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1 **Title:** Microphytobenthos as a source of labile organic matter for denitrifying microbes

2

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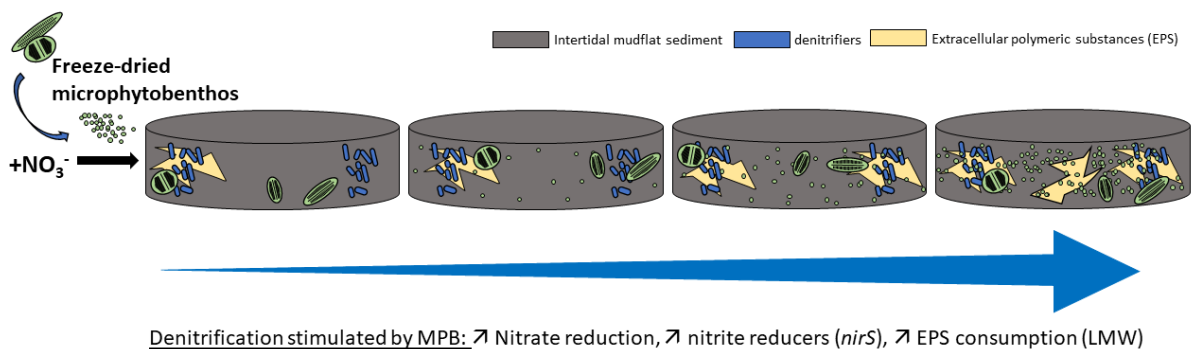
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9

10 **Graphical abstract:**



11

12

13 **Abstract:**

14 Nitrogen loads in natural waters remain elevated in populated and agricultural areas with serious impact
15 on estuarine and coastal ecosystems. Intertidal sediments can play a significant role in attenuating the
16 high nitrogen levels in water via microbial nitrate reduction, in general dominated by denitrification.
17 These heterotrophic processes are heavily mediated by both the quantity and quality of organic matter
18 available. Benthic microalgae were experimentally investigated as organic carbon source for
19 denitrifying microbes in intertidal mudflat sediments from the Seine Estuary (France). Dry
20 microphytobenthos (including algae and their extracellular polymeric substances) were added to
21 sediments and nitrate reduction rates were monitored over a two-week period using anoxic controlled
22 flow-through reactor approach. Our results show that microphytobenthos addition resulted in
23 significantly higher nitrate reduction (67 to 332% increase), highly related to the added amount of
24 microphytobenthos. Moreover, increase of the low molecular weight carbohydrates consumption (11 to
25 39%) highlight the measurable contribution of extracellular polymeric substances to the carbon
26 consumption during nitrate reduction. The addition of microphytobenthos increased the abundance of
27 nitrite reductase genes, especially those encoding the *nirS* gene (43 to 152% increase) while nitrous
28 oxide reductase genes (*nosZ* gene) remained constant. Microphytobenthos appeared to favor complete
29 denitrification as suggested by an increase in *nirS* and a decrease in clade II *nosZ* gene copy numbers.
30 This study confirms experimentally the assumption that microbes use microalgae and particularly labile

31 extracellular polymeric substances as a carbon substrate for nitrate reduction. These results reinforce the
32 impact played by microphytobenthos in intertidal mudflats by highlighting their role on denitrifying
33 microbes and nitrate removal from water.

34

35 **Keywords:**

36 Microphytobenthos; Nitrate reduction rates; denitrifying microbes; intertidal sediment; extracellular
37 polymeric substances.

38

39 **1. Introduction**

40 Estuaries cover less than 0.35 % of Earth's surface (Costanza et al. 1997), but are some of the
41 most productive ecosystems (Geider et al. 2001). The stability and long-term management of estuarine
42 ecosystems are threatened by anthropogenic pressure, particularly nutrient loading (Erisman et al. 2008;
43 Barbier et al. 2011; Porter et al. 2013; Zhang et al. 2021). This often leads to undesirable and widespread
44 disturbances such as harmful algal blooms or altered food web dynamics (Le Moal et al. 2019). Whereas
45 phosphorus inputs significantly decreased since the 70s due to improvement of waste water treatment
46 (Liu et al. 2008), nitrogen pollution remains elevated in populated and agricultural areas (Grizzetti et al.
47 2011; Ukaogo et al. 2020; Zhang et al. 2021). These differences lead to an imbalance in stoichiometric
48 ratios of nitrogen and phosphorous, impacting autotrophic communities, decreasing overall biodiversity
49 and consequently primary and secondary productivity in estuarine and coastal ecosystems (Hillebrand
50 et al. 2014). Many key services, essential for regulation and support of estuarine systems, are provided
51 by shallow water habitats such as salt marshes and mudflats (Jacobs et al. 2013; Mandal et al. 2021).
52 Intertidal mudflats sequester organic carbon (Jickells and Rae 2005), recycle nutrients, contribute widely
53 to the autochthonous estuarine primary production by benthic microalgal assemblages, known as
54 microphytobenthos (MPB), providing nutrition to countless resident and migrating animals (Mayor et
55 al. 2018; Passarelli et al. 2018; Hope et al. 2020). For decades, the role of intertidal sediments in
56 attenuating the high nitrogen loads in estuaries has been studied (Trimmer et al. 1998; Magalhães et al.
57 2002; Wang et al. 2009; Wadnerkar et al. 2019). However, due to intense hydrodynamics and
58 anthropogenic disturbances such as artificialization of banks, port and town extensions, construction of
59 roads and bridges and sediment removal, reduce mudflat areas and could thus limit these key services
60 (Lesourd et al. 2001, 2016). Especially since, in the context of climate warming, the ecological values
61 of mudflats are also threatened by the upcoming increase in extreme climatic events and sea level rise
62 (van der Wegen et al. 2017).

63 The removal of nitrogen in intertidal estuarine sediments, mainly performed by microorganisms,
64 can be attributed to several distinct nitrogen transforming processes. Among them, denitrification
65 (Tiedje et al. 1983) and anaerobic ammonium oxidation (anammox, Van de Graaf et al. 1995; Strous et
66 al. 1999; Kuenen 2008) allow inorganic nitrogen reduction into inert N₂ gas, completely eliminating
67 nitrogen from the system. In comparison, dissimilatory nitrate reduction to ammonium (DNRA, Tiedje

68 1988; Decleyre et al. 2015a) transforms nitrate (NO_3^-) into ammonium (NH_4^+), retaining inorganic
69 nitrogen in the system. These nitrogen transformations occur in anoxic environments in the presence of
70 NO_3^- . Whereas anammox is an autotrophic process, DNRA and denitrification are heterotrophic
71 processes for which the carbon quantity (Starr and Gillham 1993) and quality (Hume et al. 2002;
72 Sirivedhin and Gray 2006; Dodla et al. 2008; Stelzer et al. 2014) in the sediment plays an important
73 role. Processes allowing a complete reduction of inorganic nitrogen into N_2 are especially important
74 since they avoid retention of inorganic nitrogen in systems. Overall, denitrification in coastal sediments
75 has been shown to be dominate over anammox (Zhou et al. 2014). It has been estimated that almost 50%
76 of the nitrogen that enters streams and rivers is denitrified within the estuary before it reaches the coast
77 (Galloway et al. 2004). Denitrification is performed in 4 sequential reactions from NO_3^- to N_2 with nitrite
78 (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) as intermediates. The first, NO_3^- reduction, is not
79 specific to denitrification and can be carried out by a wide range of microorganisms. The second, NO_2^-
80 reduction to NO is only performed by denitrifying microbes (Zumft 1997). This reaction is catalyzed by
81 two different types of NO_2^- reductases encoded by the *nirS* or *nirK* genes (for a review see Kuypers et
82 al. 2018). The final step of denitrification allows reduction of N_2O , a powerful greenhouse gas which is
83 catalyzed by the nitrous oxide reductase encoded by the *nosZ* gene (Scala and Kerkhof 1998; Rich et al.
84 2003; Rösch and Bothe 2009).

85 The impact of carbon quality related to vegetation type on denitrification has been demonstrated in
86 wetland and mangrove sediments (Bastviken et al. 2007; Fernandes et al. 2016). In intertidal areas,
87 carbon sources are characterized by a large variability in composition and degradability, with terrestrial
88 plants and macrophyte carbon that are more recalcitrant than labile sources such as MPB (Bouillon and
89 Boschker 2006). The role of MPB in nitrogen turnover has been studied and demonstrated with
90 laboratory incubations of natural sediments (Sundbäck and Miles 2002; Tobias et al. 2003), benthic
91 chamber measurements in the field (Eyre et al. 2013), *in situ* ^{15}N pulse-chase experiments (Eyre et al.
92 2016) or pyrosequencing and qPCR (Decleyre et al. 2015b). Additionally, MPB assemblages produce
93 extracellular polymeric substances (EPS), to facilitate their vertical migration and sediment adhesion,
94 which are highly labile (De Brouwer et al. 2006; Shnyukova and Zolotareva 2017). It was hypothesized
95 that microbes preferentially use EPS as carbon sources (Bouillon and Boschker 2006; Bellinger et al.
96 2009; Taylor et al. 2013). Whereas these studies highlighted the important role of MPB for N
97 assimilation and as organic carbon source for NO_3^- reduction processes, the cumulative N-assimilation
98 and N-reduction processes did not allow the exact quantification of the MPB's role as organic carbon
99 source for each specific process. The addition of organic carbon in the form of MPB was shown to
100 stimulate NO_3^- reduction rates and modify the denitrifying community composition (Decleyre et al.
101 2015b; Babbin et al. 2016). In order to specifically determine the role of organic carbon originating from
102 MPB on denitrification, an experimental approach is required controlling processes like photosynthesis
103 or nitrification that can in turn influence denitrification (Bartoli et al. 2021).

104 In this context, the aim of the present study was to experimentally quantify the impact of MPB and
105 the associated carbon (EPS) on the anoxic NO₃⁻ reduction processes. Therefore, varying quantities of
106 MPB biomass were added to estuarine sediment sampled in an intertidal mudflat of the Seine estuary
107 and the efficiency of microbial NO₃⁻ reduction was studied in anoxic and dark conditions by using flow-
108 through reactors (Laverman et al. 2006). In addition to rate measurements, the abundance of key
109 denitrifying genes was determined (*nirK/nirS* and *nosZ*) to estimate the role of MPB addition on the
110 denitrifier community. We hypothesized that a higher availability of labile carbon from MPB increases
111 NO₃⁻ reduction rates and enhances growth of microbes involved in the denitrification process.

112

113 **2. Material and methods**

114 **2.1. Study site and sampling**

115 The Seine estuary is a typical example of elevated riverine nutrient fluxes from the Parisian
116 megapole resulting in eutrophication on the English Channel coast (Billen and Garnier 2007; Billen et
117 al. 2007; Morelle 2020). In this estuary, the potential of intertidal sediments carrying out NO₃⁻
118 elimination was shown to be highly related to the presence of MPB (Laverman et al. 2021). However,
119 the extent of the mudflats in the downstream Seine estuary has been reduced by a factor of 3 since 1975
120 and by a factor of 5 since 1875 which limits the surface colonized by MPB and their potential ecological
121 role.

122 Sediment and MPB were sampled in April 2019 on the northern intertidal mudflat of the Seine
123 estuarine mouth, Normandy, France (49°27'01.6''N; 0°12'20.0''E; WGS84). Sediment was collected
124 during low tide by carefully manually pushing 10 Plexiglass® rings into the sediment until they were
125 filled with the sediment surface. The rings have an inner diameter of 4.2 cm, a thickness of 0.4 cm and
126 a height of 2 cm. The rings with sediment were transported in anoxic bags and stored at 4 C until the
127 start of the experiment. Initial sediment (IS) characterization was done on sediment from two of the
128 rings. Sediment was homogenized and 5 mL was stored at -80 C until the determination of initial *nirS*,
129 *nirK* and *nosZ* gene copy numbers. The remaining sediment was freeze-dried (24 h) and part was
130 conserved for further quantification of photopigment and EPS contents. The other part of the freeze-
131 dried sediment was ground and conserved for further elemental and isotopic analyzes. MPB (*i.e.* the top
132 2 mm of the colonized sediment) was collected on the same site and freeze dried (24 h) before its
133 utilization in the flow through reactor (FTR) experiment. Thereby, the added form of MPB thus
134 corresponds to a MPB/sediment mix originating from the top 2 mm of the sediment surface covered
135 with a natural assemblage of MPB.

136

137 **2.2. Flow through reactor experiment**

138 The 8-remaining sediment rings were used to perform a FTR experiment to evaluate the
139 potential rates of NO₃⁻ reduction. Four distinct conditions including the control were performed in
140 duplicate per condition resulting in 8 reactors. Sediment from each ring was removed, weighed, and

141 homogenized, then MBP were added in the form of freeze-dried powder directly to the sediment. MPB
142 additions corresponded to control (no addition), 1, 5, and 10 % total sediment weight (hereafter named
143 M0, M1, M5, and M10). These values were chosen in order to reach higher but realistic amounts of chl
144 *a* observed in sediments of intertidal mudflats (Underwood and Kromkamp 1999). After being amended
145 with MPB and carefully homogenized, a sample of each replicated condition was kept and freeze-dried
146 (24 h) for initial characterization, while the remaining amended sediment was redistributed inside each
147 plexiglass ring. Two filters, a 0.2 μm pore size PVDF (Durapore®) membrane filter and a glass fiber
148 backing filter (1.2 mm thick, 4.7 cm diameter, PALL Corp., NY), were placed over the sediment surfaces
149 at both ends of the Plexiglas® rings. The resulting sediment reactor cell was then enclosed by POM
150 (poly-oxy methylene) Delrin® caps with in and outflow channels. A detailed description of the FTR
151 design and the determination of potential rates can be found in Laverman et al. (2006, 2012). To ensure
152 NO_3^- availability throughout the experiment, each ring was supplied by an inflow solution of 10mM
153 potassium nitrate (KNO_3^-). Inflow solutions corresponded to artificial sea water where salinity was
154 adjusted at 30 with NaCl to match the salinity measured in water from the sampling site. In addition, the
155 inflow pH was adjusted to match the ambient conditions ($\text{pH} = 8$) using NaHCO_3 (0.2 g L^{-1}). As the
156 process targeted in this study is anoxic (denitrification), all the input solutions were kept anoxic by
157 purging them with O_2 -free N_2 for at least 5 minutes before the start of the experiment. Moreover, the
158 experiment was performed in dark conditions to limit the photosynthetic activity and avoid inhibitory
159 effects or competition between diatoms and bacteria which would have influenced NO_3^- reduction rates
160 (Bartoli et al. 2021). The experiments were run at constant temperature ($21 \pm 2 \text{ C}$) for a two-week period
161 after a 16-hour settle time in experimental conditions. The two-week period was chosen based on
162 previous work indicating sufficient time for the microbial community to change upon the imposed
163 variables (Bååth et al. 1988; Laverman et al. 2015).

164 For 357 hours (15 days), the reactor cells were supplied with input solutions at a constant flow rate
165 ($Q = 2 \pm 0.15 \text{ mL h}^{-1}$) using a peristaltic pump (Gilson minipuls). The sediment inside the reactors was
166 thus saturated with a saline anoxic NO_3^- solution during the entire duration of the FTR experiment. The
167 reactor outflow samples were collected in centrifugation tubes at 4-hour intervals during the day (9am-
168 1pm, 1pm-5pm) resulting in 2 sample collections per experimental day. The night outflow solutions
169 (5pm-9am) were discarded. Each collected sample was conserved at 4 C and analyzed for ammonium
170 (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) concentrations using a Gallery™ Discrete Analyzer
171 (ThermoFisher scientific, Waltham, Massachusetts, USA).

172 The rates of reduction or production of dissolved inorganic nitrogen (DIN) in the flow-through
173 reactors ($\text{nmol cm}^{-3} \text{ h}^{-1}$) were calculated using the equation $R = (\Delta C \times Q) / V$ where Q is the volumetric
174 flow rate, V the reactor volume (27.7 cm^3) and ΔC the difference in concentration of the solute between
175 the outflow and inflow solutions. Net NO_3^- reduction rates were calculated by substituting the measured
176 difference in NO_3^- concentration between inflow and outflow (ΔC). As NH_4^+ or NO_2^- were not supplied

177 throughout the inflow solutions, net NH_4^+ and NO_2^- release rates were directly determined using the
178 outflow concentrations instead of ΔC .

179 At the end of the FTR experiment, the sediment from each reactor was homogenized and used for
180 final characterization. As described for the initial sediment samples and initial MPB amended sediment,
181 5 mL of sediment was kept at -80 C for estimation of the densities of the denitrifying communities. The
182 remaining sediment was freeze-dried and a part was kept for quantification of photopigment and EPS
183 contents. The remaining freeze-dried sediment was ground and used to perform elemental and isotopic
184 analyzes.

185

186 **2.3. Characterization of denitrifying communities.**

187 DNA was extracted from sediment (from 3 to 4.5 g wet) with the NucleoBond[®] RNA Soil kit and
188 its DNA co-elution set (Macherey Nagel) according to the manufacturer's instructions and subsequently
189 quantified and checked for purity by 260/280 (around 1.8) and 260/230 (around 2) ratio analysis with a
190 NanoDrop 1000 Spectrophotometer (Thermo Fisher scientific). Genes encoding for enzymes implied in
191 the denitrification process were enumerated via quantitative PCR following recommendations of Bustin
192 et al. (2009). The following primer pairs were used: three pairs for NO_2^- reductase genes, one for *nirS*
193 gene (*nirSC1F*, ATCGTCAACGTCAARGARACVGG, *nirSC1R*, TTCGGGTGCGTCTTSABGAAS
194 AG, (Wei et al. 2015)) and two for *nirK* gene (cluster I, *NirKC1F*, ATGGCGCCATCATGGTNYTNCC,
195 *NirKC1R*, TCGAAGGCCTCGATNARRTTRTG and cluster II, *NirKC2F*, TGCACATCGCCAACG
196 GNATGTWYGG, *NirKC2R*, GGCGCGGAAGATGSHRTGRTCNA, (Wei et al. 2015)). Two
197 nitrous oxide reductase *nosZ* genes were used (clade I: *nosZ1840F(2F)*,
198 CGCRACGGCAASAAGGTSMSST and *nosZ2090R(2R)*, CAKRTGCAKSGCRTGGCAGAA,
199 (Philippot et al. 2013), clade II: *1153_nosZ8F*, CTIGGICCIYTKCAYAC and *1888_nosZ29R*,
200 GCIGAICARAAITCBGTRC, (Jones et al. 2013)). Several clades or clusters were targeted in order to
201 be the most exhaustive. PCR reactions were performed using the SensiFAST SYBR NO-ROX mix
202 (Meridian Bioscience) in six replicates using a Wafergen SmartChip Real-time PCR system (Takara)
203 for *nirS* and *nirK* genes and in three replicates using a LightCycler[®] 480 (Roche) for *nosZ* genes. DNA
204 template quantities were normalized by dilution to reach 30 ng μL^{-1} . Negative controls, using ultra-pure
205 water instead of templates, were performed in order to check if there was no contamination; positive
206 controls, corresponding to plasmids containing a cloned gene of interest, allowed to determine copy
207 numbers. Cycle number at which the fluorescence reaches a threshold (*Ct*) was determined with the
208 second derivative maximum analysis method. Amplification efficiencies were determined using a
209 calibration curve (gene copy number versus the *Ct* value) of positive controls by performing qPCR of
210 10-fold dilutions of them; absolute quantification (as gene copy number from a *Ct*) was recovered using
211 the same calibration curve. The quantification limit was defined as fluorescent signals with at least 5 *Ct*
212 below the signal of the negative controls.

213

214 **2.4. Photopigments and EPS content**

215 Chlorophyll *a* (chl *a*) and phaeopigment content in MPB and sediment samples were determined
216 according to Lorenzen (1967). The pigments were extracted from 0.5 g of sediment suspended in 10 mL
217 acetone (90%) for 12 hours in the dark at 4 C. After centrifugation (4 C, 3000 rpm, 5 min), fluorescence
218 of the supernatant was measured using a Uvikon XL© spectrophotometer (Secomam, Aqualabo, France)
219 before and after acidification (10 µL HCl 0.3 M per milliliter of sample).

220 EPS were extracted from 0.5 g of dried sediment in 15 mL centrifugation tubes with 5 mL of distilled
221 water (MilliQ). After 1 hour of incubation at 35 C under continuous mixing (30 rpm), tubes were
222 centrifuged (4 C, 3000 rpm, 10 min). Supernatants containing the EPS were collected in a new
223 centrifugation tube. Low and high molecular weight EPS (LMW and HMW) were separated by
224 incubation in ethanol (80% final concentration) for 16 hours at -20 C. HMW EPS were precipitated by
225 cold ethanol and after centrifugation (4 C, 3000 rpm, 30 min), the supernatant which contain LMW EPS,
226 was collected in a new tube while HMW EPS remained in the pellet. Supernatants and pellets were then
227 dried at 50 C. The dried samples were resuspended in 1.16 mL distilled water for carbohydrate and
228 protein quantification (1 mL and 160 µL respectively). Total carbohydrate content was determined using
229 the phenol–sulfuric acid assay with glucose as a standard (Dubois et al. 1956) and protein content was
230 determined using the Bradford assay reagent (Bio-Rad) with bovine serum albumin (BSA) from Sigma-
231 Aldrich as standard (Bradford 1976). For carbohydrates, absorbance was read, after 40 minutes of
232 incubation, using a Uvikon XL© spectrophotometer (Secomam, Aqualabo, France) at 485 nm. For
233 proteins, absorbance was read, after 5 minutes of incubation, using a microplate reader SAFAS Xenius
234 XL© (SAFAS Monaco) at 590 nm. All results are expressed in µg per gram of dry sediment weight (µg
235 gDSW⁻¹).

236

237 **2.5. Elemental and isotopic analyzes**

238 A sub-sample of freeze-dried and crushed sediment from each replicated condition was placed
239 into a tin capsule and used to determine the N_{tot}. Another sub-sample was acid-treated with 2N HCl to
240 remove the carbonate and was subsequently rinsed with deionized water. The carbonate-free sub-sample
241 was dried at 60 C, and manually ground before being placed into a tin capsule for the determination of
242 C_{org} and δ¹³C. Contents and isotopic compositions of total organic carbon and total nitrogen (C_{org}; N_{tot};
243 δ¹³C) were determined by Elemental Analysis –Isotope Ratio Mass Spectrometry (EA-IRMS) Vario
244 pyro cube (Micromass Isoprime). The standard gas was calibrated in relation to the international
245 standard (PeeDee Belemnite-190PDB). N_{tot} and C_{org} were expressed as percentage of the dry sediment
246 mass and the C_{org}/N (molar:molar) was calculated.

247

248 **2.6. Statistical analysis**

249 For each tested condition (M0, M1, M5, and M10), the potential significant variability of
250 measured rates throughout the duration of the experiment was tested by one-way repeated measures

251 analysis of variance (RM-aov) using Sigma-Plot 12 software. Shapiro-Wilk normality tests and Bartlett
252 equal variance tests were used, and in the non-parametric cases, RM-aov on ranks were performed. Both
253 RM-aov were followed by a Tukey-test in order to discriminate differences. In addition, to discriminate
254 significant differences between the tested conditions throughout the experiment, Split-plot ANOVA
255 (Split-plot aov) were performed on R software (version 4.0.2).

256 In order to distinguish the effect of the MPB addition on nitrogen transformation, considering
257 an adaptation period of two weeks for the microbial community, the rates during the last day of the
258 incubations (2 samples per replicate resulting in 4 values) were compared to the other parameters
259 measured at the end of the experiment (chl *a*, C:N ratio, $\delta^{13}\text{C}$, gene copy numbers). After testing the
260 normality and equal variance tests as previously mentioned, a one-way analysis of variance (aov)
261 followed by a Tukey-test were performed; a Kruskal-Wallis test followed by a Dunn test were performed
262 if application conditions were not met.

263 A principal component analysis was performed using the package “FactoMineR” in R on the data
264 frame represented by the differences measured between the initial and the final values measured.
265 Correlation tests were then realized to interpret the PCA plot.

266

267 **3. Results**

268 **3.1. Sediment characteristics**

269 Sediment used in this experiment had mean silt (particles < 63 μm) and water content of 25.6
270 % and 40.5 %, respectively. The addition of freeze-dried MPB induced an increase in nitrogen and
271 carbon contents in sediment (table 1) resulting in an increasing C/N ratio from 10.3 to 10.9. The MPB
272 addition also led to an increase of labile carbon, as illustrated by the increase of EPS contents, and in an
273 increase of the chl *a* content of 42%, 202%, and 402% respectively in M1, M5, and M10 compared to
274 M0. The $\delta^{13}\text{C}$ showed higher values for M5 and M10 (on average -24.4 ± 0.4) than for M0 and M1 (on
275 average -25.5 ± 0.4).

276 After the 357 hours incubation of the different reactors under anoxic conditions and with
277 continuous supply of NO_3^- , the different organic carbon contents (total C_{org} , Chl *a*, EPS) in sediments
278 showed lower values in comparison to values before incubation (table 1). The greatest difference in
279 sediment characteristics before and after the experiment was observed for LMW carbohydrate EPS
280 (table 1), which decreased by 61 -71% over the course of the incubations. Similarly, HMW carbohydrate
281 EPS and HMW proteins also decreased by 14-41% and 22-64%, respectively. In contrast, the LMW
282 proteins increased over the course of the incubation by 7-30%. The chl *a* content also decreased by 4-
283 38% while the phaeopigment content increased by 13-178%. Nitrogen and carbon contents decreased
284 by 16-25% and 18-27% respectively leading to low variation of the C:N ratios up to 7%. The $\delta^{13}\text{C}$ only
285 decreased by 0.4-1.3%.

286

287 **3.2. Nitrate reduction and nitrite release rates**

288 The constant supply of NO_3^- to the sediment in the control condition (M0) resulted in net NO_3^-
289 reduction rates with an average of $150 \pm 3 \text{ nmol NO}_3^- \text{ cm}^{-3} \text{ h}^{-1}$ during the first 24 hours of experiment.
290 These rates then decreased significantly (RM-aov; p-value < 0.001) over the duration of the 357 hours
291 of incubation (fig. 1-A) down to $19 \pm 6 \text{ nmol NO}_3^- \text{ cm}^{-3} \text{ h}^{-1}$ the last day of experiment (fig. 2-A). In
292 parallel, the net NO_2^- release rate increased over the first 100 hours to a maximum value of $35 \pm 4 \text{ nmol}$
293 $\text{NO}_2^- \text{ cm}^{-3} \text{ h}^{-1}$ (fig. 1-B). After that, the NO_2^- release rates decreased to an average of $14 \pm 3 \text{ nmol}$
294 $\text{NO}_2^- \text{ cm}^{-3} \text{ h}^{-1}$ the last day of experiment (fig. 2-B).

295 The same NO_3^- supply with addition of MPB to the sediment resulted in significantly higher net
296 NO_3^- reduction rates for each condition correlating positively with the amount of MPB added (Split-plot
297 aov; p-values < 0.001). In comparison to the control M0, the net NO_3^- reduction rates significantly
298 increased (RM-aov; p-values < 0.001) over the first 100 hours of incubation to a maximum of 276, 383,
299 and 400 $\text{nmol NO}_3^- \text{ cm}^{-3} \text{ h}^{-1}$ respectively for M1, M5, and M10 (fig. 1-A). These rates corresponded
300 respectively to an increase of 85%; 156% and 267% compared to M0. Then, net NO_3^- reduction rates
301 significantly decreased (RM-aov; p-values < 0.001) down to 71 ± 10 , 97 ± 1 , and $140 \pm 26 \text{ nmol}$
302 $\text{NO}_3^- \text{ cm}^{-3} \text{ h}^{-1}$ at the end of the experiment respectively for M1, M5, and M10, but remaining significantly
303 higher than M0 (aov; p-value < 0.001; fig. 2-A). The NO_2^- release rates were also significantly higher
304 in amended conditions than in M0 (Split-plot aov; p-values < 0.001) over the 100 first hours of
305 incubation reaching maximal values of 124 ± 11 , 119 ± 20 , and $148 \pm 17 \text{ nmol NO}_2^- \text{ cm}^{-3} \text{ h}^{-1}$ respectively
306 for M1, M5 and M10 (Fig. 1-B). During the rest of the experiment, NO_2^- release rates decreased and no
307 significant differences were recorded the last day of the experiment (Kruskal-Wallis; p-values = 0.44,
308 0.07, and 0.45 respectively for M1, M5, and M10) with values of 15 ± 3 , 14 ± 4 , and $32 \pm 5 \text{ nmol}$
309 $\text{NO}_2^- \text{ cm}^{-3} \text{ h}^{-1}$ respectively for M1, M5, and M10 (fig. 2-B).

310

311 **3.3. Estimation of carbon consumption for denitrification**

312 By considering the C_{org} originated from the added MPB (C_{org} content of 4%), it was estimated that
313 its addition has led to an increase of 22, 108, and 216 mg of C_{org} from MPB origin (respectively in M0,
314 M1, M5, M10). With an average NO_3^- reduction rate of $73 \text{ nmol cm}^{-3} \text{ h}^{-1}$, it was estimated that a total of
315 approximately 10 mg N- NO_3^- was reduced in the non-amended control reactor (27.7 cm^3), over the
316 duration of the experiment (357 hours). In the same way, approximately 17, 28, and 44 mg of N- NO_3^-
317 were reduced respectively for M1, M5, and M10. Knowing that 5 moles of carbon are used during the
318 complete denitrification process when 4 moles of nitrogen are reduced to nitrogen gas ($4\text{NO}_3^- + 5\text{CH}_2\text{O}$
319 $+ 4\text{H}^+ \rightarrow 5\text{CO}_2 + 2\text{N}_2 + 7 \text{H}_2\text{O}$), and assuming that denitrification was the only N-reducing process, on
320 average 11, 18, 30, and 47 mg of carbon were respectively used for denitrification in M0, M1, M5, and
321 M10.

322 Considering the total amount of carbon consumed during the two-week experiment (table 1; 510,
323 380, 590, and 490 mg), it can be thus be estimated that approximately 2.1, 4.7, 5.1, and 9.7% of the
324 organic carbon degraded during the two-weeks incubation could have been used for denitrification

325 (respectively in M0, M1, M5, M10). In addition to this, as it was the added MPB carbon that drove the
326 increase in net NO_3^- reduction rates, it is hypothesized that the majority of the added MPB was used in
327 M1 (82%) and approximately 28 and 22% in M5 and M10.

328 Knowing that the proportion of carbon in glucose represents 46.7% of the molecular weight and that
329 carbohydrate EPS were expressed in glucose equivalents, it was estimated that LMW carbohydrate EPS
330 represented between 0.77 to 1.14% of the total carbon consumed while HMW carbohydrates EPS
331 represented between 0.27 and 0.35% for the different conditions (M0 to M10). By considering that all
332 consumed EPS were used for denitrification, it was roughly estimated that LMW carbohydrates
333 represented 36, 24, 17, and 12% of the carbon consumed for denitrification (18, 30, and 47 mg of carbon
334 previously estimated) and 14, 8, 2, and 3% for HMW carbohydrates.

335

336 **3.4. Ammonium release rate**

337 The control sediment (M0) showed a mean net NH_4^+ release rate of $7 \pm 4 \text{ nmol NH}_4^+ \text{ cm}^{-3} \text{ h}^{-1}$ with no
338 significant variability (RM-aov; p-value = 0.52) along the duration of the experiment (fig. 3). In MPB
339 amended sediments, significantly higher net NH_4^+ release rates were recorded for each condition (Split-
340 plot aov; p-value < 0.05 for M1 and p-values < 0.001 for M5 and M10), positively correlated to the
341 amount of MPB added (fig. 3). More specifically, after addition of MPB, a significant increase of net
342 NH_4^+ release rates (RM-aov; p-values < 0.05) was recorded over the first 100 hours of incubation up to
343 a maximum of 18 ± 1 , 73 ± 2 , and $127 \pm 14 \text{ nmol NH}_4^+ \text{ cm}^{-3} \text{ h}^{-1}$ respectively for M1, M5, and M10.
344 After that, net NH_4^+ release rates decreased over the duration of the experiment. The last day of the
345 experiment, the net NH_4^+ release rates were not significantly different for M0, M1, and M5 (Kruskal-
346 Wallis; p-values = 0.48 and 0.13 respectively for M1 and M5) which presented on average values of 1.6
347 ± 0.5 , 1.7 ± 0.7 and $8.7 \pm 1.2 \text{ nmol NH}_4^+ \text{ cm}^{-3} \text{ h}^{-1}$ respectively (fig. 4). In contrast, M10 showed a
348 significant higher net NH_4^+ release rates (Kruskal-Wallis; p-value < 0.05) with $13.9 \pm 2.6 \text{ nmol NH}_4^+$
349 $\text{cm}^{-3} \text{ h}^{-1}$ (fig. 4).

350

351 **3.5. Microbial denitrifying communities**

352 Approximately 3x more DNA was extracted from sediments at the end of the two week incubation
353 (between 13.8 and 19.3 $\mu\text{gDNA gDSW}^{-1}$; table 2) than at the beginning (4.3 $\mu\text{gDNA gDSW}^{-1}$). Using
354 quantitative PCR (qPCR) to measure the gene abundance, the initial sediment sample showed on average
355 $6.3 \times 10^7 \pm 1.7 \times 10^7$ copies of *nir* gene gDSW^{-1} and on average $3.7 \times 10^9 \pm 0.6 \times 10^9$ copies of *nosZ* gene
356 gDSW^{-1} . Compared to the initial sediment, after the 357 hours of incubation, the numbers of *nir*
357 increased two orders of magnitude (from 10^7 to 10^9) and one order of magnitude for *nosZ* gene copy
358 numbers (table 2). With the MPB addition, the increase of *nir* genes (fig. 5-A) was even higher with an
359 increase of 52%, 101%, and 160% compared to M0 respectively for M1, M5, and M10. For the *nosZ*
360 genes (fig. 5-B), no difference was observed between treatments. Both functional genes exhibited a
361 change in gene contribution over the course of the incubation: for *nir* genes, the *nirS* contribution

362 increased in default of *nirK* while for *nosZ* genes, clade I *nosZ* increased in default to clade II *nosZ*
363 (table 2; fig. 5).

364

365 **3.6. Relationship between variables during the incubation experiment**

366 A principal component analysis (PCA) was performed on the values representing the differences
367 between the parameters before and after experimentation for sediment characteristics (table 2; C, N, Chl
368 a, and EPS consumed besides phaeopigment and protein LMW EPS produced. In addition, the amount
369 of NO_3^- reduced (NR) and the quantities of NH_4^+ and NO_2^- released (AR and NiR) during the
370 experimental time were added as well as the difference in the abundance of denitrification-related genes
371 (*nirK*, *nirS*, clade I *nosZ*, and clade II *nosZ*).

372 The first and second dimension of the PCA explained 83.7% of the total inertia (fig. 6). The first
373 dimension, with 57.06% of the variance showed the highest contributions for the LMW carbohydrates
374 consumption, NO_3^- reduction, NH_4^+ and NO_2^- release and the denitrifying gene abundances. In detail,
375 the LMW carbohydrates accounted for 11.6%, NO_3^- reduced for 11.6%, NH_4^+ and NO_2^- released
376 respectively for 11.2% and 9% while the microbial denitrifying communities accounted for 8.5%,
377 11.7%, 6.4%, and 9.7% respectively for *nirK*, *nirS*, clade I *nosZ*, and clade II *nosZ*. The second
378 dimension (26.65% of variance) showed the highest contributions for the sedimentary nitrogen and
379 carbon consumed (19.1% and 14.2% respectively), the quantity of HMW EPS (19.2% and 15.8%
380 respectively for carbohydrates and proteins), chlorophyll *a* consumed (15%). The third dimension
381 (16.3% of variance) showed the highest contribution for the phaeopigments released (33.5%).

382 The correlation tests allowed interpretation of the PCA plot showing significant positive
383 correlations between the number of *nirS* gene copies number and both the NO_3^- reduced (Pearson corr.
384 coef. = 0.99; p-value < 0.01; $R^2 = 0.99$) and the NH_4^+ released (Pearson corr. coef. = 0.97; p-value <
385 0.05; $R^2 = 0.95$). In contrast, the *nirS* gene copies number showed a negative correlation with the LMW
386 carbohydrates (Pearson corr. coef. = - 0.99; p-value < 0.01; $R^2 = 0.99$). As a consequence, the LMW
387 carbohydrates showed negative correlations with both the NO_3^- reduced (Pearson corr. coef. = -0.98; p-
388 value < 0.05; $R^2 = 0.97$) and the NH_4^+ released (Pearson corr. coef. = -0.96; p-value < 0.05; $R^2 = 0.92$)
389 while a positive correlation was recorded between the NO_3^- reduced and the NH_4^+ released (Pearson
390 corr. coef. = 0.99; p-value < 0.05; $R^2 = 0.98$). A negative correlation was also observed between the
391 clade II *nosZ* gene copies number and the NO_2^- released (Pearson corr. coef. = -0.95; p-value = 0.05; R^2
392 = 0.90). The other Pearson correlations tested between variables were not significant (p-values > 0.05).

393

394 **4. Discussion**

395 Our results confirm by using a controlled flow through reactor (FTR) experiment that NO_3^- reduction
396 in intertidal sediments is limited by labile carbon availability, and highlight the important contribution
397 of the microphytobenthos (MPB) on NO_3^- reduction rates and on the denitrifying microbial
398 communities.

399 The chlorophyll *a* concentrations obtained upon addition of MPB in this study (between 8 and
400 29 $\mu\text{g chl } a \text{ gDSW}^{-1}$) were higher than the biomass usually recorded on the sampled mudflat ranging
401 from 0.2 to 11.2 $\mu\text{g chl } a \text{ gDW}^{-1}$ (Morelle et al. 2020). Nevertheless, these values were lower than the
402 range of MPB biomass measured up to 80 $\mu\text{g chl } a \text{ gDSW}^{-1}$ in some other European and American
403 estuarine systems (Underwood and Kromkamp 1999). As in a majority of estuarine systems, NO_3^-
404 concentrations are elevated (between 0.5 and 7 mg L^{-1}) in the Seine estuarine waters (Morelle et al.
405 2018). Thereby, our results suggest that the microbial activity is especially limited by the availability of
406 the labile carbon (MPB) in associated mudflats than by NO_3^- availability. Indeed, the highest addition
407 of MPB led to a maximal NO_3^- reduction rate of 5.6 $\text{mgN-NO}_3^- \text{ L}^{-1} \text{ h}^{-1}$ (400 $\text{nmol NO}_3^- \text{ cm}^{-3} \text{ h}^{-1}$).
408 Moreover, by confirming that bacteria preferentially use decomposing MPB and labile extracellular
409 polymeric substances as a carbon substrate for NO_3^- reduction, our results confirm that the diminution
410 of natural mudflat surface which entail decline of the microphytobenthos growth (Lesourd et al. 2016;
411 Morelle et al. 2020) must have drastically reduce the potential NO_3^- removal capacities in estuaries.

412

413 **4.1. Microphytobenthos stimulates nitrate reduction**

414 The increase in $\delta^{13}\text{C}$ observed in M5 and M10 confirmed the addition of MPB in sediment which
415 is known to present a higher $\delta^{13}\text{C}$ than intertidal sediment (Lee et al. 2021). The highest addition of MPB
416 performed in this study (10% total sediment weight) led to a threefold increase of NO_3^- reduction rates
417 (up to 267%) compared to the control condition. With MPB addition corresponding to 1% and 5% of
418 the total sediment weight, the net NO_3^- reduction rates showed an increase of 85% and 156%. These
419 increases highlight the important contribution of the MPB to NO_3^- reduction which could even represent
420 between 22% (M10) and 82% (M1) if denitrification is considered as the only NO_3^- reduction process
421 and responsible for the carbon degradation. As the increase in net NO_3^- reduction rates were observed
422 during the first 100 hours of the experiment before net NO_3^- reduction rates decreased, it confirms the
423 rapidly and labile nature of the major compounds of the MPB. MPB biomass represents indeed a labile
424 carbon source, without recalcitrant compounds like cellulose and lignin (Kawaida et al. 2019). MPB
425 contributes to sediment organic matter through carbohydrates, proteins, phospholipid linked fatty acids,
426 hydrolysable amino acids, or pigments including chlorophyll (Hardison et al. 2013).

427 The EPS present in MPB add a significant value for heterotrophic processes since they contain
428 labile organic carbon (Van Duyl et al. 1999; Welker et al. 2002; Tobias et al. 2003). Indeed, in addition
429 to an overall decrease in organic carbon with MPB addition, we observed a decrease in both
430 carbohydrate and protein EPS (on average 68% and 28% for LMW and HMW carbohydrates and
431 approximately 46% of the HMW proteins). Previous studies suggested that the LMW fraction is the
432 most labile and immediately used by bacteria rather than HMW polymers which require more energy in
433 producing exoenzymes for hydrolytic activity (Sundh 1992; Welker et al. 2002). This could explain the
434 higher decrease of LMW rather than HMW carbohydrates. Moreover, the quantities of LMW
435 carbohydrates consumed were significantly higher in amended sediments while the decrease in amount

436 of HMW carbohydrates showed no significant differences with the control. These results combined with
437 the increase in NO_3^- reduction rates suggest that EPS, and especially LMW carbohydrates, are being
438 used as a carbon substrate for NO_3^- reduction in this experiment. If considering denitrification as the
439 only NO_3^- reduction process, our result even suggest that carbohydrates EPS could contributed between
440 14 and 50% to the carbon consumption for denitrification. It is more complicated to estimate the
441 contribution of protein EPS, but this fraction of EPS often represents a minor part of the total EPS
442 content (Morelle et al. 2020) and thus must represent a smaller part of the consumed carbon. Especially
443 since in our experiment the LMW proteins EPS increased by about 22%. This could be the result of the
444 degradation of HMW proteins since LMW compounds are considered as the hydrolytic results of HMW
445 EPS breakdown (Orvain et al. 2014). The protein production could also be related to the development
446 of the microbial biomass which could also have secreted EPS and especially proteins to form a biofilm
447 matrix (Gerbersdorf and Wieprecht 2015). Overall, it seems that carbohydrates were more easily used
448 than protein EPS which is in agreement with previous results that consider carbohydrates as the most
449 readily available organic fraction and labile compounds from MPB (Welker et al. 2002). Nevertheless,
450 our results show that a large amount of carbon disappears without NO_3^- being used as an electron
451 acceptor since the amount of carbon used is higher than the amount required for the performed NO_3^-
452 reduction. Part of this can be explained by the use of carbon with other electron acceptors present in the
453 sediment (iron oxides) or fermentation (Abell et al. 2009), whereas another part of the organic carbon
454 may have leached from the sediment.

455 Our results also suggest that other degraded MPB cell components contribute to the carbon
456 budget of these sediments. As NO_3^- reduction rates increased, chl *a* content decreased and phaeopigment
457 content increased. The increase in phaeopigments, the degraded form of chl *a*, suggests an increase of
458 the algal biomass degradation which can then be used as carbon source for NO_3^- reduction. This agrees
459 with a previous study, showing a stimulation of denitrification rates by the addition of senescing algal
460 biomass providing N and labile C to denitrifiers in agricultural headwater stream sediments (McMillan
461 et al. 2010). However, the MPB added was a natural biofilm that may have contained other sources of
462 carbon not from microalgae origin which could have been used as carbon source for degradation. The
463 increased NH_4^+ release in the higher MPB treatments (M5 and M10) suggests that more organic matter
464 was decomposed in these treatments, as NH_4^+ is a primary product of organic matter decomposition
465 (Lemley et al. 2014). The higher net NO_3^- reduction rates measured in M1 compared to M0, whereas the
466 net NH_4^+ release rates were similar, suggests the use of organic carbon releasing less NH_4^+ such as
467 glucose or other organic compounds lacking an amino ($-\text{NH}_3^+$) group thus resulting in relative low
468 ammonification. Overall, these results confirm experimentally the assumption that microbes use both
469 decomposing MPB (Nielsen et al. 2004) and labile EPS as a carbon substrate for NO_3^- reduction (Tobias
470 et al. 2003; Hope et al. 2020).

471 In parallel to denitrification, part of the NO_2^- produced upon NO_3^- reduction could have been
472 partly reduced to NH_4^+ via DNRA or used through the anammox process. DNRA conserves nitrogen in

473 the ecosystem as NH_4^+ , therefore not resulting in the production of N_2O . The net $\text{NH}_4^+:\text{NO}_3^-$ rates ratio
474 illustrates either the transformation of NO_3^- into NH_4^+ or the NH_4^+ released after degradation of organic
475 matter. After the 2-week incubation, the percentage of NH_4^+ produced per NO_3^- reduced increased with
476 the proportion of MPB added (2%, 9% and 10% respectively for M1, M5, and M10) but was comparable
477 to M0 (9%). The reduction of NO_3^- , via DNRA would lead to an elevated ratio of NH_4^+ produced per
478 NO_3^- reduced. A rough estimate shows that considering a C/N ratio ranging between 9 and 12 for the
479 organic matter and added biomass, an organic carbon molecule oxidized would release between 0.08
480 and 0.11 molecules of NH_4^+ . Per molecule NO_3^- reduced to N_2 , 1.25 molecules of carbon are oxidized
481 resulting in 0.1 to 0.14 NH_4^+ molecules produced per NO_3^- reduced or a ratio of 10 to 14%. This is in
482 good agreement with the net $\text{NH}_4^+:\text{NO}_3^-$ rates ratios (between 9 to 10%) determined, indicating that
483 DNRA is unlikely to play an important role in these benthic sediments. The anammox process could
484 also have partly used the NH_4^+ and NO_2^- released and have produced N_2 but this study does not allow us
485 to quantify this process.

486

487 **4.2. The impact of microphytobenthos on microbial abundance and denitrification efficiency.**

488 Our results confirm that microbes use an important part of the available carbon from MPB
489 (Soares and Rousk 2019) which is reinforced by the correlations observed at the end of the experiment
490 between the copy numbers of *nirS* gene and both the LMW carbohydrates EPS and the NO_3^- reduced.
491 The increase in microbial DNA extracted between the initial sediment and sediments incubated for 2
492 weeks under NO_3^- reducing conditions, including the non MPB-amended control, prove increase of the
493 microbial biomass by means of DNA quantification (Semenov et al. 2018). Moreover, the significant
494 increase in microbial activity as net NO_3^- reduction rates upon the addition of NO_3^- and MPB, with a
495 strong impact of the amount of added MPB as shown in Figure 2A, confirms the growth of NO_3^-
496 reducers. This was also reflected by the increase in the number of functional gene copies for *nirK/nirS*
497 and *nosZ* which indicates active growth of denitrifying microbes in these estuarine sediments (Smith et
498 al. 2007).

499 Whereas the NO_2^- reductase gene copy numbers (*nir*) show a significant increase with the MPB
500 additions, the total nitrous oxide reductase (*nosZ*) gene copy numbers remain unchanged (Table 2,
501 Figure 5). Interestingly, the increase in NO_2^- reductase gene copy numbers is mainly due to an increase
502 in *nirS* gene copies, whereas *nirK* gene copies significantly decreased. The denitrifiers possessing *nirS*
503 genes developed and grew faster than those possessing *nirK* genes upon the addition of the MPB
504 biomass. This shows that the imposed conditions (anoxic, NO_3^- supply) and the increasing amounts of
505 organic carbon in the form of MPB favored the growth of organisms harboring *nirS* gene. A more
506 important abundance of *nirS* gene correlated to high NO_3^- load was already observed by Lee and Francis
507 (2017) in different sediments of the San Francisco Bay. This result of the current study corroborates that
508 these variables (anoxia, NO_3^- supply, MPB) play a significant role in the capacity of the sediment to
509 reduce NO_3^- via denitrification (Papaspyrou et al. 2014). It has been suggested that bacteria possessing

510 *nirS* genes are generally complete denitrifiers rather than *nirK* containing organisms (Graf et al. 2014);
511 this might indicate that the bacterial growth upon MPB biomass stimulated those capable of full
512 denitrification. This would be in line with the slight increase in gene copy number of clade I *nosZ* which
513 are more likely to be complete denitrifiers, as 83% of genomes with clade I *nosZ* also possess *nir* genes
514 (Graf et al. 2014; Hallin et al. 2018). In comparison, microbes harboring clade II *nosZ* genes are also
515 called “non-denitrifier” nitrous oxide reducers as these microbes often lack NO_3^- and NO_2^- reductase
516 genes (Bertagnolli et al. 2020). The stimulation of organisms carrying out full denitrification during the
517 incubation period and related to the addition of organic carbon would require a metagenomics analysis,
518 allowing reconstruction of genomes and confirmation of an increase of ‘complete denitrifiers’.

519 Compared to the initial sediment, *nir* copy numbers at the end of the incubation increased two
520 orders of magnitude (from 10^7 to 10^9) and one order of magnitude for *nosZ* copy numbers at the end of
521 the incubation. However, gene copy numbers of *nosZ* exceeded those of *nirS* and *nirK* in both the initial
522 and incubated sediment. Whether the difference in gene copy numbers of the different reductases can
523 be translated in different activities is unclear. The increase of NO_2^- reductases upon the production of
524 NO_2^- appears efficient as little NO_2^- accumulated. The subsequent production and reduction of nitrous
525 oxide gas was not determined but the high abundance of *nosZ* gene copy numbers (compared to *nir* gene
526 copy numbers) leads to believe that they were initially sufficient to ensure the reduction of nitrous oxide.

527 The total number of *nir* genes (*nirK* and *nirS*) showing increasing values in relation with the
528 amount of added MPB (fig. 5) highlights that presence of organic carbon from microphytobenthic origin
529 positively influenced the growth of NO_2^- reducers and their potential reduction to NO. Considering that
530 the amount of NO_2^- produced is a trigger for NO_2^- reductases, the increase of NO_3^- reduction rates (or
531 total NO_2^- release rates) is in good agreement with the increase of the total number of *nir* gene copy
532 numbers. This is reinforced by the net NO_2^- release rates which remain low along the experiment
533 ($< 100 \text{ nmol NO}_2^- \text{ cm}^{-3} \text{ h}^{-1}$), implying that most of the NO_3^- reduced to NO_2^- is reduced further.
534 Difference between the total and net NO_2^- release rates suggests that 66 – 72 % of the NO_2^- was further
535 reduced to NO (N_2O and N_2) or other (e.g. NH_4^+). This calculation considers the initial accumulation of
536 NO_2^- which is a common phenomenon usually explained via the induction of NO_2^- reduction genes upon
537 an increase in NO_2^- (Betlach and Tiedje 1981). At the end of the two-week incubation, the proportion of
538 NO_3^- reduced to NO_2^- (estimated by the net $\text{NO}_2^-:\text{NO}_3^-$ rates ratio), and thus not further reduced to N_2 or
539 NH_4^+ , was higher in the non-amended sediment (on average 80 %). This ratio was lower when MPB
540 was added, between 14 to 23% for the different additions, indicating a higher reduction of NO_2^- into N_2
541 or NH_4^+ . This is also in good agreement with the increasing numbers of *nirS* gene copy numbers and a
542 slight increase of the clade I *nosZ* genes indicating a significant role and increase of denitrification upon
543 the addition of MPB biomass as carbon source.

544

545 **5. Conclusion**

546 This study experimentally demonstrated, in Seine estuary sediments, that microphytobenthos plays
547 a crucial role in benthic nitrogen fluxes via nitrate reduction and especially denitrification. This crucial
548 role being made through the use, by denitrifiers, of the labile extracellular polymeric substances secreted
549 by the MPB which might contribute up to 50% of the carbon used for denitrification. But also, through
550 the use of decomposing microphytobenthic components. In addition, the results showed that addition of
551 microphytobenthos led to a significant shift in the denitrifier community composition favoring complete
552 denitrifiers and the complete reduction of NO_3^- into N_2 leading to efficient nitrogen removal from waters.
553 Beyond the role played by intertidal mudflats and microphytobenthos on nitrogen cycling, the potential
554 role of adjacent intertidal salt marshes to carry out NO_3^- elimination processes was also highlighted in
555 this estuary (Laverman et al. 2021). These results are thus all the more important in the context of climate
556 change since the increase in extreme climatic events and sea level rise represent a serious threat to the
557 presence of many estuarine intertidal mudflats, adjacent salt marshes and their associated ecological
558 values.

559

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569

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Table 1. Sediment characteristics before the FTR experiment and differences observed after the FTR experiment (357-h of incubation), for the unamended sediment (M0) and the 3 MPB amended sediments (M1, M5 and M10) all with a constant nitrogen supply (10 mM KNO₃). With the percentage of nitrogen (N) and carbon (C), the corresponding weight of carbon inside a reactor (g react⁻¹), the chlorophyll a and phaeopigment content (chl a and phaeo in µg gDSW⁻¹), and the EPS contents for low and high molecular weight carbohydrates and proteins (in µg gDSW⁻¹). The quantity of carbon per reactor was calculated considering the mean weight of 54.1g of sediment inside reactors

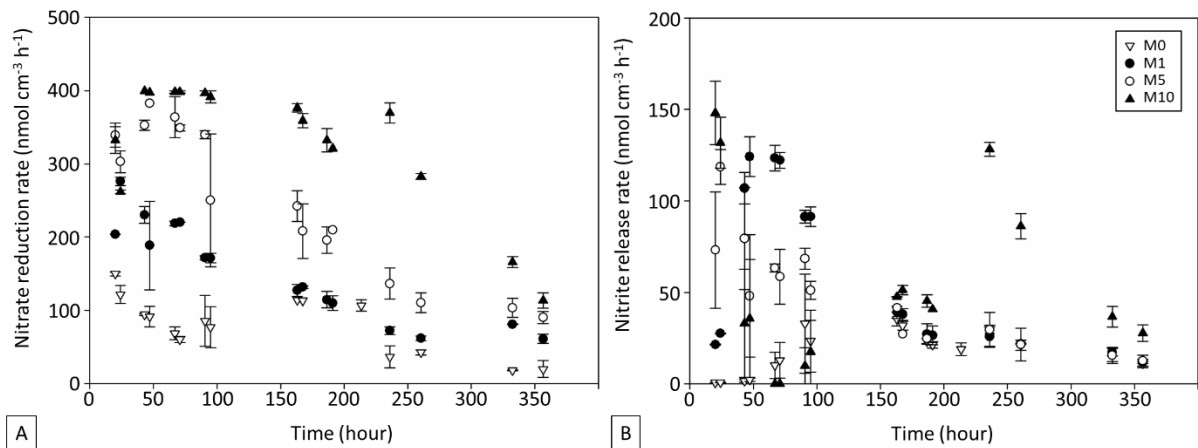
	Elementary Analyses				Photopigments		EPS (µg gDSW ⁻¹)				
	N (%)	C (%)	C g react ⁻¹	δ ¹³ C	C:N	Chl a (µg gDSW ⁻¹)	Phaeo (µg gDSW ⁻¹)	Carbohydrates LMW	Carbohydrates HMW	Proteins LMW	Proteins HMW
Sediment characteristics before experimentation											
M0	0.43	3.81	2.06	-25.5	10.3	5.7	12.9	255.0	147.1	5.5	11.5
M1	0.43	3.85	2.08	-25.6	10.4	8.1	31.5	260.0	154.6	5.7	11.6
M5	0.44	4.01	2.17	-24.8	10.6	17.2	39.1	279.8	184.9	6.3	11.9
M10	0.45	4.21	2.28	-24.0	10.9	28.6	48.6	304.5	222.8	7.1	12.2
Differences observed after the experiment											
M0	-0.10 (-23%)	-0.95 (-25%)	0.51	-0.1 (-0.4%)	-0.2 (-2.2%)	-2.15 (-38%)	+23.1 (+178%)	-156.3 (-61%)	-60.2 (-41%)	+0.4 (+07%)	-6.7 (-58%)
M1	-0.08 (-19%)	-0.71 (-18%)	0.38	-0.1 (-0.4%)	+0.0 (+0.2%)	-2.38 (-29%)	+4.1 (+13%)	-173.3 (-67%)	-53.6 (-35%)	+1.7 (+30%)	-7.4 (-64%)
M5	-0.11 (-25%)	-1.09 (-27%)	0.59	-0.1 (-0.4%)	-0.3 (-2.9%)	-0.63 (-4%)	+6.5 (+17%)	-199.1 (-71%)	-25.9 (-14%)	+1.3 (+20%)	-2.6 (-22%)
M10	-0.07 (-16%)	-0.90 (-21%)	0.49	+0.3 (+1.3%)	-0.7 (-6.9%)	-6.39 (-22%)	+18.5 (+38%)	-217.3 (-71%)	-51.3 (-23%)	+2.1 (+30%)	-5.2 (-42%)

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Table 2. Average of the abundance of the microbial denitrifiers quantified via *nir* (±sd; n=12) and *nosZ* gene copies (±sd; n=6). With the quantity of extracted DNA per ng of dry sediment, for the *nir* genes, the contributions of *nirK* and *nirS* and for *nosZ* genes, the contributions of the clades I & II *nosZ*.

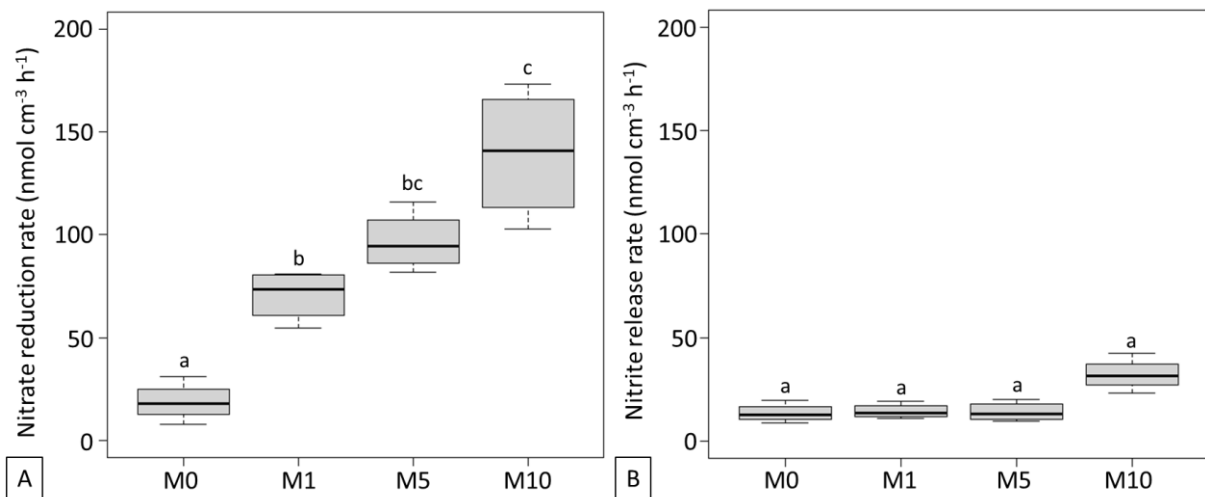
	µg ext. DNA gDSW ⁻¹	Nb of <i>nir</i> gene copies gDSW ⁻¹	% of <i>nirK</i>	% of <i>nirS</i>	Nb of <i>nosZ</i> gene copies gDSW ⁻¹	% of Clade I <i>nosZ</i>	% of Clade II <i>nosZ</i>
IS	4.3	6.3×10 ⁷ ± 1.7×10 ⁷	11.8	88.2	3.7×10 ⁹ ± 0.6×10 ⁹	73.4	26.6
M0	13.8	6.2×10 ⁹ ± 3.2×10 ⁹	6.6	93.4	30.9×10 ⁹ ± 1.3×10 ⁹	77.9	22.1
M1	15.8	9.5×10 ⁹ ± 3.1×10 ⁹	5.6	94.4	27.3×10 ⁹ ± 2.7×10 ⁹	80.6	19.4
M5	19.3	12.5×10 ⁹ ± 4.0×10 ⁹	2.7	97.3	28.0×10 ⁹ ± 5.5×10 ⁹	82.6	17.4
M10	18.6	16.2×10 ⁹ ± 7.0×10 ⁹	1.6	98.4	34.3×10 ⁹ ± 5.1×10 ⁹	87.2	12.8

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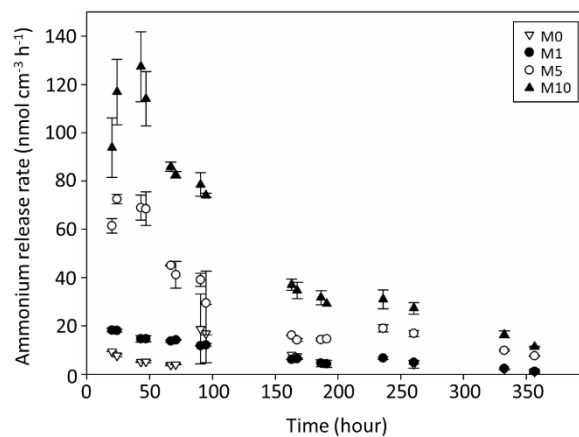
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Figure 1. Average (A) nitrate reduction rates and (B) nitrite release rates along the 357 hours of incubation ($n=2$) for the unamended sediment (M0) and the 3 MPB amended sediments (M1, M5 and M10) all with a constant nitrate supply (10 mM KNO_3).



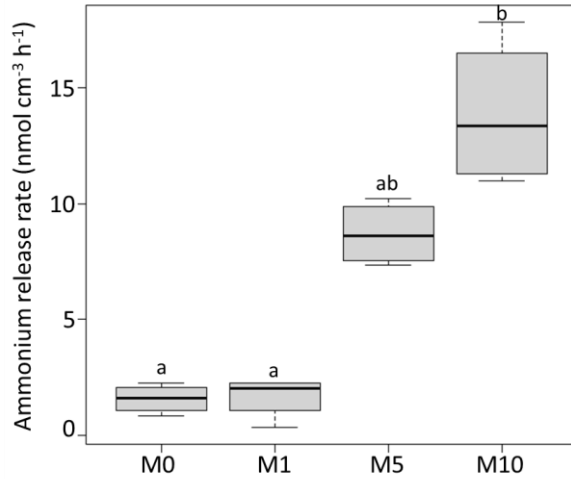
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Figure 2. A. Boxplot of nitrate reduction rates (A) and nitrite release rates (B) at the end of the experiment (332- 356 hours; $n=4$) for the unamended sediment (M0) and the 3 MPB amended sediments (M1, M5 and M10) all with a constant nitrate supply (10 mM KNO_3). The letters above the boxes correspond to the statistical results of an anova followed by a post-hoc test; a different letter represents a significant difference between treatments.



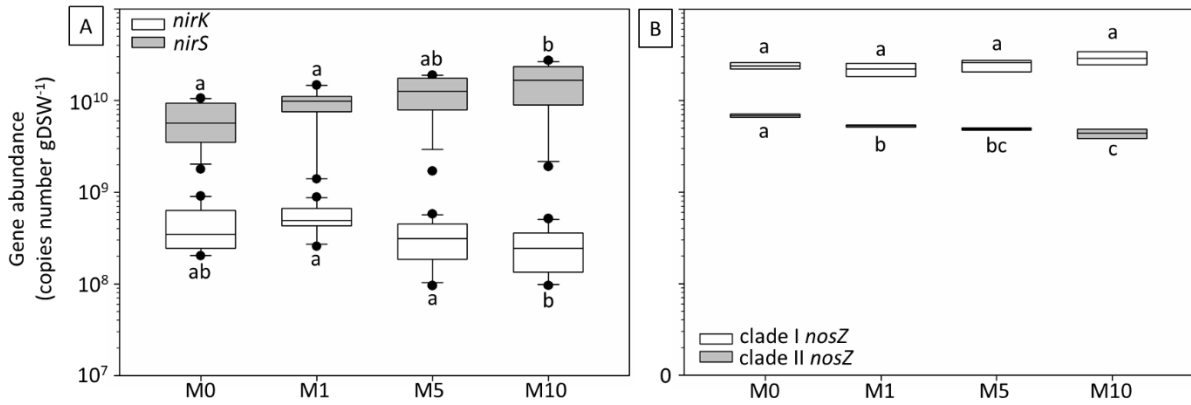
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Figure 3. Average ammonium release rates along the 357 hours of incubation ($n=2$), for the unamended sediment (M0) and the 3 MPB amended sediments (M1, M5 and M10) all with a constant nitrate supply (10 mM KNO_3).



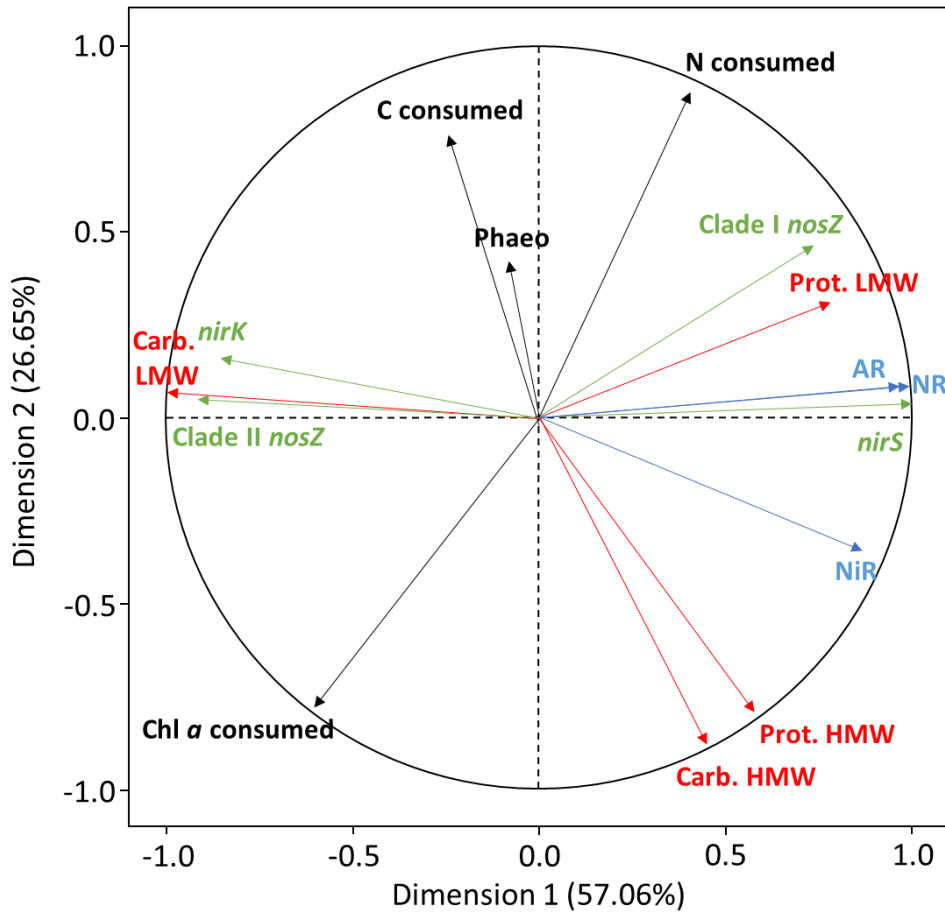
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Figure 4. Average ammonium release rates at the end of the experiment (332- 356 hours; $n=4$), for the unamended sediment (M0) and the 3 MPB amended sediments (M1, M5 and M10) all with a constant nitrate supply (10 mM KNO_3).



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Figure 5. Denitrification genes abundance for (A) the *nir* genes (*nirK* in white and *nirS* in grey; $n=12$) encoding the reduction of nitrite to nitric oxide and (B) The *nosZ* genes (*nosZ* clade I in white and *nosZ* Clade II in grey, $n=6$) encoding the nitrous oxide reductase, for the unamended sediment (M0) and the 3 MPB amended sediments (M1, M5 and M10) all with a constant nitrogen supply (10 mM KNO_3).



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Figure 6. A. Principal component analyse (PCA) on values representing the differences between before and after incubation in the FTR (table 2). With the percentage of nitrogen (N) and carbon (C) consumed, the quantity of phaeopigment produced (Phaeo); the quantity of chlorophyll a (Chl a), and EPS consumed (Carb. HMW; Carb. LMW; Prot. HMW) or produced (Prot. LMW); the microbial denitrifying communities (difference in relative abundance of nirK, nirS and nosZ clade 1&2); the quantity of nitrate consumed (NR), and the quantities of nitrite and ammonium released (NiR and AR respectively).