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Response of the ammonia oxidation activity of microorganisms in surface sediment to a controlled sub-seabed release of CO_2

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Abstract

The impact of a sub-seabed CO_2 leak from geological sequestration on the microbial process of ammonia oxidation was investigated in the field. Sediment samples were taken before, during and after a controlled sub-seabed CO_2 leak at four zones differing in proximity to the CO_2 source (epicentre, and 25 m, 75 m, and 450 m distant). The impact of CO_2 release on benthic microbial ATP levels was compared to ammonia oxidation rates and the abundance of bacterial and archaeal ammonia *amoA* genes and transcripts, and also to the abundance of nitrite reductase (*nirS*) and anammox hydrazine oxidoreductase (*hzo*) genes and transcripts. The major factor influencing measurements was seasonal: only minor differences were detected at the zones impacted by CO_2 (epicentre and 25 m distant). This included a small increase to ammonia oxidation after 37 days of CO_2 release which was linked to an increase in ammonia availability as a result of mineral dissolution. A CO_2 leak on the scale used within this study (< 1 tonne d^{-1}) would have very little impact to ammonia oxidation within coastal sediments. However, seawater containing 5 % CO_2 did reduce rates of ammonia oxidation. This was linked to the buffering capacity of the sediment, suggesting that the impact of a sub-seabed leak of stored CO_2 on ammonia oxidation would be dependent on both the scale of the CO_2 release and sediment type.

Keywords: CO_2 leakage, marine sediment, ammonia oxidation, ATP, *amoA*, *hzo*

1. Introduction

CCS technology has the potential to reduce CO_2 emissions from fossil fuel power stations by 80–90% (Holloway, 2007) and the IPCC recognize that effective CCS could play a substantial role in mitigation, potentially reducing CO_2 emissions by 45 % by 2050 (Metz et al, 2005). However, concerns over the environmental impact of CO_2 leakage from sub-seabed

48 storage are hindering the development of CCS technologies. Assessments of the impact of
49 CO_2 leakage from sub-seabed storage sites within the marine environment are essential, both
50 to aid our understanding of the potential risks involved and to allow the development of novel
51 technologies useful for monitoring CO_2 leaks. If CO_2 leakage occurs from geological storage
52 or due to pipeline failure, it has the potential to create considerable localized reductions in
53 seawater pH (Blackford et al. 2008; 2009). Studies of the impact of elevated CO_2 have
54 demonstrated several negative impacts of elevated CO_2 on marine organisms, including
55 changes in metabolic activity, fertility, growth and survival, particularly for calcifying species
56 (Gattuso & Hansson, 2011). Impacts to the microbially-driven biogeochemical cycling of
57 nutrients have also been predicted to occur (Gehlen et al. 2011), in particular the nitrogen
58 cycle (Hutchins et al. 2009).
59 Elevated CO_2 has been shown to result in a clear decrease in the first step in
60 nitrification, ammonia oxidation, within open ocean waters (Huesemann et al., 2002, Beman
61 et al., 2011, Kitidis et al., 2011). Ammonia oxidation in the open sea was inhibited by
62 lowering pH by 0.05 –0.5 units (Heusemann et al., 2002; Beman et al., 2011, Kitidis et al.,
63 2011). This is thought to be related to the effect of pH on the balance of ammonia (NH_3) to
64 ammonium (NH_4^+): a decrease in pH favors the formation of ammonium (Bell et al., 2007,
65 2008, Wyatt et al. 2010). As ammonia oxidizers are thought to use ammonia rather than
66 ammonium (Suzuki et al., 1974, Stein et al., 1997), this is expected to impact nitrification at
67 decreased pH. In contrast, elevated levels of CO_2 have been shown to enhance rates of both
68 carbon and nitrogen fixation for some diazotrophs (Hutchins et al. 2007; Fu et al. 2008).
69 Together, this may shift the balance of N available for oceanic phytoplankton, favoring those
70 more able to uptake NH_4^+ (Beman et al. 2011). However, nitrification rates were highest at
71 low pH at an estuarine site suggesting that the negative impacts of elevated CO_2 on pelagic
72 nitrification may be restricted to open ocean waters (Fulweiler et al. 2011).

The impact of elevated levels of CO_2 and reduced pH on benthic nitrification is also known to be complex (Kitidis et al., 2011; Laverock et al., 2013), with elevated CO_2 possibly impacting the bioirrigating behaviour of bioturbating macrofauna species, and reducing the concentration of ammonia and oxygen available for nitrification (Laverock et al. 2013), or altering the balance of ammonia oxidizing microbes (Tait et al. 2014a). Similarly, a series of mesocosm studies on the impact of elevated CO_2 on sediment-water nutrient fluxes within sediment mesocosms containing bioturbating macrofauna have also indicated possible impacts to nitrification (Widdicombe and Needham, 2007; Widdicombe et al. 2009). However, Kitidis et al. (2011) showed that the benthic ammonia oxidation within the surface of a range of sediments was not impacted by CO_2 concentration. One possible explanation was thought to be the high pH buffering capacity of sediments. Alternatively, pH in sediments is known to fluctuate due to the supply and nature of organic matter, the action of bioturbating fauna and also transport processes such as solute diffusion or advection (Kitidis et al., 2011, Zhu et al. 2006). It may be that the ammonia oxidizing microbes residing within sediments are already adapted to the low, fluctuating pH conditions within sediments. A third possibility is that a change in the composition of the ammonia oxidizing community might allow nitrification to continue under lower pH conditions (Tait et al., 2014a). Nitrification is carried out by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA). Cultured representative of ammonia oxidizing archaea have a much greater substrate affinity for ammonia than bacteria (Martens-Habbena et al. 2009), and may therefore have a competitive advantage over bacteria under lower pH conditions. For example, the abundance of AOA ammonia monooxygenase subunit A (*amoA*) transcripts increased with decreasing pH within an Arctic benthic mesocosm, whereas the abundance of both AOB *amoA* transcripts and genes decreased (Tait et al., 2014a). Within many soils, AOA have also been shown to be more dominant than AOB (Leininger et al. 2006; Nicol et al. 2008; Yao et al.,

98 2011) and were more important for ammonia oxidation in strongly acidic soil (Zhang et al.,
99 2012). It is possible that a CO_2 related pH change may be an important influence on the ratio
100 of AOA to AOB (Bouskill et al., 2012; Bowen et al., 2013; Tait et al., 2014a).

101 Within coastal sediments, much of the nitrite and nitrate used by denitrifying
102 bacteria is derived from nitrification (Joye and Anderson, 2008). However, in a study of the
103 impact of elevated pCO_2 on the N cycling community in an Arctic sediment there was a
104 decrease in the abundance of *nirS* transcripts with increasing pCO_2 (Tait et al. 2014a) and
105 although there was a possible trend of decreasing denitrification with increasing pH, the high
106 degree of variability amongst replicate cores resulted in the conclusion that there was no
107 difference to denitrification rates between pCO_2 treatments (Gazeau et al. 2014). The study of
108 Tait et al. (2014a) also reported an increase in the abundance of *Planctomycetes*-specific 16S
109 rRNA most closely related to known the anammox genus *Scalindua*, and this was linked to a
110 decrease in the flux of nitrite at pH 7.2 (Gazeau et al. 2014). In addition, the benthic
111 mesocosm study of Widdicombe and Needham (2007) recorded a decreased release of nitrite
112 within sediment cores incubated at elevated levels of CO_2 which may have been linked to an
113 increase in annamox activity. Together, these studies imply the process of anammox may also
114 be impacted by elevated levels of CO_2.

115 Within the present study, we assessed the effects of discharged CO_2 from the sea bed
116 in Ardmucknish Bay (west coast of Scotland) on benthic ammonia oxidation through
117 measurements of potential ammonia oxidation activity. These measurements were compared
118 with the abundances of genes and their transcripts of archaeal and bacterial *amoA*, nitrite
119 reductase (*nirS*) and hydrazine oxidoreductase (*hzo*), a key enzyme in the anammox process,
120 catalyzing the oxidation of the intermediate hydrazine (N_2H_4) to dinitrogen (N_2).

121

122 **2. Materials and methods**

123 **2.1 CO_2 leakage experiment**

124 Our experiment constituted part of the artificial CO_2 leakage experiment from the
125 sub-seafloor in Ardmucknish Bay located on the west coast of Scotland (Blackford et al.,
126 2014). A horizontal borehole was drilled from the shore to unconsolidated sediments 250 m
127 offshore, and carbon dioxide injected through a pipe to 11 m below the seabed in 12 m of
128 water. Gas release began on the 16th May and ended on the 22nd June: a total of 4.2 tonnes of
129 CO_2 was injected into the overlying sediments. Full details of the experimental design can be
130 found at Taylor et al. (a) (manuscript in review). Samples were collected prior to the start of
131 CO_2 release (DP), on days 14 and 36 during CO_2 release (30th May and 21st June, D14 and
132 D36) and after injection ceased on 28th June, 10th July and 19th September (R43, R55 and
133 R126, respectively). Samples were collected from four zones (Zone 1: Epicentre, Zone 2: 25
134 m distant, Zone 3: 75 m distant, Zone 4: 450 m distant). These were chosen due to their
135 similarities in bathymetry and ecological conditions (*e.g.* similar sediment types, water depths
136 and water column nutrient concentrations, temperature, oxygen and salinity).

137 Measurements of overlying water physico-chemical parameters can be found in
138 Lichtschlag et al. (manuscript in review). Briefly, bottom temperature rose from 9 ºC to 13 ºC
139 throughout the course of the experiment. Salinity varied between 29 and 34 due to mixing of
140 lower salinity waters from Loch Etive and higher salinity water transported into Ardmucknish
141 Bay by tidal currents (Taylor et al. (a) manuscript in review). Seasonal variations in
142 concentrations of dissolved oxygen, phosphate, silicon, ammonia and nitrate/nitrite were
143 apparent. There was also some evidence of the release of silicon and ammonium at zone 1
144 during the latter stages of CO_2 release and early recovery phase: this was associated with an
145 overall increase in the dissolution of minerals at these time-points, including potentially toxic
146 metals (Lichtschlag et al. manuscript in review). Bottom water nutrient concentrations can be
147 found in Supplementary Table 1.

148

149 **2.2 Microbial ATP level as a background measure of microbial activity**

150 ATP determination was carried on the five replicate sediment samples collected at
151 each zone. ATP content was measured using an ATP Biomass Kit HS (Biothema) according to
152 the manufacturer's instructions. Briefly, approximately 1g sediment was added to a weighed
153 7 ml bijou tube containing 4 ml sterilised water, and reweighed. Samples were then shaken by
154 hand, placed in a sonicating water bath (2 minutes, 40% intensity) and the sediment allowed
155 to settle. Fifty µl supernatant was added to a cuvette, followed by 50 ul Extractant B/S and
156 finally 400 ul reconstituted ATP reagent HS. The cuvette was inserted into a Deltatox®
157 instrument and the light emission recorded as relative light units (RLU).This was adjusted
158 according to the weight of sediment added to the bijou and the data were reported in relative
159 light units (RLU) per gram of wet sediment.

160

161 **2.3 Nitrification rate measurement**

162 ***Field potential***

163 The materials for the analysis were collected from the oxidized layer, as nitrification
164 is an aerobic activity. The oxygen penetration depth into the sediment in Ardmucknish Bay
165 was measured by a Clark-type microelectrode (Taylor et al., (b) manuscript in review). Values
166 ranged from 0.2 to 0.4 cm at depth throughout the experiment, confirming that nitrification
167 was occurring in the surface of the sediment.

168 Sediment samples were manually collected by divers using two acrylic cores (50 mm
169 in diameter). At the same time, bottom water was also collected with a Niskin sampler at each
170 zone. Each core was sealed with rubber plugs and transported back to the laboratory. The top
171 10 mm sediment was sectioned and used as the material for the assay of the ammonia
172 oxidation rate. Two g sediment was put into a 225 ml glass vial. The vial was filled with 219

ml bottom water, 1 ml of 20 mM ^{15}N labeled NH_4Cl solution added to give a final concentration of 90 μM $^{15}NH_4Cl$, and plugged with butyl rubber and sealed with an aluminum cap. The triplicate sealed vials were incubated for 40 – 48 hours at 10 °C under dark condition. Samples were taken at 0, 12, 20-24, and 40-48 h. The suspensions were centrifuged at 4,000 rpm for 10 minutes at 10 °C, the supernatant was filtered (Millipore Millex, pore size 0.22 μm), and the filtrates frozen at -20 °C.

The concentration of NH_4^+-N, NO_2^--N, and NO_3^--N in the filtrate samples were measured using an auto-analyzer AACS-2 (BL tech Co. Ltd., Japan). Nitrogen stable isotope ratio of nitrite and nitrate within each sample was measured following the method of Isobe et al. (2011), except that the nitrate was reduced with a Cd-Cu column to nitrite. Thereafter, the generated NO_2 was reduced to N_2O using the azide method of Mcllvin and Altabet (2005). The concentration and ^{15}N atom% and ^{14}N atom% of produced N_2O were measure with the gas chromatograph equipped with a quadrupole mass spectrometer (GC/MS-QP2010 Plus, Shimadzu, Kyoto, Japan) with a CP-PoralPLOT Q-HT column (25 m X 0.32 mm, Varian, USA). Ammonia oxidation rate was calculated with the formula (Beman et al., 2011; see Supplementary Materials) and standardized as per dry sediment weight.

The above manipulation was carried out 4 times (DP, D14, D36 and R126); however the protocol was slightly modified for the DP and R126 samples. For the pre-CO_2 release (DP) samples, sectioned sediments suspended with the bottom seawater were aerated with air just before the assay to standardize the oxygen concentration at each station. Twenty ml of suspended sample was put into a 225 ml vial and the vial was filled with the aerated bottom water from each zone and 1 ml of 20mM $^{15}NH_4Cl$ solution. For R126 samples, sediment cores were collected from zone 1 and zone 4 only.

Verification of inhibitory effect of CO_2 (CO_2 exposure in laboratory)

198 To verify that a high concentration of CO_2 had an inhibitory effect on ammonia
199 oxidation within Ardmucknish Bay sediments, the top 1 cm sediment was collected from
200 zone 1 and mixed with bottom water. The sediment was allowed to settle and the suspended
201 fraction divided in to three glass bottles of 500 ml. Vials were exposed to high CO_2
202 concentrations (5 % and 20 % of CO_2) for 48 hours at 10 °C by bubbling the controlled gas
203 mixtures at 100 ml per minute, or air. After the pre-incubation, 20 ml of the sample was
204 placed into a 225 ml vial and the vial filled with the bottom seawater, previously exposed
205 with same gas as the sediment, and 1 ml of 20 mM $^{15}NH_4Cl$ added to each vial. The
206 incubation was carried out for 48 hours at 10 °C in the dark, and nitrification rates measured
207 as described above.
208
209 **2.4 Archaeal and bacterial *amo*A abundance**
210 ***Nucleic Acid extraction and cDNA synthesis***
211 At each zone, five replicate sediment samples were manually collected by divers
212 using five 50 ml syringes with the end cut off. Each syringe was sealed with a rubber bung
213 and transported back to the lab were the top 1 cm was sectioned and frozen at -80 °C. RNA
214 and DNA were extracted from 2 g sediment using the MoBio RNA PowerSoil Total RNA
215 Isolation Kit with the DNA elution accessory kit according to the manufacturer's instructions.
216 Nucleic acids were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies,
217 Delaware). Typical yields were 10 µg RNA and 25 µg DNA g^{-1} sediment. The RNA was
218 reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) with 0.2 µg of
219 RNA and the supplied random primers.
220
221 ***qPCR and RT-qPCR***
222 An ABI 7000 sequence detection system (Applied Biosystems, Foster City, USA)

223 and QuantiFast SYBR Green PCR Kit (Qiagen) was used for all qPCR and RT qPCR
224 experiments. For each sediment sample, the abundance of Thaumarchaeal and bacterial amoA
225 transcripts, nitrite reducer (*nirS*) and hydrazine oxidoreductase (*hzo*) genes and transcripts
226 was determined using the following primers: archaeal *amoA*: Arc-amoA-for and
227 Arch-amoA-rev (Wuchter et al. 2006); bacterial *amoA*: amoAF1 (Rotthauwe et al. 1997) and
228 amoAR2 (Hornek et al. 2006); *nirS*: NirS1F and NirS3R (Braker et al. 1998); *hzo*: HzoF1 (Li
229 et al. 2010) and hzocl1R2 (Schmid et al. 2008). Reaction mixtures contained either 50 ng
230 DNA or 1 µL cDNA. The primer concentrations, annealing temperatures and cycle conditions
231 are listed in Supplementary Table 2. Assays contained a standard curve containing 10^2 to 10^8
232 amplicons μl^{-1} cDNA or DNA. DNA standard curves for each primer pair were constructed
233 using cloned sequences. For RT qPCR, standard curves were produced from cDNA following
234 prior in vitro transcription of cloned sequences using the Ampliscribe T7 Flash kit (Epicentre)
235 following methodologies described by Smith et al. (2006). Gene and transcript numbers were
236 quantified via comparison to standard curves using the ABI Prism 7000 detection software.
237 Automatic analysis settings were used to determine the threshold cycle (CT) values and
238 baselines settings. Each assay was preformed twice with similar results each time. The
239 no-template controls were below the threshold in all experiments. For each standard curve,
240 the slope, y intercept, co-efficient of determination (r^2) and the efficiency of amplification
241 were determined as follows:

242 archaeal amoA genes: $r^2 = 0.999$, y intercept = 34.28, E = 100 %;

243 bacterial amoA genes: $r^2 = 0.999$, y intercept = 36.19, E = 92.4 %;

244 *nirS* genes: $r^2 = 0.999$, y intercept = 33.98, E = 107.7 %;

245 *hzo* genes $r^2 = 0.999$, y intercept = 32.7, E = 103.1 %;

246 archaeal amoA transcripts: $r^2 = 0.998$, y intercept = 33.29, E = 103.5 %;

247 bacterial amoA transcripts: $r^2 = 0.999$, y intercept = 34.79, E = 97.2 %;

248 *nirS* transcripts: $r^2 = 0.992$, y intercept = 34.3, E = 105.81 %;

249 *hzo* transcripts $r^2 = 0.995$, y intercept = 33.03, E = 100.1 %.

250 Here, E shows the amplification efficiency.

251 **2.5 Statistics**

252 The impact of proximity to the CO_2 release site and time, and interactions between
253 the two on measurements of ATP abundance, potential nitrification rates and from log(x +
254 1)-transformed abundance gene and transcript abundance data was determined using
255 PERMANOVA (Anderson et al. 2008). This non-parametric approach was chosen as the data
256 did not meet the normal distribution required in parametric testing. Significant terms were
257 investigated further using pairwise comparisons with 999 permutations (Anderson et al.
258 2008) for the gene and transcript abundance data. Due to the lower number of replicates used
259 for assays of nitrification rate measurements (n = 3), significant differences between zones at
260 specific time points was investigated using Tukey-Krammer honest significant difference
261 (HSD) or Wilcoxon rank sum tests. Pearsons rank correlations were used to investigate
262 possible relationships between the abundance of bacterial but not archaeal *amoA* genes and
263 transcripts per gram sediment and seasonal changes to bottom water ammonium
264 concentrations.

265

266 **3. Results**

267 **3.1 ATP content**

268 Seasonal differences in ATP abundances were evident (PERMANOVA Pseudo-F =
269 7.86; $p = 0.001$) (Figure 1); measurements were lower on D14 (end of May) and on R126
270 (mid-September). There was also evidence of differences between zones (PERMANOVA
271 Pseudo-F = 2.91; p = 0.005). There was a trend for a decrease in ATP content from zone 1 to
272 3 at this field site throughout the experiment, although the difference was only statistically

273 significant between zone 1 and 3 at DP and D14 (PERMANOVA pairwise tests p = 0.045 and
274 p = 0.015, respectively). The range of ATP concentrations throughout the experiment was
275 18,410 - 88,073 RLU g^{-1} at zone 1, 13,370 – 73,798 RLU g^{-1} at zone 2 and 14,248 – 297,800
276 RLU g^{-1} at zone 3. The ATP concentration in reference zone 4 (16,521 – 41,395 RLU g^{-1})
277 typically lay within this range of data observed at zone 1 - 3. The only other significant
278 difference in ATP concentration was at zone 2 on D36 (273,798 RLU g^{-1}), which was higher
279 than the reference zone 4 at this time-point (37,682 RLU g^{-1} p=0.02). It should be noted that
280 there was considerable variability between replicate cores at each site which is why, for
281 example, the apparently large difference between zone 3 and all other zones at R43 is not
282 significant.
283
284 **3.2 Nitrification rate**
285 ***Field potential***
286 The potential activity of ammonia oxidation was measured at each zone before,
287 during and after CO_2 release. Ammonia oxidation rates at zone 1 were relatively stable during
288 the experiment period, averaging 0.044 μmol g^{-1} dry sediment h^{-1} (Figure 2). The ammonia
289 oxidation rate at zone 4 on DP (0.081μmol g^{-1} dry sediment h^{-1}) was the highest rate recorded,
290 but rates decreased through time (Figure 2). Statistical differences among the stations were
291 apparent on DP (PERMANOVA Pseudo-F = 5.31; p = 0.023), D14 (Pseudo-F = 16.47; p =
292 0.013) and D36 (Pseudo-F = 28.38; p = 0.001). The activity at zone 2 on DP (0.021 μmol g^{-1}
293 dry sediment h^{-1}) was lower than that at zone 1 (Turky-Krammer HSD p = 0.025) but that at
294 zone 4 on DP was higher than zone 1(p = 0.021). On D14, ammonia oxidation rates at zone 2
295 and zone 3 were similar to zone 1. Activities at zone 1 were significantly higher than those at
296 zones 2 and 3 on D36 (p = 0.01 and p <0.001, respectively). However, the activity at zone 4
297 was not significantly different from that at zone 1 on D36 (p=0.09). The activity at zone 4

298 decreased with time elapsed and that on R126 was lower than that of zone 1 (Wilcoxon rank
299 sum test, χ^2=3.857, p <0.05).
300
301 ***Verification of inhibitory potential of CO_2 on nitrification***
302 The pH in NBS scale of the pre-incubation samples exposed with 5 % and 20 % CO_2
303 was 6.1 and 5.5, respectively. Although the potential ammonia oxidation rates of the control
304 was maintained at 0.044 μmol g^{-1} dry sediment h^{-1}, rates in the sediments exposed with 5 %
305 and 20 % CO_2 were significantly reduced (ca. 0.001 μmol g^{-1} dry sediment h^{-1}) (Figure 3).
306
307 **3.3 Archaeal and bacterial amoA abundance**
308 ***Abundance of N cycling genes and transcripts***
309 The abundance of archaeal and bacterial ammonia oxidizing (*amoA*), bacterial nitrite
310 reductase (*nirS*), and anammox hydrazine oxidoreductase (*hzo*) genes and transcripts were
311 measured before, during and after CO_2 release (Figure 4). Bacterial *amoA* gene abundance
312 was higher than archaeal *amoA* gene abundance, averaging 4.09 x 10^5 copies g^{-1} sediment for
313 bacterial *amoA* genes and 4.84 x 10^4 copies g^{-1}sediment for archaeal *amoA* genes. For both
314 archaeal and bacterial *amoA* genes, differences in abundance are evident through time
315 (two-way PERMANOVA for archaea: Pseudo-F = 5.76; p = 0.001 and for bacteria: Pseudo-F
316 = 4.73; p = 0.001). For bacteria, peaks in abundance were apparent during pre-exposure (10^{th}
317 May) (Figure 4a) and for archaea (Figure 4b), in samples taken at the end of the exposure
318 period (19^{th} September). However, there were no differences between zones for either
319 archaeal (Pseudo-F = 2.38; p = 0.051) or bacterial (Pseudo-F = 1.26; p = 0.259) *amoA* gene
320 abundance.
321 Comparing *amoA* gene relative abundances with 16S rRNA abundances (Tait et al.
322 manuscript in review) also indicated the ratio of bacterial and archaeal *amoA*:16S rRNA

genes varied seasonally, with bacteria peaking during pre-exposure (Figure 4c: Pseudo-F = 7.67; $p = 0.001$) and archaea peaking on the 21st June (D36) (Figure 4d; Pseudo-F = 3.66; $p = 0.002$). Weakly significant differences in the ratio of archeal *amoA*:16S rRNA genes between zones (Pseudo-F = 2.41; $p = 0.038$) were due to a lower ratio on D36 at zone 1 compared to zone 4).

RT-qPCR measurements of archaeal and bacterial *amoA* transcripts also indicated higher abundances of bacterial *amoA* transcripts (averaging 2.46×10^5 copies g^{-1} sediment) than archaeal *amoA* transcripts (averaging 6.63×10^4 copies g^{-1} sediment). Again, seasonal changes to the abundance of *amoA* transcripts occurred for both bacteria (Pseudo-F = 14.73; $p = 0.001$) (Figure 4e) and archaea (Pseudo-F = 15.62; $p = 0.001$) (Figure 4f), with decreased expression apparent at the end of June/July for bacteria, and increased expression for archaea in July and September. There were no differences in the abundance of bacterial *amoA* transcripts between zones (Pseudo-F = 1.46; $p = 0.14$), but differences were detected for archaeal *amoA* transcripts (Pseudo-F = 3.21; $p = 0.008$). This was due to a decrease in abundance at zone 1 on R43 and R55. The abundance of bacterial but not archaeal *amoA* genes and transcripts g^{-1} sediment correlated with seasonal changes to bottom water ammonium concentration (Supplementary Table 1) (genes: $\rho = 0.878$; $p = 0.001$ and transcripts: $\rho = 0.855$; $p = 0.001$).

The abundance of *nirS* genes also differed through time, with a peak in abundance occurring in late June (Pseudo-F = 3.72; $p = 0.003$). There was also a decrease in abundance at zone 1 on D36 and R43 (Figure 4g; Pseudo-F = 6.1; $p = 0.002$). However, although there were also temporal changes to the abundance of *nirS* transcripts (Pseudo-F = 19.03; $p = 0.001$) there were no differences between zones (Pseudo-F = 0.74; $p = 0.521$) (Figure 4h).

Again the abundance of *hzo* genes varied seasonally (Pseudo-F = 4.61; $p = 0.001$) but differences between zones were not detected (Pseudo-F = 1.2; $p = 0.298$) (Figure 4i).

However, the abundance of both *hzo* transcripts varied both seasonally (Pseudo-F = 11.28; p = 0.001) and with proximity to the CO_2 exposure site (Pseudo-F = 2.64; p = 0.036): this was due to an increase in abundance at zone 2 during the later stages of CO_2 release (D36) (Figure 4j).

4. Discussion

Gas bubble plumes were observed at the seafloor within hours of commencing CO_2 injection. The CO_2 bubbles dissolved in the water column and resulted in a reduction in CO_2 partial pressure in bottom water, varying from 380 – 1,500 µatm depending on injection rate and tidal effects (Blackford et al. 2014). Maximum reduction of pH on the surface sediment was 0.84 at zone 1 compared with zone 4 (Taylor et al., (b) manuscript in review).There was also evidence that this high CO_2 plume in the water column was advected to zone 2 due to tidal circulation (Atamanchuk et al. manuscript in review). Along with the bubbling of CO_2 into the water column, there was also evidence that CO_2 diffused up through the sediment. However, CO_2-induced chemical changes in the upper 25 cm of sediment pore waters were not observed until the last week of the CO_2 injection period (Blackford et al., 2014). Such chemical changes in pore water persisted for two weeks after CO_2 release ended. This included an increase in dissolved inorganic carbon (DIC), pore water alkalinity and calcium ions but an increase in pore water acidity during D36 and R43. pH fell from 7.7 to 7.5 and then increased again just before CO_2 released ended (Blackford et al. 2014). Together this suggested that the elevated levels of CO_2 resulted in rapid dissolution of calcium carbonate present within the sediments. At the same time, there was co-release of cations including Ca, Sr, Li, Fe and Mn, and concentrations of the nutrient elements including dissolved silicon and ammonium increased (Lichtschlag et al. manuscript in review).

The ammonia oxidation rates measured within this study are within the ranges

373 measured at other coastal sites (Mortimer et al. 2004; Magalhães et al. 2005; Caffrey et al.
374 2007; Tait et al. 2014b). A previous study using a range of different sediment types
375 demonstrated elevated levels of CO_2 had no impact on rates of benthic ammonia oxidation
376 (Kitidis et al. 2011). We have also demonstrated that both benthic nitrification and the
377 abundance of archaeal and bacterial *amoA* genes and transcripts per gram sediment were
378 predominantly unaffected by the sub-seabed release of CO_2. The main response detected for
379 all measurements were either seasonal in nature, possibly due to sediment heterogeneity or
380 differences in the physico-chemical parameters between the four different sites selected for
381 study. A dominance of seasonal effects on microbial abundance and activity over CO_2 effects
382 has previously been observed in a terrestrial CO_2 release experiment (Morales and Holben,
383 2013). However, there was evidence of increased dissolution of ammonium at zone 1 during
384 the latter stages of CO_2 release. At zone 1, ammonia concentration on D35 reached an
385 average of 178 (±68) μmol L^{-1}, whereas concentrations at zone 4 throughout the period
386 studied averaged 89 (±31) μmol L^{-1} (Lichtschlag et al. manuscript in review). Interestingly,
387 this coincides with a slight increase in ammonia oxidation rates at zone 1 at this time-point
388 (Figure 2). Ammonia oxidation increased slightly (by 30– 59 %) at zone 1 when compared to
389 zones 2 and 3, suggesting that increased availability of substrate may have resulted in
390 increased ammonia oxidation regardless of CO_2 concentration.
391 The ability of benthic ammonia oxidizers to withstand elevated levels of CO_2 and
392 reduced pH is thought to be associated with the high pH buffering capacity of marine
393 sediments. This was apparent in the deeper Ardmucknish Bay sediments where, despite the
394 high level of CO_2 detected at depth, pH increased to 7.8 on R43 as the rise in DIC was
395 buffered by the dissolution of sediment calcium carbonate (Lichtschlag et al., manuscript in
396 review). Thus, it would appear that the pH buffering ability of the sediments at Ardmucknish
397 Bay neutralized the reduced pH of the pore water. The greatest impact detected within the top

25 cm examined occurred at the bottom of this layer. Although these impacts were not as prominent within surface sediments (Lichtschlag et al., manuscript in review), buffering of pH changes may also have occurred within the top 1 cm sediment examined within this study. However, the particulate inorganic carbon content of the surface sediments was within the range of 0.102 – 0.168 % (Supplementary Table 3). It is not known how long the sediments in Ardmucknish Bay would be expected to buffer the increased levels of CO_2. When the sediment collected from zone 1 on R126 was exposed with high concentration CO_2 (5 % and 20 % which correspond to pH 6.2 and 5.5), ammonia oxidation activities almost disappeared (Figure 3). Thus, the nitrifying microbes were sensitive to higher concentrations of CO_2 (5 % CO_2, pH 6.2). Such high concentrations (30 mmol kg^{-1}) of DIC were actually observed at 15 – 20 cm below the surface at R43 on zone 1 (Blackford et al., 2014). If such a concentration of DIC rose to the surface, or if a higher concentration of CO_2 was leaked from sub-seabed storage, reduction to benthic ammonia oxidation may become apparent.

We speculated that benthic ammonia oxidizers may respond to elevated levels of CO_2 and reduced pH by a shift in the balance of archaeal and bacterial ammonia oxidizers to favour the former. This was detected in an Arctic benthic mesocosm experiment (Tait et al. 2014a) and was thought to be linked to the finding that archaea have a higher substrate affinity for ammonia than bacteria (Martens-Habbena et al. 2009). However, the abundance of bacterial *amoA* genes and transcripts and the abundance of archaeal *amoA* genes did not differ with proximity to the CO_2 release site. Only minor differences to the abundance of archaeal *amoA* transcripts during the initial recovery phase (R43) were detected at the CO_2 release site which may be linked to the high dissolution of minerals at this time point, including several heavy metals at this time point (Lichtschlag et al. manuscript in review). Tait et al. (2014a) also showed a shift in the composition of the active AOB community occurred within sediment cores adjusted to pH 7.8 and 7.7. Unfortunately, we do not have

any data to determine whether a change in archaeal or bacterial ammonia oxidizing community occurred as a result of CO_2 exposure, and so this possibility cannot be ruled out. T-RFLP analysis of 16S rRNA PCR products indicated a rapid shift in the composition of the active community at both zone 1 and 2 (Blackford et al. 2014).

Several studies have now shown AOA to be much more abundant than AOB, and that AOA are the dominant ammonia oxidizers in pelagic systems, (Coolen et al. 2007; Beman and Francis 2006; Wuchter et al. 2006, Mincer et al. 2007; Bouskill et al. 2012; Horak et al. 2013). However, the dominance of AOA over AOB within sediments is less clear with some studies reporting higher abundances of archaea (Caffrey et al., 2007; Bernhard et al., 2010; Abell et al., 2010; Tait et al., 2014b), whereas others have reported higher incidence of bacteria, as observed in this study (Caffrey et al., 2007; Mosier and Francis 2008; Wankel et al. 2011; Zheng et al. 2014). The relative contribution of AOA and AOB to ammonia oxidation was also not clear: neither the abundance of bacterial or archaeal *amoA* genes nor their transcripts correlated with the ammonia oxidation rates. However, it is interesting to note that the abundance of bacterial but not archaeal *amoA* genes and transcripts per gram sediment correlated with seasonal changes to bottom water ammonium concentration, suggesting that this group responded to changing substrate concentrations.

As much of the nitrate and nitrite required for denitrification in coastal sediments is provided by nitrification (Joye and Anderson, 2008), a reduction in nitrification could therefore be expected to also impact denitrification. A study modelling the impact of ocean acidification within the North Sea also suggested that a reduction in nitrification would lead to a reduction in denitrification (Blackford and Gilbert, 2007). This has the potential to lead to eutrophication, but as noted by Blackford and Gilbert (2007), this may be negligible in already eutrophic waters. In this study, measurements of *nirS* genes and transcripts indicated a significant drop in the abundance of *nirS* genes per gram sediment during the latter stages

448 of CO_2 release and the initial recovery phase (Figure 4g). This pattern is identical to
449 measurements of bacterial 16S rRNA genes per gram sediment at zone 1 at these time points
450 (Pearson correlation: $\rho = 0.644$, $p = 0.001$; Tait et al., manuscript in review), suggesting that
451 the decrease in abundance may be due to an overall decrease in bacterial numbers rather than
452 a direct impact on nitrite reducers. This decrease was thought to be associated with the
453 increased dissolution of minerals, including several toxic metal species, at these time points
454 (Lichtschlag et al. manuscript in review).

455 Previous mesocosm experiments have suggested the decreased efflux of nitrite at
456 low pH may be due to an increase in anammox activity (Widdicombe and Needham, 2007;
457 Widdicombe et al. 2009), and there was also an increase in the abundance of
458 *Planctomycetes*-specific 16S rRNA, the majority of which grouped with known anammox
459 bacteria, in sediments exposed to elevated levels of CO_2 (Tait et al., 2014a). In this study, the
460 increase in the abundance of hydrazine oxidoreductase (*hzo*) transcripts at zone 2 during the
461 latter stages of CO_2 exposure, also suggest an increase in anammox activity (Figure 4j). Zone
462 2 was at a distance of 25 m from zone 1 and was intermittently exposed to high CO_2 seawater
463 due to tidal circulation (Atamanchuk et al. manuscript in review). This site may have
464 benefited from the more moderate increased CO_2 concentrations without the associated
465 release of toxic metals measured at zone 1. However, the numbers of transcripts were low in
466 abundance, and we do not know if this would have impacted anammox activity or the flux of
467 ammonium and nitrite from the sediments. More studies are required to determine the
468 response of anammox bacteria to the sub-seabed release of CO_2.

469 Adenosine triphosphate is known as the intracellular transfer of energy and is often
470 used as an index of microbial biomass (Jenkinson et al., 1979, Karjalainen et al., 2001) or as
471 an index of biological activity. Interestingly, on DP and D13 there was a decreasing trend in
472 ATP abundance from zone 1 to zone 3, indicating metabolic activity was possibly higher

473 nearer the release site at these time-points. However, when pCO_2 concentrations of the
474 sediments were at their highest, only a slight increase in ATP abundance was detected during
475 the latter stages of CO_2 release (D35) at 25 m from the release site at zone 2 (Figure 1),
476 similar to the increase in *hzo* transcript abundance described above. Previous work carried out
477 at a terrestrial CO_2 vent found that ATP increased at moderately elevated CO_2 concentrations
478 but not at the highest CO_2 concentrations near the vent (Beaubien et al., 2008). Again this
479 may be due to a combination of a moderate increase in CO_2 concentration with the absence of
480 toxic metal release. Alternatively, this may be indicative of increased organic matter
481 availability at this time-point. Using benthic chambers, Ishida et al. (2013) detected increased
482 ATP abundance in fjord sediment following exposure to 5,000 μatm pCO_2, and this was
483 thought to be due to the growth of microorganisms adapted to the high pCO_2 conditions,
484 possibly due to a decline of the predation pressure from meiobenthos (Ishida et al., 2013).
485 However, no CO_2 impact was recorded on other biological components away from the
486 injection site at any stage, including to the abundance or community composition of
487 macrofauna (Widdicombe et al. manuscript submitted) and meiofauna (Jeroen Ingels,
488 personal communication).
489 Also apparent in this study were differences between the zones used to examine the
490 potential extent of the effects of the CO_2 release. For example, surface sediment ATP content
491 fluctuated seasonally, but there were very little significant change detected between zones
492 (Figure 1). This may have been due in part to the high degree in variability between replicate
493 samples at each site, probably reflecting a patchy distribution of organisms in these sediments.
494 In addition, the potential ammonia oxidation rate at zone 4 was higher than that at zone 1 on
495 DP and D14 (Figure 3), although there were no significant differences among the abundances
496 of bacterial and archaeal *amoA* transcripts at all zones on DP and D14 (Figure 2). Although
497 we measured similar levels of ammonium and oxygen at all four sites, the variability may

498 have been due to lower quantities of organic matter at zone 4. High sediment organic matter
499 content has been shown to suppress rates of ammonia oxidation in lakes (Strauss and
500 Lamberti, 2000). Alternatively, the higher ammonia oxidation rates may be due to differences
501 in the community structure of ammonia oxidation microbes at zone 4 and zone 1.
502 A full scale assessment of potential monitoring sites is required prior to CO_2 storage to
503 compare sediment types and identify localized differences in physico-chemical parameters.
504

505 **5. Conclusion**

506 Previous experiments have shown elevated levels of CO_2 impact pelagic but not benthic
507 ammonia oxidation (Huesemann et al., 2002., Beman et al., 2011, Kitidis et al., 2011). This
508 CO_2 release experiment shows only moderate impact on benthic ammonia oxidation within a
509 coastal sediment. Contrary to previous experiments, we have indicated that sub-seabed
510 release of CO_2 may result in the release of ammonia from sediments resulting in an increase
511 in ammonia oxidation. In addition, only moderate impacts to the abundance of N cycling
512 genes and transcripts were detected: seasonal and site variations on the abundance and
513 activity were the main signature in the shallow coast. We can conclude that a leak of CO_2 on
514 the scale used within this study (< 1 tonne day^{-1}) would have very little impact to ammonia
515 oxidation within coastal sediments. However, impact would be dependent on both the scale of
516 the CO_2 release and the type of sediment: we have shown that seawater containing 5 % CO_2
517 did reduce rates of ammonia oxidation, and this was linked to the buffering capacity of the
518 sediment. It remains to be tested if this would also be the case in sediments within deeper
519 waters: if oxygen is available, a tipping point, dependent on sediment type and a major
520 release of CO_2, thus exceeding the buffering capacity of the sediment, would have to occur
521 before benthic nitrification is impacted.

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Legends to Figures

Figure 1

Median ATP contents in the sediment of four sites (zone 1 = epicentre; zone 2 = 25m distant, zone 3 = 75m distant and zone 4 = control, 450m distant) during the experiment. An asterisk and markings indicate where a significant difference was observed between two zones within samples collected on the same day ($p < 0.05$ PERMANOVA pair-wise comparisons).

Figure 2

The potential ammonia oxidation rates at the pre-injection (DP), 14 days after the injection starting (D14), the end of the injection (D36), and the end of the recovery period (R126). Numbers in parentheses show the day of year. Error bars are standard deviations. Assays of zone 2 and zone 3 were not examined at R126. Significant differences from zone1 at specific time-points are indicated by * $p < 0.05$, ** and $p < 0.01$ (PERMANOVA Turkey-Krammer HSD analysis or Wilcoxon rank sum tests).

Figure 3

The potential ammonia oxidation rates of zone 1 exposed artificially to high concentration CO_2 (5% and 20%) in the laboratory at R126. Significant differences from zone1 at specific time-points are indicated by * $p < 0.05$, ** and $p < 0.01$ (Wilcoxon rank sum tests).

Figure 4

The abundance g^{-1} sediment of (A) bacterial *amoA* genes, (B) archaeal *amoA* genes, (C) bacterial *amoA*:16S rRNA genes, (D) archaeal *amoA*:16S rRNA genes, (E) bacterial *amoA* transcripts, (F) archaeal *amoA* transcripts, (G) *nirS* genes, (H) *nirS* transcripts (I) *hzo* genes and (J) *hzo* transcripts measured using qPCR and RT-qPCR, before, during and after CO_2

782 exposure at each of four zones. Five separate cores were collected from each zone at each
783 time point. Error bars are 95 % confidence intervals and significant differences from zone 1
784 at specific time-points are indicated by * $p < 0.05$, ** and $p < 0.01$(PERMANOVA pair-wise
785 comparisons).