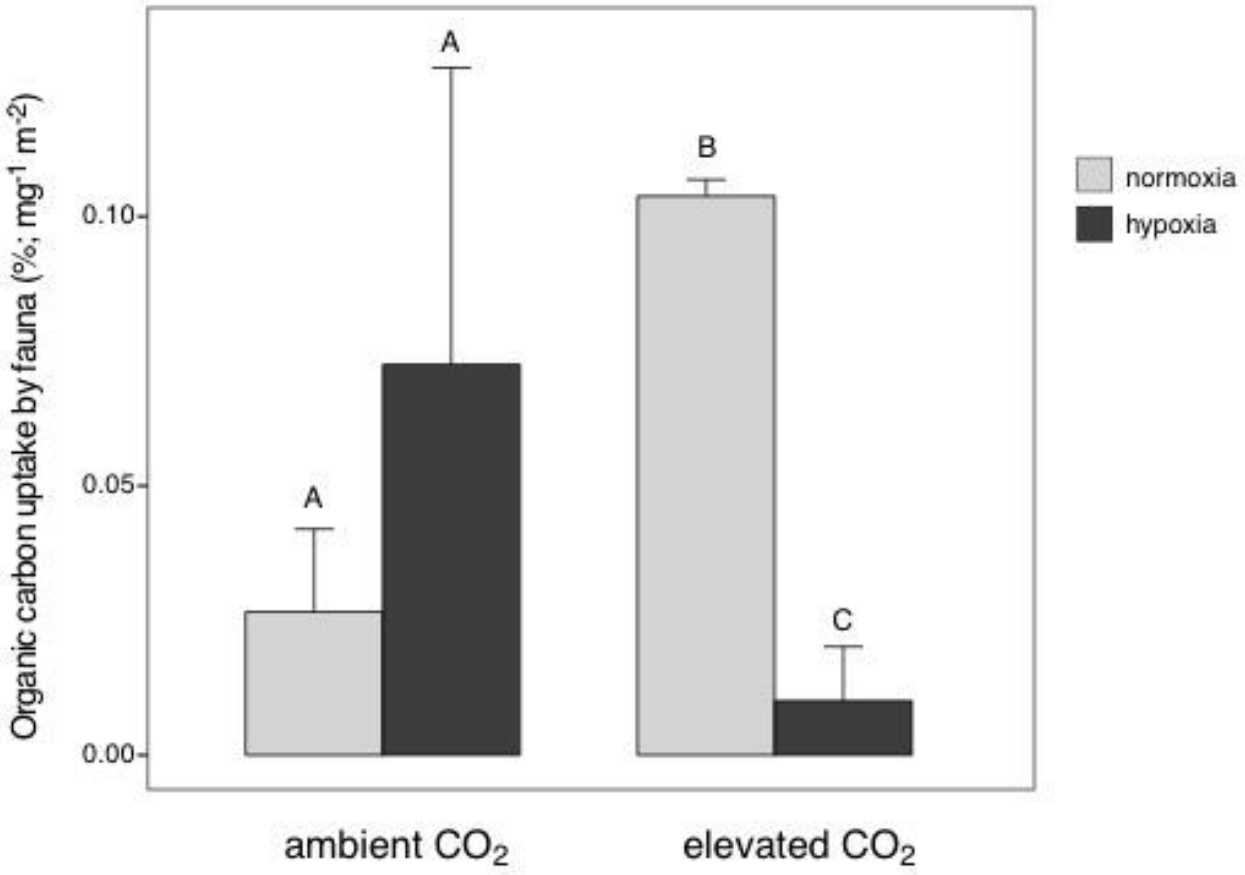

377
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379 **Fig. 1.** Organic carbon incorporation (mean ± SE) in fauna tissue (%; mg^{-1} m^{-2}) under different

380 combination of [CO_2] (ambient, elevated CO_2) and [O_2] (normoxia, hypoxia).

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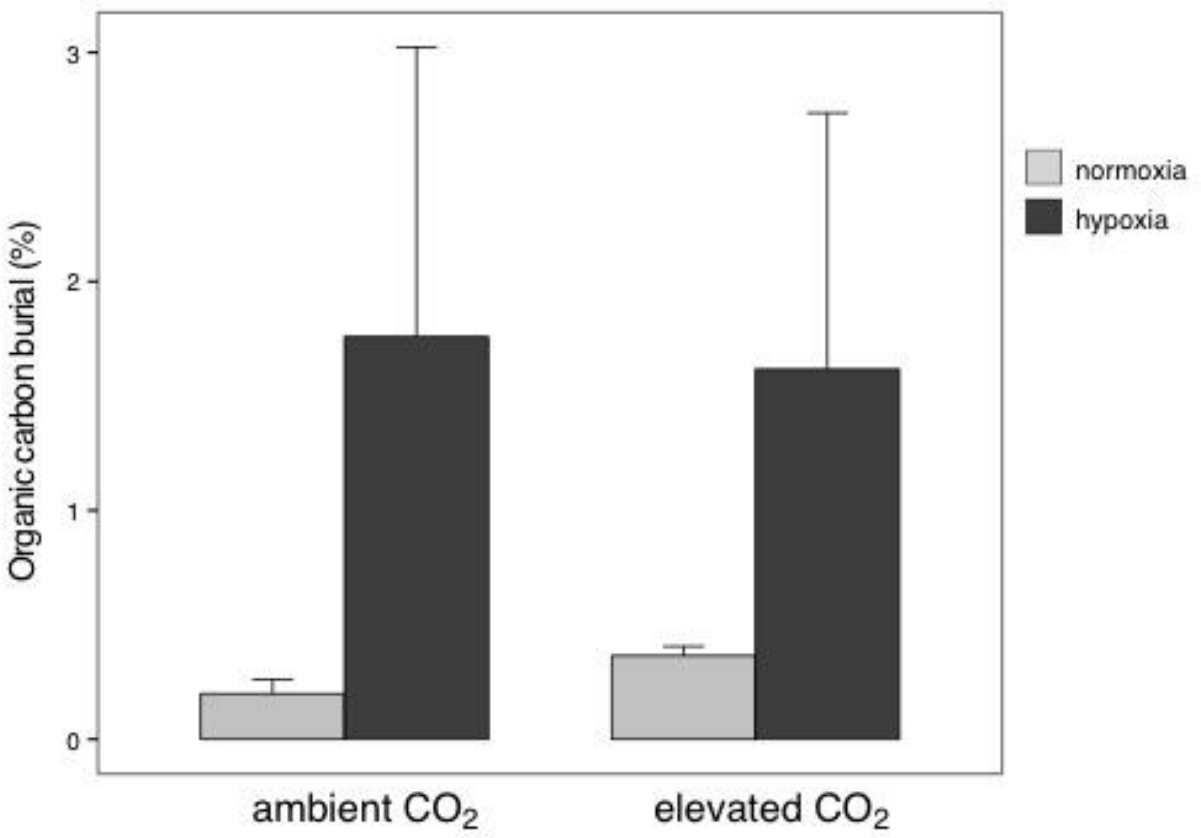

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384 **Fig. 2.** Organic carbon incorporation (mean ± SE) in sediment layers (%; 2-6 cm layer) under
385 different combination of [CO_2] (ambient, elevated CO_2) and [O_2] (normoxia, hypoxia).

386

387 There was no significant difference in carbon faunal uptake among different sources of algal
388 detritus or between ambient and elevated CO_2 treatments (Table 1b; Fig. 3). However, faunal uptake
389 of control algal detritus tended to increase under elevated CO_2 levels (Fig. 3).

390 Under enhanced CO_2, sediment burial (layer 2-6 cm) of organic carbon from algal detritus
391 previously exposed to elevated CO_2 was greater than that from algal detritus maintained at ambient
392 CO_2 conditions (Table 1b; Fig. 4). We found no accumulation of organic carbon in the 0-2 cm and
393 6-10 cm sediment layers for cores exposed to elevated CO_2 with algal detritus previously exposed
394 to elevated CO_2 (Fig. S2 in supplementary information).

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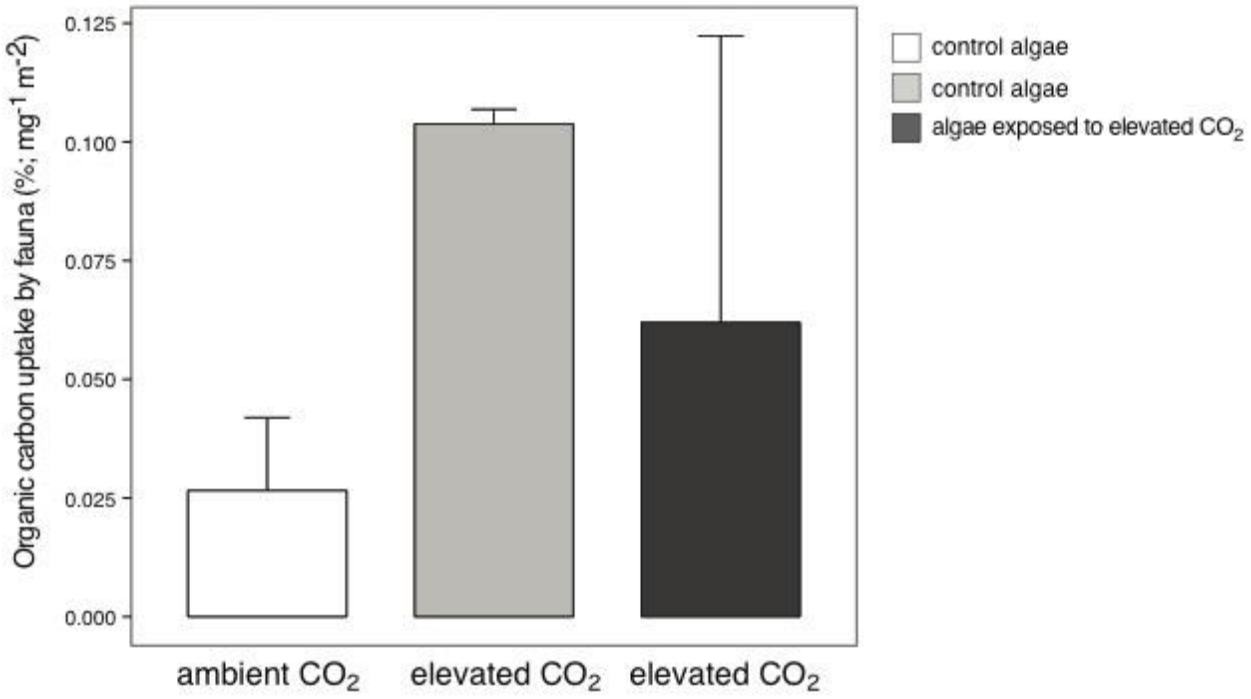

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397

398 **Fig. 3.** Organic carbon incorporation (mean ± SE) in fauna tissue (%; mg^{-1} m^{-1}) exposed to ambient

399 CO_2/control algal detritus, elevated CO_2/control algal detritus and elevated CO_2/algal detritus

400 exposed to elevated CO_2 (respectively *white*, *light grey* and *dark grey* bars)

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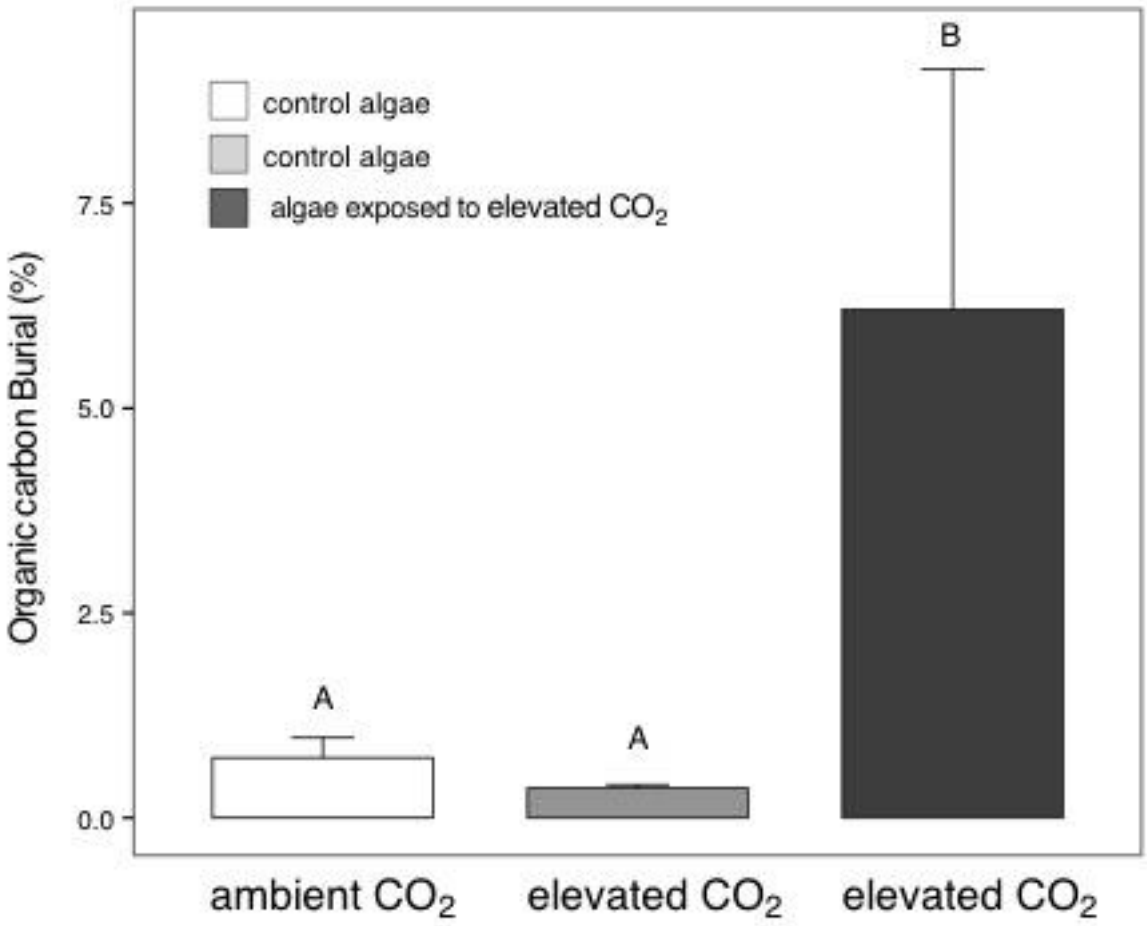
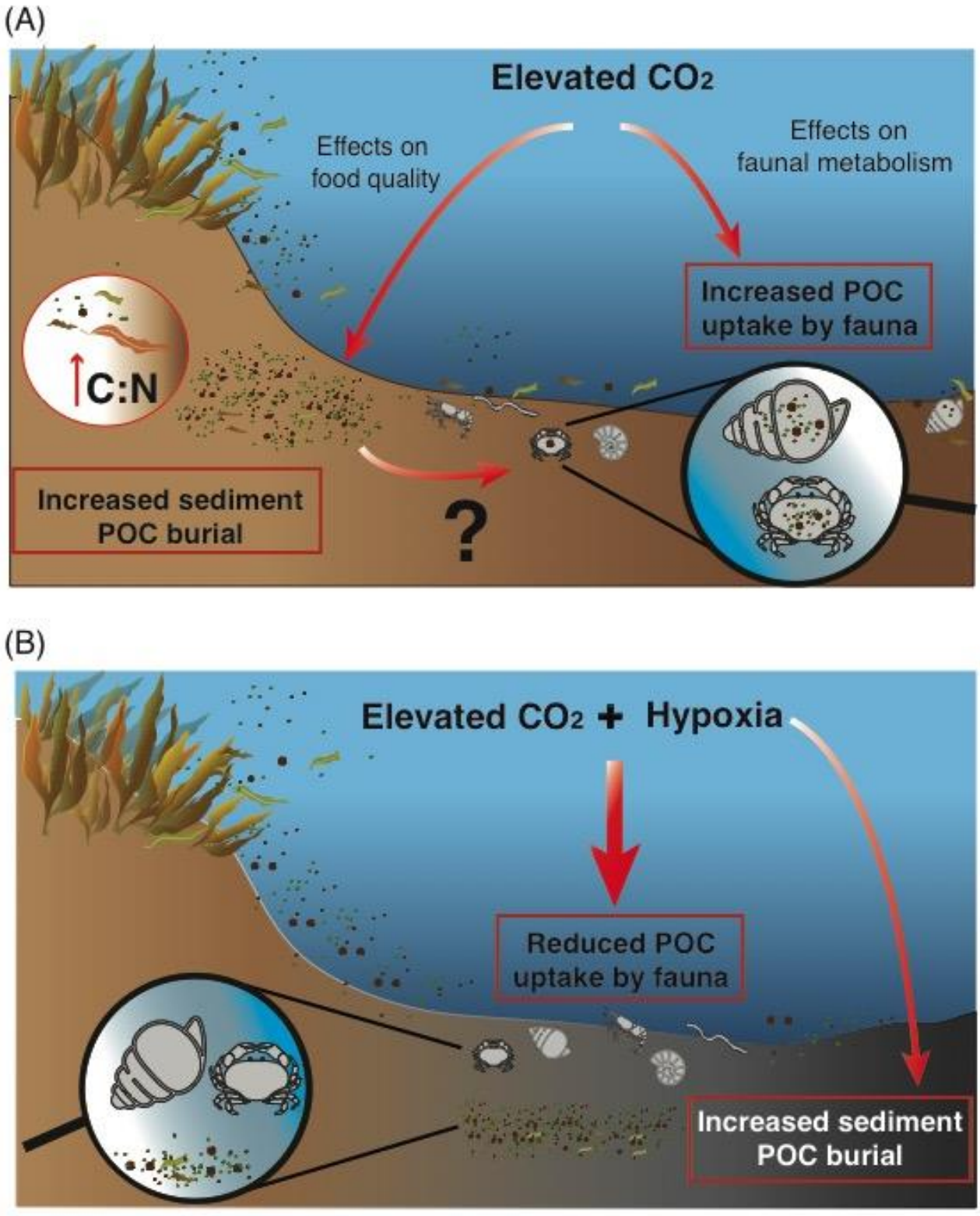


Fig. 4. Organic carbon accumulation (mean ± SE) in sediment (%; 2-6 cm layer) exposed to ambient CO_2/control algal detritus, elevated CO_2/control algal detritus and elevated CO_2/algal detritus exposed to elevated CO_2 (respectively *white*, *light grey* and *dark grey* bars).



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409 **Fig. 5. Schematic illustration showing the effects of elevated seawater CO_2 and hypoxia on**
410 **fauna mediated particulate organic carbon (POC) fluxes in coastal seabed, based on results**
411 **from the mesocosm experiment**. **A)** Increased POC uptake by fauna, when consumers were

exposed to elevated CO_2 condition (direct effects of elevated CO_2 on faunal metabolism); enhanced POC burial in the sediment and high variability (question mark) in the POC uptake by fauna, when both consumers and resources (algal detritus) were exposed to elevated CO_2. **B)** Hypoxia hindered the POC uptake by fauna at elevated $CO_2$and increased the POC burial in the sediment, when consumers were exposed to the combined effects of elevated CO_2 and low oxygen concentration.

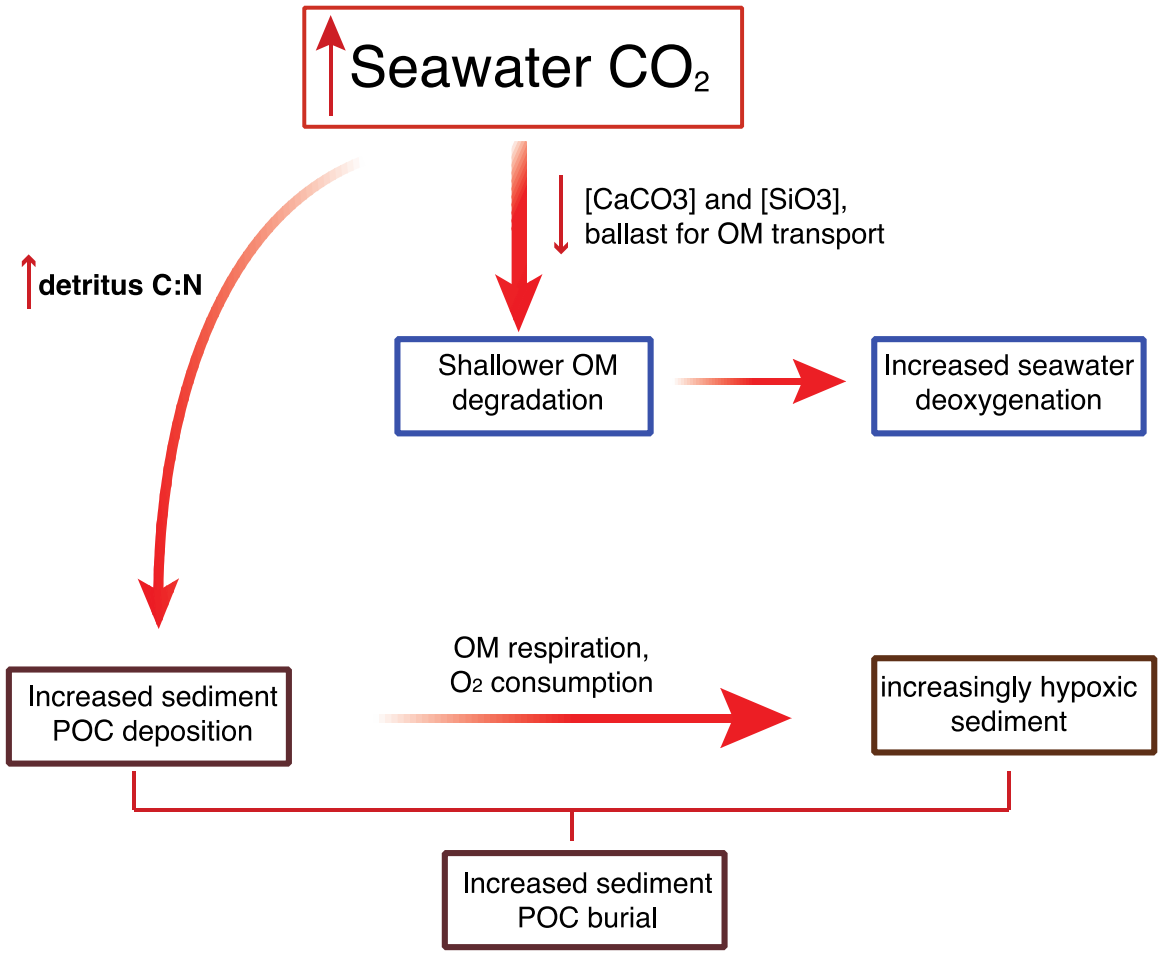

Fig. 6. Diagram showing the potential impacts of elevated seawater CO_2 on biogeochemical cycles, either by changing the stoichiometric ratio of organic detritus, which arrives to the seabed, or reducing organic matter (OM) transport through the water column. Blue frames indicate processes in the water column; brown frames indicate processes at the seabed.

4. Discussion

Both elevated CO_2 and hypoxia significantly influenced the flux of organic carbon in marine sediments, as mediated by benthic biota. Infauna responded to elevated CO_2 by increasing the uptake of algal detritus at normal O_2 concentrations, but not when exposed to hypoxia (Fig. 5a,b). This suggests that metabolic depression may occur in marine invertebrates exposed to the combination of hypoxia and elevated CO_2. As coastal areas with low O_2 and high CO_2 have increased globally and will continue to expand under future OA scenario, our results may suggest a limited ability of benthic communities to sustain normal mediation of important carbon cycling processes both under present and under future ocean conditions.

OA can negatively affect benthic marine invertebrates, either directly, by altering physiological processes (Pan *et al.*, 2015, Wang *et al.*, 2018, Widdicombe & Spicer, 2008) or indirectly, via modification of food web interactions (Duarte *et al.*, 2016, Kamya *et al.*, 2017, Queirós *et al.*, 2015). Previous studies have shown that elevated CO_2 can result in reduced growth rate, disruption of extracellular acid-base balance, alteration of metabolism, lethargy and modification of individual level trade-offs in energy consuming processes of invertebrates, across different taxonomic groups (Kroeker *et al.*, 2010, Portner & Farrell, 2008, Widdicombe & Spicer, 2008). Some species are able to maintain normal level of physiological activity under elevated CO_2, although increasing metabolic rates and, thus, oxygen consumption (Pan *et al.*, 2015, Queirós *et al.*, 2015, Stumpp *et al.*, 2012, Widdicombe & Spicer, 2008, Wood *et al.*, 2008). For instance, Wood *et al.* (2008) found increased respiration and calcification rates and decreased arm muscle mass of the brittlestar *Amphiura filiformis* under elevated CO_2, indicating a trade-off between the maintenance of skeletal integrity and locomotion. Other studies have reported positive effects on the physiology (*e.g.* growth, calcification and metabolic rate) of molluscan species exposed to elevated CO_2 when resources were abundant, suggesting that food availability can mediate the susceptibility of marine invertebrates to OA (Pansch *et al.*, 2014, Ramajo *et al.*, 2016a, Thomsen *et al.*, 2013). In our study, elevated CO_2 significantly increased the organic carbon uptake by fauna at normoxia, suggesting

451 that infaunal invertebrates were able to compensate short-term negative effects of elevated CO_2
452 through enhanced food intake.

453 Elevated CO_2 can additionally affect the relationship between consumers and resources
454 indirectly, by changing the nutritional quality of food (Duarte *et al.*, 2016, Falkenberg *et al.*, 2013,
455 Kamya *et al.*, 2017, Stiling & Cornelissen, 2007). We tested for this relationship and found that,
456 under elevated CO_2 condition, the uptake of fauna fed with algal detritus grown at elevated CO_2
457 (higher C:N ratio) was highly variable and did not differ from that of algae previously maintained at
458 ambient CO_2 (Fig. 5a). Consumers can respond to changes in food nutritional quality at enhanced
459 CO_2 either by preferentially consuming algae with higher nutritional quality (Falkenberg *et al.*,
460 2013, Kamya *et al.*, 2017) or by increasing consumption of less nutritional food (*i.e.* compensatory
461 feeding) (Cruz-Rivera & Hay, 2001, Duarte *et al.*, 2011, Duarte *et al.*, 2014), resulting in a species-
462 specific feeding behaviour of consumers (Tomas *et al.*, 2015). The lack of a clear response of
463 infauna to altered resource quality could also be due to the slight, thought significant, increase in
464 algal C/N ratio (~13%) under short-term elevated CO_2 condition, compared to those recorded in
465 longer-term elevated CO_2 experiments in terrestrial systems (Stiling & Cornelissen, 2007). The
466 duration of our experiment (four weeks) was appropriate to detect the isotopic signal of traced
467 carbon in primary consumers, whilst reducing changes in the measured response variables due to
468 subsequent processing of labelled materials within the food web (Middelburg, 2014, Queirós *et al.*,
469 2019). Further studies are, however, needed to evaluate how the persistence of elevated CO_2
470 conditions predicted under future climate scenarios can directly or indirectly modify resource-
471 consumer relationships.

472 Importantly, once elevated CO_2 was applied with hypoxia, no increased carbon uptake by
473 fauna was observed, suggesting limited capacity of marine invertebrates to cope with both stressors
474 in combination. Feeding activity is a very oxygen demanding process and accounts for a large
475 proportion of an organism's energy budget (Sokolova, 2013). Under hypoxia, the oxygen required
476 by marine organisms to support energetically costly processes, such as feeding, assimilation and

digestion of food, is not met by ambient oxygen supply. This means that also animals more tolerant to OA could be negatively affected by elevated CO_2 when concurrently exposed to hypoxia (Miller *et al.*, 2016, Portner *et al.*, 2005, Tomasetti *et al.*, 2018). This suggests that in well-mixed shelf coastal systems, as simulated in our study, even short-term hypoxic events may compromise the ability of marine invertebrates to deal with future ocean conditions. Indeed, benthic invertebrate contribution to sedimentary carbon cycling could be currently impaired in coastal areas exposed to low levels of oxygen and pH due to strong vertical stratification and high nutrient loading. Despite predictions of an expansion of these hypoxic areas as a consequence of climate changes (Melzner *et al.*, 2012), very few studies have, to date, investigated the cumulative effects of hypoxia and acidification on benthic communities (Gobler & Baumann, 2016). The combined effects of these stressors have been shown to reduce the survivorship and growth in bivalves (Gobler *et al.*, 2014), reduce growth rate in abalone (Kim *et al.*, 2013) and cause metabolic depression in different species of invertebrates, such as sipunculids, echinoderms, and crustaceans (Portner *et al.*, 2005, Steckbauer *et al.*, 2015). Our results suggest that, in combination, elevated CO_2 and hypoxia may limit the ability of benthic communities to mediate globally important carbon fluxes on the seabed (Middelburg, 2018, Snelgrove *et al.*, 2018).

Organic carbon accumulation was detected at the intermediate layer of sediment (2-6 cm), while we found no accumulation of carbon in the shallower and deeper sediment layers, regardless of experimental conditions. Organic matter arriving at the sediment surface may be subjected to many different processes. For instance, the organic carbon ingested by fauna may be egested back to the sediment and transferred through the food web or accumulated into deeper layers of the sediment. The carbon uptake by fauna and bacteria seems be strongly related to their biomass (Would et al. 2016). In addition, at any trophic levels, organic carbon can be metabolised and re-mineralised through fast degradation (Wood et al. 2009; Gantikaki et al. 2011). In our experiment, algal detritus added to the surface sediment was assimilated by fauna, which is particularly abundant in the top 0-2 cm layer, and then transferred to the underlying sediment layer (2-6 cm).

503 The detection of carbon accumulation further away from the sediment-water interface may also be
504 due to the lower abundance of animals found in the deeper part of sediment cores, thereby the
505 remaining carbon was not consumed by animals and remained in the sediment. This result
506 highlights the importance of faunal mediation towards carbon cycling, with mixing between
507 sedimentary carbon pools and the overlying water reduced to those layers where fauna were more
508 abundant.

509 We report here that hypoxia tended to increase organic carbon burial in the 2-6 cm layer,
510 regardless of CO_2 concentration (Fig. 5b), possibly as a consequences of alterations on infaunal
511 assemblage functioning (Keil, 2017). Previous experimental studies, using carbon-labelled
512 phytodetritus as a tracer, have shown that, under normoxia, both animals and microbes can
513 assimilate labile carbon directly and respiration is generally the major fate of added labelled carbon
514 (Woulds *et al.*, 2016). Hypoxia may cause metabolic depression, reduced activity or lethargy in
515 marine invertebrates (Galic *et al.*, 2019, Levin *et al.*, 2009), thereby indirectly promoting the
516 organic carbon preservation in marine sediments. For instance, Jessen et al. (2017) have recently
517 shown that low oxygen negatively affected faunal diversity and activity (*i.e.* bioturbation) and
518 promoted microbial anaerobic processes, resulting in a significant increase of the sediment organic
519 carbon burial. To date, however, most studies estimating carbon fluxes on the seabed are still
520 largely focused on physical and biogeochemical processes (Middelburg, 2018, Snelgrove *et al.*,
521 2018). As recently highlighted in Queirós *et al.* (2019), continuing to ignore the vital mediation of
522 seabed carbon cycling by invertebrates may likely limit our understanding of how the global ocean
523 carbon cycle occurs, what processes and ecosystem components are involved, and what is their
524 resilience under a changing ocean climate.

525 Elevated CO_2 concentration in seawater did not affect organic carbon burial in our
526 experiment directly. The effects of elevated CO_2 on carbon sequestration in marine sediments are
527 still unclear. Some laboratory studies have found an increase in microbial degradation of organic
528 matter under elevated CO_2 that could lead to lower carbon sequestration under elevated CO_2

529 (Grossart *et al.*, 2006, Piontek *et al.*, 2013). However, the concurrent increase of primary production
530 under elevated CO_2 could reduce microbial degradation of organic matter, resulting in negligible
531 effects of OA on organic carbon burial. For instance, in a recent study, Zank et al. (2017) found no
532 effects of elevated CO_2 on the concentration and molecular composition of organic carbon, despite
533 a clear effect of phytoplankton on organic matter production, suggesting no change in the amount of
534 organic matter in coastal systems under elevated CO_2 condition. In contrast, in our study, elevated
535 CO_2 significantly increased the sediment deposition of algal detritus previously exposed to elevated
536 CO_2, likely as a consequence of its decreased nutritional value (*i.e.* higher C:N ratio, Fig. 5a). This
537 is in accordance with previous work (Riebesell *et al.*, 2007), where an increase in C:N ratio of
538 primary-producer tissues (about 16% at 700 µatm CO_2 level) was also observed under elevated
539 CO_2, due to an overconsumption of dissolved inorganic carbon, leading to an increase in the export
540 of particulate organic carbon. Stoichiometric changes of exported organic matter at elevated CO_2
541 could have a major impact on biogeochemical cycles (Fig. 6) (Andrews *et al.*, 2017, Hofmann &
542 Schellnhuber, 2009). Most of the oxygen consumed during organic matter respiration is used to
543 oxidize carbon rather than nitrogen, thus resulting in excess oxygen consumption in deep water
544 (Oschlies *et al.*, 2008). In addition, elevated CO_2 may limit the sinking speed and transport of
545 organic matter through the water column, by reducing the production of calcareous ($CaCO_3$) and
546 siliceous (SiO_2) minerals, which provide ballast for the transport of organic carbon in deep water
547 (Hofmann & Schellnhuber, 2009). This could, ultimately, result in shallower organic matter
548 remineralisation and further expansions of O_2 depletion zones (Hoffman et al. 2009; Andrew et al.
549 2017). Importantly, the combined effects of elevated CO_2 and hypoxia may slow down the
550 mineralization of organic matter, likely increasing the burial of enhanced organic carbon production
551 in marine sediments. On the other hand, expansion of oxygen depleted zones may increase
552 denitrification and loss of fixed nitrogen, potentially impact nitrogen cycling and ocean productivity
553 (Kalvelage *et al.*, 2013). Our results highlight how changes in resources, in addition to consumers,

554 may affect important processes determining ocean carbon cycling, and that foodweb interactions are
555 key to predict ecosystem-level impacts of climate change.
556 In summary, the results of our experiment show that elevated CO_2 and episodic hypoxic
557 events may affect net sequestration of organic carbon in coastal systems through the modification of
558 relevant faunal mediated pathways and resource quality. To the best of our knowledge, this is the
559 first study experimentally investigating the combined effects of these two stressors on faunal
560 mediated carbon fluxes on a well-mixed coastal seabed. Episodic events of hypoxia, as simulated in
561 our study, have been commonly documented in coastal systems, following intense depositions of
562 organic matter at the seabed (Tait *et al.*, 2015, Zhang *et al.*, 2015). However, it is noteworthy that
563 the persistence of low O_2 may also be driven by other seasonal and interannual cycles, depending on
564 different processes, such as hydrodynamic conditions of the water body, thermal stratification and
565 nutrient loads (Breitburg *et al.*, 2018). Hypoxic areas, such as the Western Baltic Sea, the coasts of
566 Japan and China or the Gulf of Mexico, are currently affected by coastal acidification, due to
567 heterotrophic degradation of organic matter (Melzner *et al.*, 2012, Thomsen *et al.*, 2013). Thus,
568 evaluating the combined effects of hypoxia and elevated CO_2 on marine life is essential for
569 understanding how marine ecosystems respond to these conditions under both current and future
570 climate conditions. In addition, further studies could also evaluate the impacts of future ocean
571 acidification scenario on biological and biogeochemical processes in these coastal hypoxic systems
572 already exposed to low O_2 and high CO_2 conditions. The capacity of marine organisms to sustain
573 physiological processes under stress (*e.g.* reproduction, growth, calcification, locomotion) may
574 determine their survival under a changing climate (Widdicombe & Spicer, 2008). Increase of food
575 uptake is a strategy that has been observed across taxa, and reflects higher metabolic costs to the
576 individual associated with stress response pathways (Queirós *et al.*, 2015, Thomsen *et al.*, 2013).
577 Our results indicated that this compensatory mechanism may be impaired under hypoxia, possibly
578 weakening the ability of marine invertebrates to cope with elevated [CO_2] and potentially reflects
579 that higher metabolic costs will come at the expense of increased O_2 uptake rates in aerobes.

580 Alternatively, a decrease in feeding rates could also represent a mechanism for marine organisms to
581 deal with the exposure to elevated CO_2 and low O_2, by reducing aerobic metabolism and thus O_2
582 requirement (*i.e.* metabolic depression) (Pörtner *et al.*, 2004, Rosa & Seibel, 2008). This could
583 result in reduced growth rates and altered behaviour (Galic *et al.*, 2019, Gobler *et al.*, 2014,
584 Tomasetti *et al.*, 2018). Thus, hypoxia and elevated CO_2, in combination, may impair the key role
585 of infaunal assemblages in determining carbon fluxes at the sediment-water interface and their
586 contribution toward carbon sequestration (Queirós *et al.*, 2019). In addition, changes in organic
587 matter quality due to elevated CO_2 could increase the export of organic carbon in marine sediments
588 and the expansion of low O_2 concentration, ultimately altering ecosystem functioning, including
589 nitrogen cycling and ocean productivity at global scales (Hofmann & Schellnhuber, 2009,
590 Kalvelage *et al.*, 2013, Levin, 2018).

591 In this light, management actions aimed to reduce local stressors (*e.g.* eutrophication-driven
592 hypoxia and coastal acidification) can be considered a good strategy for mitigating the impacts of
593 global climate change (*e.g.* OA) on marine community functions and biogeochemical processes. For
594 instance, although measures to reduce eutrophication can take a long time to become effective
595 (Varjopuro *et al.*, 2014), increases in seawater oxygen concentration have been documented in
596 some coastal systems, following nutrient input reduction (Kemp *et al.*, 2009). As the incidence of
597 hypoxia and elevated CO_2 are predicted to increase as a consequences of climate change (Breitburg
598 *et al.*, 2018, Gobler & Baumann, 2016), more studies are necessary to raise awareness of the
599 impacts of multiple stressors on carbon fluxes in coastal marine sediments under future climate
600 change scenarios, as well as to tune up suitable remediation strategies.
601

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