

1 **Influence of restored mussel reefs on denitrification in**
2 **marine sediments**

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14 **Abstract**

15 Globally we have lost 85% of shellfish reefs, with the concomitant loss of ecosystem
16 services. Restoration provides opportunities to regain lost services and engage society in the
17 wider ecosystem benefits of restoration. Previous research has demonstrated that the
18 functional role of shellfish can be context dependent, and we therefore measured fluxes of
19 nutrients and dissolved gases from four restored mussel reefs along a grain size gradient in
20 New Zealand as a metric of success in ecosystem function. This globally novel research
21 found that restored mussel reefs were successful in enhancing denitrification. Mussel reefs
22 can therefore be important in reducing the risk of future eutrophication, but the importance of
23 nutrient remineralisation varies in response to organism patchiness, flow conditions and
24 background nutrient concentrations. These data show that mussel reef creation can quickly
25 restore ecosystem function, but site selection to enhance specific services is important. This
26 study provides evidence of a previously unrealised ecosystem service provided by restored
27 mussel beds that allows future restoration efforts to better target areas in order to have the
28 largest impact on the removal of anthropogenic nitrogen.

29 **Keywords:** ecosystem services; denitrification; biogeochemical processes; grain size
30 gradient; restoration

31 **1. Introduction**

32 In coastal ecosystems, biogeochemical cycling of nitrogen (N), phosphorus (P), and oxygen
33 (O₂) is regulated by the benthos (Dale and Prego, 2002; Fulweiler et al., 2010), and coasts
34 and shelf ecosystems are disproportionately important in these processes (Codispoti, 2007;
35 Galloway et al., 2008; Seitzinger and Phillips, 2017). These coastal ecosystems sit at the
36 land-sea interface and are therefore influenced by a wide range of pressures from both
37 terrestrial and marine sources (Thrush et al., 2013). The coastal benthos is dominated by soft-

38 sediments (Snelgrove, 1999) and pressures (e.g. nutrient loading, hypoxia and anoxia, organic
39 matter deposition) can lead to changes in biogeochemical cycling and nutrient exchanges
40 across the sediment-water interface (Statham, 2012). In this study, we measure changes in
41 ecosystem service delivery associated with mussel (*Perna canaliculus*) restoration efforts that
42 are beyond the most generally recognised services of food provision and increased biogenic
43 habitat/juvenile fish refugia (Lohrer et al., 2018; Parsons et al., 2013). We explore the
44 multiple benefits of restoration efforts by testing how the presence of restored mussel beds
45 influence the ecosystem service of nitrogen pollution removal and how this changes under the
46 pressure of increased fine sediments.

47 More than 85 % of oyster reefs have been estimated to have been lost worldwide
48 (Beck et al., 2011), with a resultant loss in the multiple ecosystem services these reefs
49 provide. Whilst previous research has focused on oysters, with efforts in the USA and
50 Australia in particular, in New Zealand, green-lipped mussels (*Perna canaliculus*) were once
51 widespread throughout the inner Hauraki Gulf in particular. Intense dredging activities from
52 the 1950s to the 1960s lead to a sharp decline in these reefs, with little to no natural reefs
53 remaining today (Paul, 2012). In addition to this, the Gulf's marine environment has been
54 altered as a result of increased urbanisation from Auckland, the largest city in New Zealand,
55 and changing land use pressures from the surrounding land. Restoration efforts for green-
56 lipped mussels started in 2013 and are ongoing, with over 150 tonnes of mussels deployed so
57 far to form reefs of varying sizes. These reefs have been purposefully placed along a
58 sediment grain size gradient, from inner harbour out to more exposed coastal island areas, to
59 determine where to focus future restoration efforts to not only to enhance their success rates
60 but also to increase the number of ecosystem services the reefs can provide. The experimental
61 placement of the initial restored reefs allows the unknown ecosystem services of mussel reefs
62 to be explored, including their influence on nutrient cycling.

63 Both grain size and the standing stock of microphytobenthos at the sediment surface
64 can alter transport rates of nutrients across the sediment-water interface. Muddier sediments
65 have lower porosity and permeability, leading to decreased water flow and diffusion across
66 the sediment-water interface. Animal-mediated advection of solutes therefore becomes more
67 important than physical advection in muddier sediments, despite macrofaunal diversity
68 usually being lower in muddy sediments (Douglas et al., 2017; Lohrer et al., 2004; Thrush et
69 al., 2004). Microphytobenthos exhibit spatially distinct patterns with sediment grain size
70 (Jesus et al., 2009; Underwood and Barnett, 2006), and can decrease solute flux by ‘capping’
71 the sediments, thus clogging the interstitial spaces, and by intercepting nutrients at the
72 sediment surface (O’Meara et al., 2017; Serpetti et al., 2016).

73 Like other bivalves, mussels ingest particulate nitrogen and organic matter from the
74 water column. Some of this is assimilated, some excreted as dissolved nitrogen and
75 phosphorus, and the remainder egested as biodeposits onto the sediment, resulting in the
76 movement of large quantities of organic material from the water column onto the sediment
77 (Newell, 2004). Nitrogen in these biodeposits can be buried or mineralised and used by
78 microbes in nitrogen cycle pathways, such as nitrification and denitrification (Smyth et al.,
79 2018). Carbon in the biodeposits can instigate another pathway to process nitrogen, DNRA,
80 which is favoured over denitrification in low nitrate conditions (Hardison et al., 2015).
81 Microbial communities living in the sediment rely on inputs of nutrients and organic matter
82 from the water column, so benthic-pelagic coupling by active filter feeders such as mussels
83 can be very influential on nutrient cycling. Mussel shells can also serve as habitat for
84 nitrifying and denitrifying bacteria (Welsh et al., 2015).

85 Nutrient cycling in sediments is a complex process, dominated by differing microbial
86 metabolic pathways which regulate the exchange of dissolved and gaseous compounds across
87 the sediment-water interface (Statham, 2012). Furthermore, due to intense competition

88 between microbes for certain molecules, nutrient cycling in sediments is often tightly
89 coupled, with waste products from one metabolic pathway used as resources in other
90 pathways (Kuypers et al., 2018). Net responses to the release of nutrients and organic matter
91 from mussels is therefore not always a simple result, with feedback loops and competition for
92 resources leading to unpredictable nutrient exchange across the sediment-water interface.

93 In this study, we used restored reefs of the same age and size to test the effect of
94 mussels on nutrient cycling, and the influence of increasing fine sediments around the
95 restored reefs on nutrient cycling. Using *in situ* benthic chambers, we measured solute fluxes
96 (nitrogen, phosphorus and oxygen) across the sediment-water interface along this restored
97 mussel reef grain size gradient. To our knowledge, this is the first time that denitrification
98 rates and nutrient fluxes have been measured *in situ* in restored mussel reefs. We predicted
99 that the restoration of mussels on the seafloor would increase rates of denitrification relative
100 to areas without mussel restoration by providing organic material, removing oxygen, and
101 increasing the availability of ammonium, similar to oyster-mediated sediment denitrification
102 (e.g. Kellogg et al., 2013; Newell et al., 2002).

103 2. Methods

104 2.1 Study region

105 This experiment was conducted in Mahurangi Harbour and Kawau Bay on the East Coast of
106 the North Island, New Zealand. The seven sites used in the experiment were similar in depth
107 but varied in sediment type from muddy sand to predominantly sand (Table 1). Three sites
108 (Martins Bay North (MBN) and South (MBS) and Motoketekete (MK)) were located in
109 Kawau Bay, and the remaining four sites (Mahurangi mid (MM), Ngaio Bay (NB), Otarawao
110 Bay (OT) and Pukapuka (PP)) were located within Mahurangi Harbour.

111 The restored mussel reefs were created in October 2016 by shovelling approximately
112 850 kg of cleaned live adult mussels off a barge to evenly cover an area of approximately 25
113 m² at each site. All mussels came from the same mussel farm and were harvested at the same
114 time so all mussels were similar in size. Experiments were carried out in March 2017 to allow
115 the reefs time to establish.

116 **Table 1.** Mean and range of site characteristics measured during chamber incubations in and out of restored mussel reefs. Sites are arranged in
 117 decreasing mud content from left to right

Site		NB	PP	MM	OT	MBN	MBS	MK
Sediment properties								
Coarse sand (% > 500 µm)	in	1.28 (0.76 – 1.73)	0.47 (0.36 – 0.78)	0.55 (0.28 - 0.85)	0.30 (0.22 – 0.39)	2.50 (1.01 – 3.29)	0.27 (0.21 – 0.31)	7.48 (6.37 – 8.70)
	out	1.23 (0.78 – 2.03)	0.42 (0.27 – 0.49)	0.19 (0.13 - 0.27)	0.22 (0.01 – 0.31)	2.70 (1.89 – 3.71)	0.31 (0.22 – 0.40)	5.69 (5.08 – 6.19)
Mud (% < 63 µm)	in	50.15 (39.88 – 59.06)	32.18 (28.26 – 35.77)	26.11 (23.32 – 30.33)	24.45 (20.17 – 28.11)	14.16 (10.62 – 19.50)	6.73 (5.25 – 9.71)	3.56 (3.19 – 3.75)
	out	50.49 (44.01-61.26)	32.97 (28.82 – 35.23)	26.02 (24.17 – 28.08)	27.49 (23.96 – 32.47)	13.23 (9.57 – 15.65)	5.36 (4.48 – 7.19)	3.32 (2.73 – 3.92)
Sediment organic matter (% loss on ignition)	in	4.93 (4.62 – 5.21)	3.87 (3.28 – 4.28)	3.06 (2.74 – 3.48)	3.19 (2.63 – 3.86)	2.81 (2.34 – 3.35)	2.03 (1.69 – 2.48)	1.97 (1.80 – 2.14)
	out	5.09 (4.42 – 5.40)	3.76 (3.38 – 4.20)	2.97 (2.84 – 3.22)	3.62 (3.32 – 3.76)	2.59 (2.25 – 2.77)	1.80 (1.78 – 1.82)	1.78 (1.70 – 1.92)
Porosity (0-2 cm, %)	in	72.51 (67.08 – 77.93)	64.78 (61.98 – 67.57)	61.78 (59.78 – 63.77)	57.53 (56.22 – 58.83)	53.39 (53.02 – 53.77)	45.08 (40.83 – 49.33)	48.11 (47.15 – 49.07)
	out	69.02 (68.58 – 69.45)	64.17 (63.98 – 64.35)	61.03 (58.32 – 63.75)	61.64 (61.00 – 62.28)	52.59 (51.90 – 53.28)	45.81 (45.20 – 46.42)	47.28 (46.97 – 47.60)
Sediment traps – total suspended solids (g)	in	-	6.85	3.32	6.81	-	27.47	5.83
	out	-	3.54	12.98	2.36	-	14.12	4.38
Microphyte biomass (µg g ⁻¹)								
Chlorophyll a (0-2 cm)	in	9.11 (8.77 – 9.46)	9.43 (7.57 – 11.29)	21.07 (20.35 – 21.78)	12.98 (12.67 – 13.30)	6.74 (6.31 – 7.17)	5.36 (4.36 – 6.36)	8.57 (8.20 – 8.94)
	out	8.66 (8.20-9.11)	9.95 (8.94 – 10.95)	20.32 (19.49 – 21.15)	15.33 (15.19 – 15.48)	6.22 (5.67 – 6.76)	4.96 (4.76 – 5.16)	7.45 (6.99 – 7.91)
Phaeophytin (0-2 cm)	in	1.58	-0.16	-7.49	-3.12	1.58	-0.10	-2.67
	out	-0.19	-0.97	-7.53	-4.11	-0.73	-0.75	-2.11
Macrofaunal abundance (core ⁻¹)								

Total number of macrofauna	in	24.75	26.25	18.50	19.00	61.75	65.00	118.75
	out	25.75	25.25	24.75	21.00	79.00	41.25	55.50
Macrofauna species richness	in	10.25	9.00	8.50	8.50	10.75	10.50	26.25
	out	10.75	10.00	8.50	8.00	12.75	7.25	16.50
Water column								
PAR ($\mu\text{mol m}^{-2} \text{s}^{-1}$)		15.66	28.19	60.23	50.26	67.93	71.91	81.13

118

119 2.2 Flux determination

120 Fluxes of dissolved O₂ and inorganic nutrients (ammonium (NH₄⁺), nitrate-plus-nitrite
121 nitrogen (NO_x, reported as nitrate) and phosphate (PO₄³⁻)) were determined *in situ* using
122 benthic incubation chambers in February 2017 at all seven sites. Fluxes of dissolved N₂ and
123 Ar gas were determined from the same chambers at four of the seven sites (samples from the
124 remaining three sites were unfortunately irreparably damaged during storage). At each site,
125 two pairs of dark and light benthic chambers (0.25 x 0.25 m, 41 L volume) were installed in
126 the centre of each mussel reef and on sediment with no mussels at least 5 m out of the reef
127 and incubated for approximately four hours. The chambers within the restored reefs were
128 placed so that each one contained an average of 15 live mussels, whilst the ones outside of
129 the reef contained no mussels. An Odyssey® Waterproof Photosynthetic Active Radiation
130 (PAR) Logger was deployed at each site for the duration of the sampling as a proxy for
131 turbidity.

132 Water samples (60 mL x 2) were taken by divers on SCUBA withdrawing water with
133 syringes via ports from each chamber just after installation and approximately four hours
134 later. To correct for water column effects, ambient water was also incubated in 1 litre light
135 and dark bottles without sediment, and these were fixed to the seafloor at each site for the
136 duration of the sampling. Dissolved oxygen was measured immediately using a Hach
137 portable dissolved oxygen probe and the samples were then filtered using Whatman GF/F
138 filters (pore size 0.7 µm), frozen (-20 °C), and stored in the dark pending further analysis.
139 Samples for analysis of N₂ and Ar gas concentrations were collected into 15 mL glass
140 exetainers, preserved with zinc chloride solution, sealed with airtight lids, and stored at
141 temperatures below incubation temperatures but above freezing until later analysis.

142 Fluxes of inorganic nutrients (NH_4^+ , NO_x and PO_4^{3-}) were determined on a Lachat
143 Quick-Chem 8000 automated flow injection analyser. N_2 and Ar gas concentrations were
144 determined using Membrane Inlet Mass Spectrometry (MIMS). Salinity and temperature
145 were recorded for calculation of gas solubility based on Hamme and Emerson (2004). The
146 N_2 :Ar technique (Kana et al., 1994) gives a denitrification rate as a net measurement of N_2
147 fluxes (i.e., gross nitrogen fixation – gross denitrification). Previous studies have shown that
148 anammox in estuaries contributes < 15 % of nitrogen removal (Hou et al., 2015) and does not
149 occur at sites such as ours with high salinity (> 19) and low nutrient levels (~ 0.2–6.0 μM
150 NO_3^- ; Rich et al., 2008). We therefore assumed that all positive fluxes of N_2 are indicative
151 of net denitrification, while a negative flux indicated net nitrogen fixation.

152 Fluxes of N_2 , O_2 , NH_4^+ , NO_x and PO_4^{3-} were calculated as difference between start
153 and end concentrations for each chamber, and calculated as:

$$154 \quad \text{Flux } (\mu\text{mol m}^{-2} \text{ h}^{-1}) = \frac{(\text{Final} - \text{Initial flux}) \times \text{Chamber volume (L)}}{\text{Sediment area (m}^2) \times \text{Duration of sampling (h)}}$$

155 These rates were corrected for the addition of replacement water drawn into each chamber at
156 the time of sampling. Concentrations of PO_4^{3-} were below or near detection limits (0.04 mg
157 L^{-1}) resulting in uncertainty and variability in flux calculations, and therefore, this nutrient
158 was not considered further.

159 Denitrification efficiency was calculated as the fraction of sediment nitrogen flux lost
160 to denitrification versus being returned to the water column as a nutrient source for further
161 use by algae (Owens, 2009):

$$162 \quad \text{Denitrification efficiency (\%)} = \left[\frac{N_2}{(N_2 + \text{NO}_x + \text{NH}_4)} \right] \times 100$$

163 *2.3 Environmental variables*

164 Following the incubations, a range of sediment properties was determined from each chamber
165 to characterise differences in sediment properties across sites and to pair environmental data
166 with response variables in statistical models. Three cores (1.9 cm diameter, 2 cm depth) were
167 collected at random in each chamber and pooled, and sediments were kept frozen and
168 lyophilised for analysis.

169 Microalgal pigment concentrations (chlorophyll *a* and phaeophytin) as a proxy for
170 microphytobenthos standing stock, were measured with a spectrophotometer (Lorenzen,
171 1967) after extraction in acetone to separate degradation products from chlorophyll *a* (Arar
172 and Collins, 1997). Grain size samples were digested with 6 % hydrogen peroxide for 48
173 hours to remove organic matter and a Calgon solution (0.5 % [mass : volume] sodium
174 hexametaphosphate) was used to break apart any aggregates. Sediments were then wet sieved
175 to measure cumulative percentages of coarse sand, medium sand, fine sand, very fine sand
176 and mud in the sediment (i.e. particle sizes > 500, 500-250, 250-125, 125-63, and < 63 μm
177 diameter, respectively; Day, 1965). Percent SOM was determined by loss on ignition from
178 dried sediment (60 °C) samples for 5.5 hours at 400 °C (Dean, 1974), and porosity from mass
179 of wet sediment/volume of wet sediment.

180 One macrofauna core (10 cm diameter x 10 cm depth) was also collected from each
181 benthic chamber following the incubation. Cores were sieved on a 500 μm mesh, preserved
182 in 70 % isopropyl alcohol, and stained with Rose Bengal. Macrofauna were sorted in the
183 laboratory, identified to the lowest possible/practical taxonomic level, and counted.

184 *2.4 Statistical analyses*

185 All statistical calculations were carried out in R (R Core Team, 2017). Between site
186 differences were examined using one-way ANOVA followed by Student-Newman-Keuls's
187 post hoc comparison ($\alpha = 0.05$). Generalised linear models (GLMs) were used to determine

188 the environmental factors controlling denitrification rates and efficiency in dark and light
189 conditions. GLMs were conducted as multiple linear regressions to investigate the
190 interactions between multiple environmental variables. To compare the relative importance of
191 explanatory factors all response (denitrification rate and efficiency) and explanatory variables
192 were standardised to run between 0 and 1. VIF scores were used to determine
193 multicollinearity, and variables with VIF values > 10 were reviewed for their inclusion in the
194 models and one was selected for inclusion in further analysis based on highest correlation
195 (Pearson's r) with denitrification or denitrification efficiency. VIF scores were recalculated
196 for the remaining variables to verify all values were < 10 . N_2 and NH_4^+ fluxes were excluded
197 from the denitrification efficiency models because they are used in the calculation of
198 efficiency. Variables were eliminated from the full model using a backward selection
199 procedure (variables significant at $\alpha = 0.15$ retained in final models; Crawley, 2014) based on
200 the Akaike Information Criterion (AIC; Akaike, 1974). The backward selection process
201 started with the full model and sequentially excluded explanatory variables based initially on
202 the correlation with the response variable and other explanatory variables.

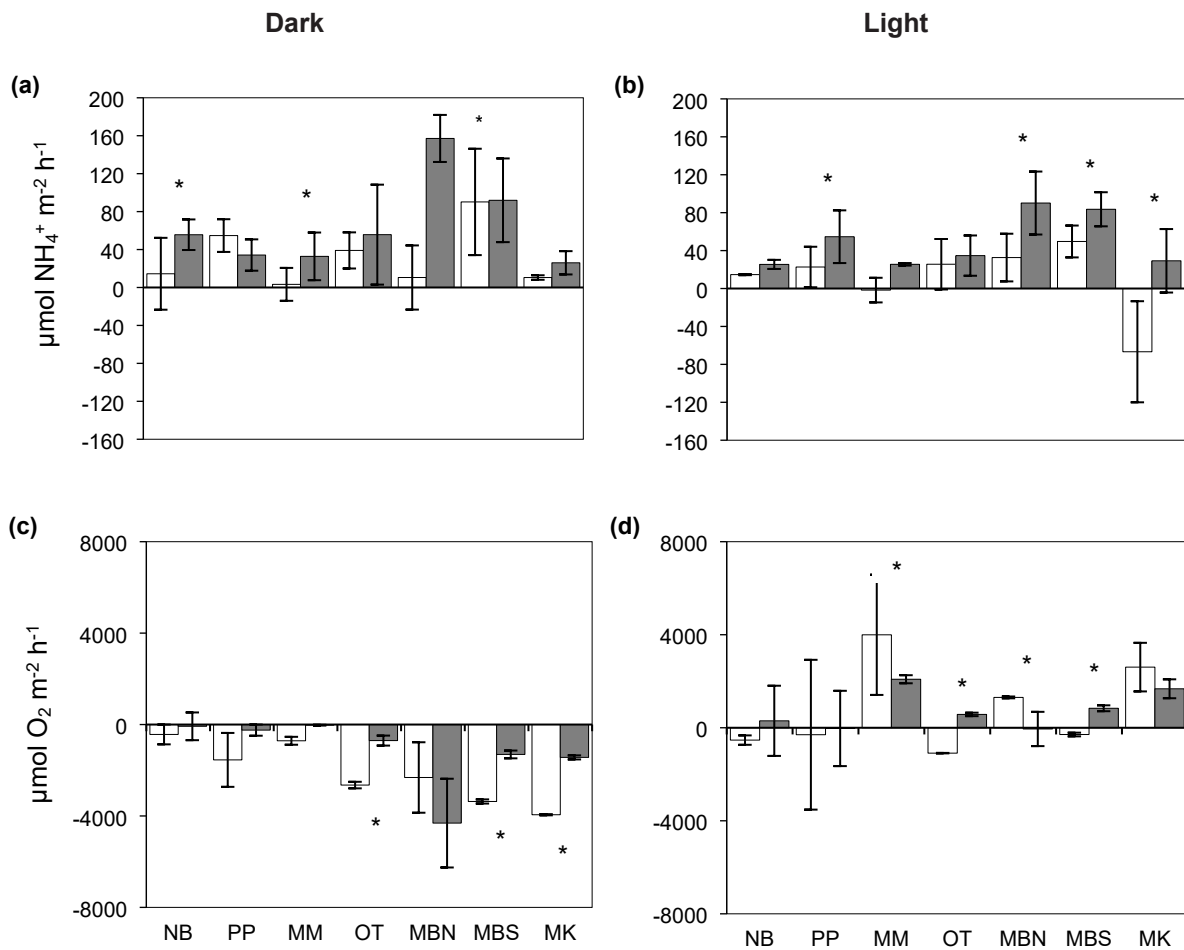
203 **3. Results**

204 *3.1 Nitrate, ammonium and oxygen fluxes*

205 Nitrate fluxes were higher in the muddier sites (NB, PP and MM) under dark conditions and
206 lower in the muddier sites under light conditions. Fluxes varied between uptake (negative
207 flux) and production (positive flux), and were very low at all sites, with the exception of high
208 uptake in the reef at site OT ($-159.46 \pm 166.77 \mu\text{mol NO}_x \text{ m}^{-2} \text{ h}^{-1}$) and high production out of
209 the reef at site MBS ($926.60 \pm 918.06 \mu\text{mol NO}_x \text{ m}^{-2} \text{ h}^{-1}$) in dark conditions, and high uptake
210 within the reef at site PP in light conditions ($-302.73 \pm 74.82 \mu\text{mol NO}_x \text{ m}^{-2} \text{ h}^{-1}$).

211 Ammonium fluxes were lower in the restored reefs than in the sediment adjacent to
212 the restored reefs at all sites in both dark and light conditions, except for site PP in the dark
213 where it was lower out of the reef ($54.73 \pm 17.25 \mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ h}^{-1}$ in reef and 34.21 ± 16.53
214 $\mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ h}^{-1}$ out of reef; Fig. 1a-b). There was production of ammonium from all
215 sediments both in and out of restored reefs in both dark and light conditions, with the
216 exception of site MK in the light, where ammonium was consumed in the restored mussel
217 reef ($-66.70 \pm 53.31 \mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ h}^{-1}$; Fig. 1b).

218 Sediment oxygen demand was higher in restored reefs at all sites except site MBN
219 under dark conditions and sites MM, MBN and MK under light conditions (Fig. 1c-d).
220 Uptake of oxygen occurred across all sites under dark conditions, both in and out of restored
221 reefs. Fluxes varied between uptake and production across all sites under light conditions.



222 **Fig. 1** Mean net fluxes of NH₄⁺ and O₂ in dark and light conditions across the sites from the
 223 upper estuary to the outer bay (left to right). *White bars* are in the restored mussel reefs, *grey*
 224 *bars* sediment out of the mussel reefs. Asterisks indicate significant differences between reefs
 225 and controls at each site ($P < 0.05$), error bars are standard error of the mean

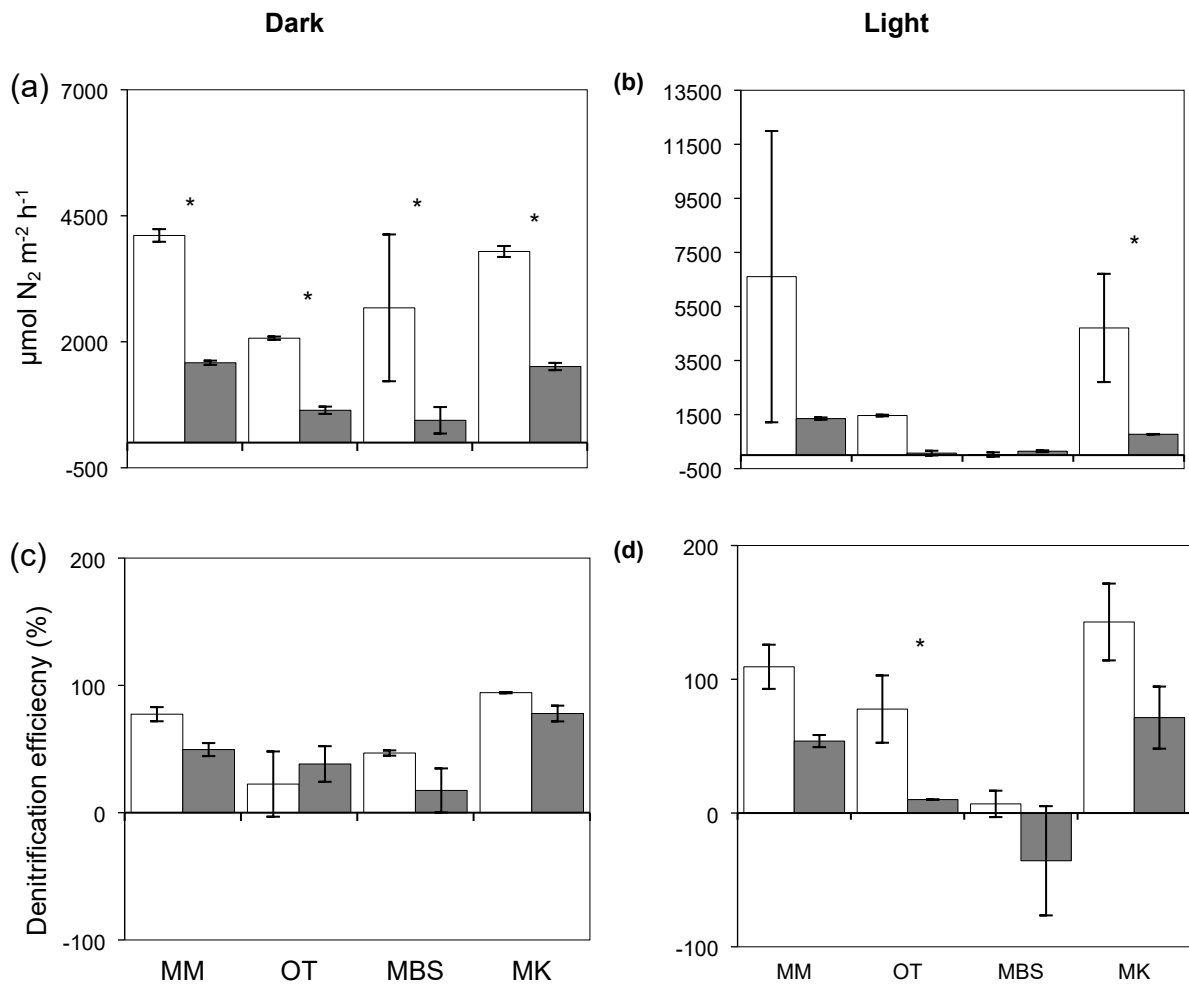
226

227 3.2 Denitrification

228 Denitrification rates were positive across all sites and treatments, indicating net
 229 denitrification was occurring (Fig. 2a-b). Net denitrification rates in the dark were
 230 significantly higher in the restored reefs than in the sediment adjacent to the restored reefs at
 231 all sites ($P < 0.05$; Fig. 2a). The presence of the restored reefs increased denitrification rates
 232 in the dark at each site by 61 %, 69 %, 83 % and 60 %, respectively. The same was true for

233 denitrification rates in the light, with increases of 80 %, 95 % and 84 % for sites MM, OT and
234 MK, respectively, though only site NK was significantly higher (Fig. 2b). Site MBS was the
235 exception, with net denitrification rates in the light not significantly different in or out of the
236 mussel reef, and net denitrification rates much lower than other sites in the light ($21.92 \pm$
237 $80.57 \mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$ in reef and $145.07 \pm 32.72 \mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$ out of reef, compared to an
238 average of $4258.05 \pm 1500.10 \mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$ in reef and $732.30 \pm 369.01 \mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$
239 out of reef at the other three sites; Fig. 2b). Consistent with our understanding of
240 biogeochemical processes, patterns in denitrification rate in the dark were affected by mussel
241 presence, ammonium and oxygen flux, and amount of coarse sediment, a proxy for sediment
242 permeability, ($R^2_{\text{adj}} = 0.75, p < 0.001$; Table 2). Patterns in the light were also affected by
243 mussel presence, oxygen flux, and amount of coarse sediment, as well as phaeophytin content
244 and sediment porosity ($R^2_{\text{adj}} = 0.91, p < 0.001$). All relationships were positive, except for
245 coarse sediment content in the light which worked to decrease net denitrification rates.

246 Denitrification efficiency was higher in the restored reefs than in adjacent sediment at
247 all sites in both dark and light conditions, except for site OT in the dark where it was higher
248 out of the reef ($22.47 \pm 25.64 \mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$ in reef and $38.25 \pm 13.99 \mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$ out
249 of reef; Fig. 2c-d). Denitrification efficiency in the dark was best predicted by coarse
250 sediment content, and mussel presence ($R^2_{\text{adj}} = 0.48, p = 0.013$), with coarse sediment
251 content the most important predictor. Denitrification efficiency in the light was best predicted
252 by, in order of importance, phaeophytin content, coarse sediment content, and mussel
253 presence ($R^2_{\text{adj}} = 0.72, p < 0.001$). All relationships were positive, with the exception of
254 phaeophytin content for denitrification efficiency in the light.



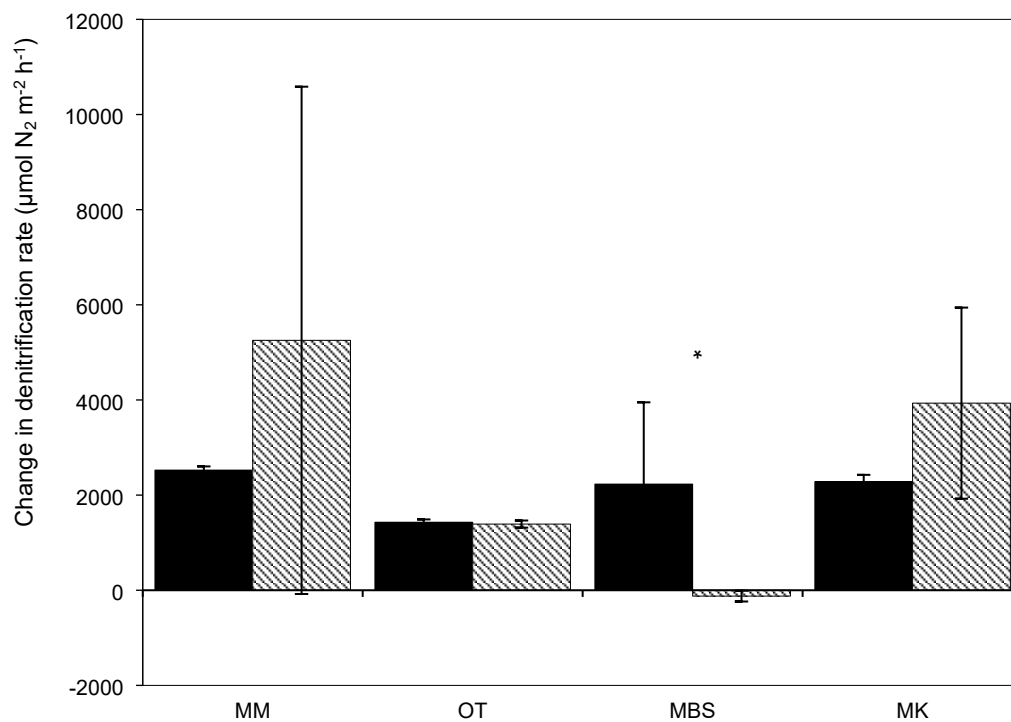
255 **Fig. 2** Mean net fluxes of denitrification rate and efficiency in dark (a and c) and light (b and
 256 d) conditions across the sites from the upper estuary to the outer bay (left to right). *White bars*
 257 are in the restored mussel reefs, *grey bars* sediment out of the mussel reefs. Asterisks indicate
 258 significant differences between reefs and controls at each site ($P < 0.05$), error bars are
 259 standard error of the mean. Note the difference in scale for plots a and b

260 **Table 2.** Predictive factors of denitrification rate and efficiency. Only variables significant at $\alpha = 0.15$ were retained in final models, following a
 261 backward elimination procedure. Standardised regression coefficients in bold are significant at $p < 0.05$, absence indicates that the parameter was
 262 not retained in the final model, and a hyphen indicates the parameter was not included in the initial model. Location = in or out of mussel bed,
 263 Mac = macrofauna abundance, Chl *a* = sediment chlorophyll *a* concentration, Phaeo = sediment phaeopigment concentration, Por = Porosity,
 264 SOM = sediment organic matter

Flux		R^2_{adj}	p	Ammonium	Nitrate	Oxygen	Coarse	Location	Mac	Chl <i>a</i>	Phaeo	Por	SOM	AIC
Denitrification rate	Dark	0.75	< 0.001	0.29		0.79	0.54	0.85						-5.08
	Light	0.91	< 0.001			0.92	-0.07	0.21			0.28	0.31		-33.28
Denitrification efficiency	Dark	0.48	0.013	-	-	0.47	0.64	0.30						1.04
	Light	0.72	< 0.001	-	-		0.39	0.22			-0.44			-14.90

266 Mussel reefs enhanced (indicated by a positive change in net denitrification rate)
 267 denitrification at all sites except for site MBS under light conditions where the change was
 268 marginally negative ($-123.15 \pm 113.29 \mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$; Fig. 3). The magnitude of the effect
 269 of the restored reefs was dependent on light condition, with similar effects seen in the dark at
 270 all sites (ranging from 1429.73 to 2525.36 $\mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$). Greater but more variable effects
 271 were seen in the light at sites MM and MK, which lay at each end of the grain size gradient
 272 (5253.02 ± 5332.00 and $3933.22 \pm 2008.44 \mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$, respectively).

273

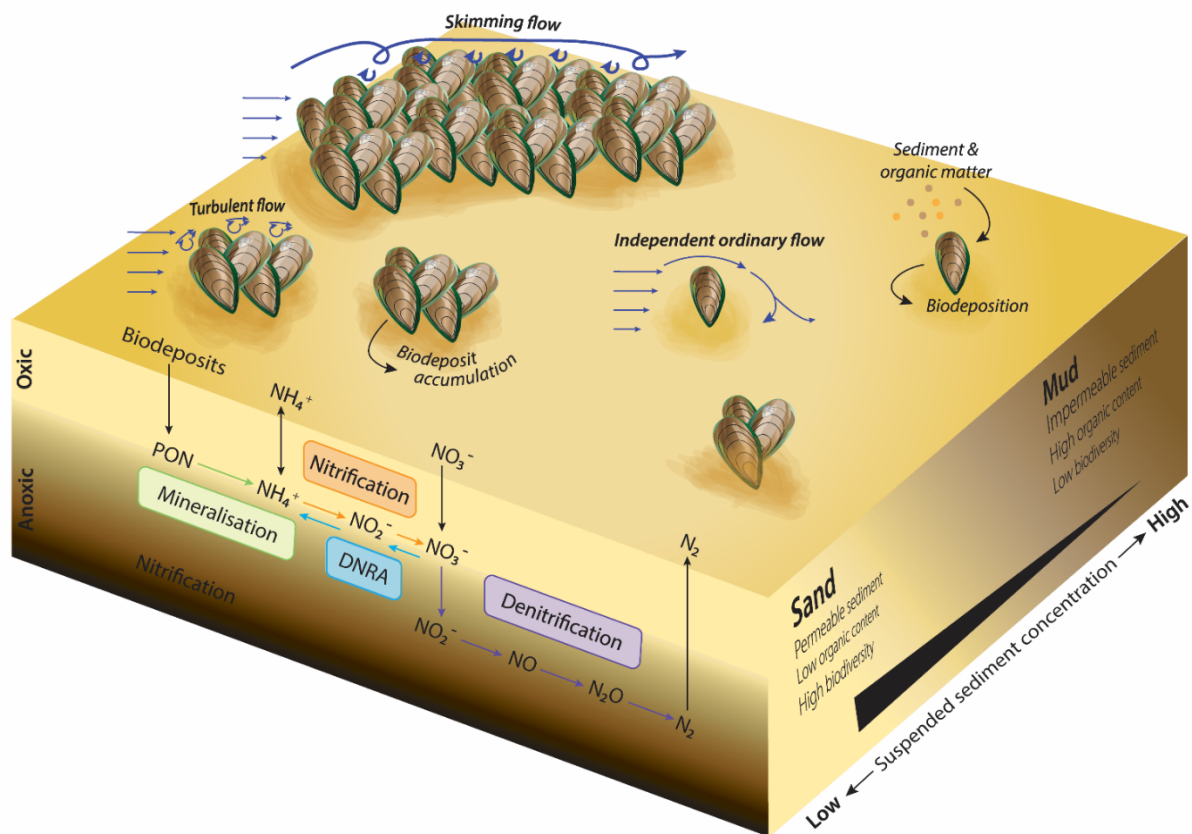


274

275 **Fig. 3** Patterns of mussel-mediated change in denitrification rate compared to sediment with
 276 no mussels at each site from the upper estuary to the outer bay (left to right). *Black bars* are
 277 rates measured in the dark, *hatched bars* are rates measured in the light. Asterisks indicate
 278 bars that are significantly different to each other ($P < 0.05$)

279 **4. Discussion**

280 This study is the first to conduct *in situ* measurements of fluxes in restored mussel reef sites.
281 Overall, our results show that the presence of restored mussel reefs enhanced denitrification
282 rates and efficiency at all sites along a grain size gradient, when compared to adjacent
283 sediments with no emergent biogenic structure. This demonstrates that by filtering particles
284 from the water and depositing organic matter as biodeposits, mussels and the microhabitats
285 they generate on the sediment positively influence denitrification in the underlying sediment
286 (Fig. 4). This allows restored reef systems to become a sink for reactive nitrogen in the
287 coastal zone, which has implications for placing restored reefs in the future to enhance this
288 important ecosystem service.



289
290 **Fig. 4** Influence of mussels on nitrogen pathways and flow dynamics

291 Our fluxes of N₂ were much higher than those previously reported for both mussel
292 and oyster reef environments (Table 3), although *in situ* measurements of denitrification in
293 restored mussel reefs have never previously been done, making for only very general
294 comparisons. *Ex situ* denitrification measurements in restored oyster habitats have resulted in
295 lower values recorded compared to *in situ* observations (compared to *in situ* Humphries et al.,
296 2016; e.g. *ex situ* Kellogg et al., 2013; Smyth et al., 2015). The most direct comparison
297 possible, an *in situ* study on denitrification rates in restored oyster reefs in Rhode Island,
298 USA, reported rates substantially lower than our observed rates ($581.9 \pm 164.2 \mu\text{mol N}_2 \text{ m}^{-2}$
299 h^{-1} compared to our average of $3180.28 \pm 987.65 \mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$; Humphries et al., 2016).
300 Our rates also exceeded those found in aquaculture farms of both mussels and oysters
301 (Christensen et al., 2003; Humphries et al., 2016; Kaspar et al., 1985), and provide valuable
302 information from under-represented low-nutrient systems (Vieillard et al., 2020).

303 **Table 3.** Comparison of results from this study with other directly measured denitrification rates in bivalve habitats. Values are averages across
 304 each study \pm standard error

Location	Species	Incubation type	Site	Denitrification rate ($\mu\text{mol N}_2 \text{ m}^{-2} \text{ h}^{-1}$)	Source
Mahurangi Harbour and Kawau Bay, North Island, New Zealand	Mussels (<i>Perna canaliculus</i>)	In situ	Restored reefs	3180.28 \pm 987.65	This study
Kenepuru Sound, South Island, New Zealand	Mussels (<i>Perna canaliculus</i>)	Ex situ	Aquaculture farms	60.57 \pm 48.69	Kaspar et al. (1985)
Tasman Bay and Beatrix Bay, South Island, New Zealand	Mussels (<i>Perna canaliculus</i>)	In situ & ex situ	Aquaculture farm	9.6 \pm 1.3	Christensen et al. (2003)
Bogue Sound, North Carolina, USA	Oysters (<i>Crassostrea virginica</i>)	Ex situ	Natural reef	\sim 87.5 \pm 21.25	Piehler and Smyth (2011)
Ninigret Pond, Rhode Island, USA	Oysters (<i>Crassostrea virginica</i>)	In situ	Aquaculture farms Restored reefs	346.1 \pm 168.6 581.9 \pm 164.2	Humphries et al. (2016)
Middle Marsh, North Carolina, USA	Oysters (<i>Crassostrea virginica</i>)	Ex situ	Restored reefs	211.65 \pm 11.61	Smyth et al. (2015)
Shoal Creek, Maryland, USA	Oysters (<i>Crassostrea virginica</i>)	Ex situ	Restored reefs	25.76 \pm 1.76	Kellogg et al. (2013)

306 Other coastal emergent biogenic habitats, such as seagrass beds, saltmarshes and
307 oyster reefs, have been found to have higher rates of denitrification compared habitats with
308 no emergent biogenic structure (Piehler and Smyth, 2011). Whilst plant-based biogenic
309 habitats produce new organic matter via photosynthesis (Smyth et al., 2015), shellfish reefs
310 made up of filter-feeders enhance benthic-pelagic coupling, subducting organic material in the
311 form of faeces and pseudo-faeces to the underlying sediment in the interstices between shells,
312 thus supplying organic nitrogen and carbon to the sediment microbial community from which
313 dissolved inorganic nitrogen can then be regenerated and removed (Joye and Anderson, 2008;
314 Newell et al., 2002). The presence of mussels can also boost biogeochemical processes in the
315 sediment by the excretion of ammonium which is then used by processes such as nitrification
316 (Norkko et al., 2001), which could explain our low rates of ammonium flux and sediment
317 organic matter in the mussel reefs.

318 Denitrification efficiency was also higher at the restored sites than at the control sites,
319 with the exception of one site in the dark. Denitrification efficiency is a measure of the
320 likelihood that nitrate and nitrite will be transformed into nitrogen gas via denitrification
321 (Kellogg et al., 2013), and is useful for comparisons between sites or systems. At some sites
322 our denitrification efficiency was approaching 100 %, comparable to intertidal oyster reefs in
323 North Carolina, USA (Piehler and Smyth, 2011; Smyth et al., 2013). In our study,
324 denitrification efficiency was positively correlated with coarse sediment content in both dark
325 and light conditions. Coarse sediments have higher permeability, and therefore higher
326 porewater nutrient release. However, sites with low water flow can increase microbial contact
327 time with available substrate pools, thus increasing processing time and production of N₂
328 (O'Meara et al., 2020), and mussel reefs alter water flow by forming clumps and solid
329 structures above the sediment. Biogenic structures such as mussels can therefore enhance
330 denitrification via multiple direct and indirect pathways.

331 Humans are increasingly impacting coastal ecosystems which are critical for a wide
332 range of ecosystem services (Spalding et al., 2014). One such impact is nutrient over-
333 enrichment which leads to eutrophication, a long-known global issue in shallow coastal
334 ecosystems (Nixon, 1995). Stressors are beginning to interact in surprising ways and
335 understanding the systems involved and any mitigations that can improve them is becoming
336 more and more crucial (Humphries et al., 2016). The value of nitrogen removal via
337 denitrification has been estimated as \$US 13 kg N⁻¹ by the North Carolina Nutrient Offset
338 Credit Program, and \$US 98.70 kg N⁻¹ from global energy and emdollar values (Piehler and
339 Smyth, 2011; Watanabe and Ortega, 2011, respectively). Using the average of these values
340 and directly scaling up for the size of a restored bed leads to an average valuation of \$US
341 1,132,897 per bed, a 73 % increase compared to the nitrogen value of the same area of
342 adjacent sediment of \$US 304,629. These estimates, they provide a useful valuation of a key
343 ecosystem service that can be crucial for swaying monetary and political support for
344 restorations.

345 In New Zealand, intensification of land use has resulted in increases in national loads
346 of nitrogen (Snelder et al., 2018) and sediment (Dymond et al., 2012) into coastal waters. Our
347 results emphasise the importance of quantifying the potential ecosystem responses to
348 intensifying stressors and the potential for targeted shellfish restoration to enhance
349 denitrification rates. Albeit that the effect size differed between sites, mussel reefs increase
350 denitrification regardless of the grain size at the site. Restored mussel reefs can therefore play
351 an important and previously unrealised role in coastal ecosystems in mitigating the effects of
352 nutrient loading by providing a key pathway to the ultimate removal of fixed nitrogen from
353 coastal systems. Restoration of mussel reefs can therefore provide ecosystem services beyond
354 the well-known benefits related to food and 3D habitat provision. A better understanding of
355 the full suite of ecosystem services attained from mussel restoration efforts also improves our

356 ability to describe restoration goals, achieve maximum benefits, and better target systems
357 based on the desired objectives of the restoration.

358

359 **Acknowledgements**

360 We thank Kaiwen Yang, Craig Norrie, Stefano Schenone, Brady Doak, Sam Parkes and Rod
361 Budd for field assistance, and Jasmine Low for assisting with figure development.

362 Funding: Mussel deployments and fieldwork was funded by the McCrae Family Foundation.

363 JRH was supported by a National Institute of Water and Atmospheric Research Doctoral

364 Scholarship and a George Mason Centre for the Natural Environment Research Fellowship,

365 and TAO by a University of Auckland postdoctoral fellowship.

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