Seasonal variation in denitrification and dissimilatory nitrate reduction to ammonia process rates and corresponding key functional genes along an estuarine nitrate gradient

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4

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23 **Running title:** Seasonal variation in estuarine nitrate-reduction

25

26 **ABSTRACT**

27 This research investigated spatial-temporal variation in benthic bacterial community structure,

28 rates of denitrification and dissimilatory nitrate reduction to ammonium (DNRA) processes

29 and abundances of corresponding genes and transcripts at three sites – the estuary-head, mid-

30 estuary and the estuary mouth along the nitrate gradient of the Colne estuary over an annual

31 cycle. Denitrification rates declined down the estuary, while DNRA rates were higher at the

32 estuary head and middle than the estuary mouth. In four out of the six two-monthly time-

33 points, rates of DNRA were greater than denitrification at each site. Abundance of gene

34 markers for nitrate-reduction (nitrate reductase *narG* and *napA*), denitrification (nitrite

35 reductase *nirS*) and DNRA (DNRA nitrite reductase *nrfA*) declined along the estuary with

36 significant relationships between denitrification and *nirS* abundance, and DNRA and *nrfA*

37 abundance. Spatially, rates of denitrification, DNRA and corresponding functional gene

38 abundances decreased along the estuary. However, temporal correlations between rate

39 processes and functional gene and transcript abundances were not observed.

40

41 **INTRODUCTION**

42   Estuarine sediments are natural environmental gradients of nutrients and salinity, and

43 significant sites of microbial diversity and activity. Bacterial diversity within these sediments

44 is often higher than in other environments (Lozupone and Knight 2007) and the bacteria

45 present drive essential nutrient cycles with direct consequences for ecosystem function.

46 Previously, we showed that the largest nutrient loads to all mainland UK estuaries were

47 attributable to nitrate: at least an order of magnitude greater than ammonium (Nedwell *et al.,*

48 2002; Earl *et al.,* 2014). Benthic microorganisms mediate the nitrate load entering coastal

49 waters via denitrification, dissimilatory nitrate reduction to ammonia (DNRA) and anaerobic

50 ammonia oxidation (annamox) processes.

51   Heterotrophic denitrification by facultatively anaerobic bacteria using nitrate to respire

52   organic matter, produces N_2 and to a lesser extent the greenhouse gas N_2O (Seitzinger *et*

53   *al.,*1988; Nedwell *et al.,* 1999; Dong *et al.,* 2002), removing up to 50% of the nitrate load

54   from estuaries (Nedwell *et al.,* 1999). Anammox, the anaerobic autotrophic oxidization of

55   NH_4^+, uses NO_2^- as an electron acceptor yielding N_2, (Strous *et al.,* 1999; Kuenen 2008).

56   Reported values of N_2 production from anammox in estuarine sediments range from 0 to 30%

57   (Brin *et al.*, 2014, Dong *et al.,* 2009, Nicholls and Trimmer 2009). Denitrification and

58   anammox are significant pathways that remove nitrate as gaseous products from ecosystems,

59   thus reducing the risk of eutrophication. In contrast, DNRA is an alternative pathway that

60   reduces nitrate and nitrite to ammonium. It is a significant, but often overlooked processes in

61   coastal benthic sediments accounting for up to 30% of nitrate reduction activity (Giblin *et al.,*

62   2013). DNRA may contribute to eutrophication by retaining biologically available nitrogen

63   within the system as NH_4^+. Thus the balance between the two predominant nitrate reduction

64   pathways of denitrification and DNRA in benthic estuarine sediments influence the nutrient

65   load entering coastal waters.

66   Both denitrification and DNRA compete for NO_3^- and NO_2^- as an electron acceptor.

67   Nitrogen transformations by denitrification and DNRA in estuarine sediments are influenced

68   by interactions between a number of factors, primarily NO_3^- and organic carbon

69   concentrations, temperature and pH. Previous studies in estuarine sediments have indicated

70   that denitrification may be favoured when nitrate concentrations are high while DNRA tends

71   to outcompete denitrification where there is high availability of organic electron donor and

72   low nitrate (King and Nedwell 1985, Dong *et al.,* 2011).

73   Denitrification and DNRA are catalyzed by a series of nitrate and nitrite reductase

74   enzymes encoded by genes whose abundance can be used as proxies in determining potential

75   for nitrate reduction within environments (Philippot and Hallin 2005). *narG* and *napA* genes

76 encode subunits of two distinct nitrate reductases (NAR and NAP) that mediate reduction of

77 nitrate to nitrite. Nitrite can enter the denitrification pathway in processes mediated by nitrite

78 reductase enzymes encoded by *nirS* or *nirK* genes (see Zumft 1997). Alternatively, in DNRA

79 nitrite is reduced to NH_4^+ by the NrfA enzyme encoded by *nrfA* (see Simon 2002).

80 Consequently, molecular analyses of *nirS/nirK* and *nrfA* genes can be used to investigate the

81 genetic potential in an environment for either denitrification or DNRA.

82   Previously, we demonstrated a decline in rates of denitrification and DNRA, and a

83 corresponding decline in abundance of genetic markers for these processes, down the nitrate

84 gradient of the Colne estuary (Dong *et al.,* 2009) at a single time point (February 2005).

85 Furthermore, previous research revealed seasonal variability in rates of denitrification (Dong

86 *et al.,* 2000); and spatial variability in rates of nitrate exchange across the sediment/water

87 interface, with highest rates in the upper Colne estuary and lowest at the mouth (Thornton *et*

88 *al.,* 2007). These studies did not however simultaneously study the fate of nitrate via benthic

89 denitrification or DNRA nor the nitrate and nitrite reducing communities driving these

90 processes over an annual period to determine seasonal effects. Therefore the aim of this study

91 was to determine rates of nitrate reduction processes linked to corresponding functional gene

92 and transcript abundances along the Colne estuary at two monthly intervals over a 12-month

93 period. Based on previous studies we propose the following hypotheses: first, the trend of

94 decreasing rates of denitrification and DNRA along the estuary gradient is seasonally stable.

95 Second, the relative importance of denitrification and DNRA will vary seasonally, with

96 DNRA higher in the summer. Third, that the abundance of key functional genes and

97 transcripts will correlate spatially and seasonally with corresponding rate processes along the

98 estuary.

99

100 **MATERIALS AND METHODS**

SITE DESCRIPTION, FIELD SAMPLING AND NUTRIENT ANALYSIS

102   Three sites along the Colne estuary, U.K. the estuary head (EH) at the Hythe, mid-

103   estuary (ME) at Alresford Creek and the estuary mouth (EM) at Brightlingsea were sampled

104   at two-monthly intervals from April 2005 to February 2006. The EH is characterised by fine

105   silt sediments (87 to 98% silt:clay <65 μM) and salinity range between 2 to 17 ppt; ME

106   sediments are fine silt (80 − 95% silt:clay <65 μM), with salinity range between 20-32 ppt

107   while EM sediments are clay with a thin layer of fine mud sand (silt:clay <65 μM) and

108   salinity ranging from 28 to 32 ppt. At each site replicate small cores of sediment (10 cm

109   length in core tubes, 3.4 cm internal diameter by 22 cm length) were taken to measure process

110   rates (n = 5 per each process). Triplicate sediment samples were also taken from the top 1 cm

111   of sediment for molecular analysis, and returned on ice to the laboratory within one hour of

112   sampling, prior to storage at -70 °C of aliquots (0.5 g wet weight) of sediment. Water samples

113   were also collected at high tide at the three sites, then samples (10 ml) were filtered through

114   glass fibre filter papers (GF/F, Whatman, UK) and frozen at −20 °C prior to subsequent

115   colorimetric nutrient analyses (Strickland and Parsons, 1972) using a segmented flow

116   autoanalyser (Skalar Analytical B.V., Breda, The Netherlands). Limits of detection for nitrate

117   and nitrite were 0.002 μM, ammonium 0.003 μM. Analytical accuracy for nutrient analysis

118   was maintained by membership of a quality assurance scheme (www.quasimeme.org).

119

120   MEASUREMENT OF RATES OF DENITRIFICATION AND DNRA

121   Denitrification rates to both N_2 and N_2O (Dong *et al.,* 2006) and DNRA rates (Dong *et*

122   *al.,* 2009) were determined simultaneously on intact sediment cores. Briefly, denitrification

123   and DNRA rates were measured by ^{15}N-labeled nitrate addition to sediment cores. Five cores

124   of sediment (~10 cm deep) were collected in Perspex tubes (3.4 cm internal diameter, 22 cm

125   length) from each site. On return to the laboratory, the cores were put in an incubation tank at

126   *in situ* water temperature and submerged in site water that was vigorously bubbled with air

127   overnight to re-equilibrate. Next day, the rates of denitrification and DNRA were measured

128   by ^{15}N labeled nitrate addition to sediment cores. After a 3-hour incubation of the cores, the

129   sediment core and the overlying water were mixed to form a slurry. Slurry samples (12.5 ml)

130   were removed for the quantification of denitrification gaseous products (N_2 and N_2O).

131   Subsamples (10 ml) of the slurried sediment cores were taken for the subsequent recovery of

132    $^{15}NH_4^+$ to determine rates of DNRA. Ammonium in the slurry samples was extracted by

133   steam distillation and ammonium gas was trapped in acid solution. Ammonium in the acid

134   solution was then absorbed onto zeolite, which was then combusted and reduced to N_2.

135   Isotope ratios of N_2 in samples were measured by isotope ratio mass spectrometry using the

136    $^{14:15}N_2$ ratio in air as a standard. DNRA rates were calculated using the isotope ratio of N_2.

137   The DNRA calculation in the previous (Dong *et al.,* 2009; Dong *et al.,* 2011) and present

138   work used the ratios of $^{14:15}NO_3^-$ in the water column, thus showing only the rate of DNRA

139   supported by nitrate from the water column.

140

141   NUCLEIC ACID EXTRACTION

142   DNA and RNA were co-extracted from 0.5 g sediment, using Lysing Matrix B tubes

143   (Bio-101) as described previously in Smith *et al.*, (2007). Briefly, to each 0.5 g sediment

144   sample, 0.5 ml of 240 mM sodium phosphate buffer (pH 8) and 0.5 ml of

145   phenol:chloroform:isoamyl alcohol (25:24:1) (pH 4) were added. Samples were lysed by bead

146   beating for 30 seconds at 2,000 rpm, and centrifuged for 10 mins at 17,563 x g. The aqueous

147   phase was added to 0.5 ml chloroform:isoamyl (24:1), mixed and centrifuged for a further 10

148   minutes at 17,563 x g. The aqueous phase was removed for DNA and RNA precipitation with

149   2.5 volumes of ice-cold ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2). Total

150   nucleic acids were pelleted by centrifugation and washed twice in ice cold 70% ethanol, air

151 dried and suspended in 100 μl of DEPC water. Total RNA was prepared by diluting a 25 μl

152 aliquot of total nucleic acids with an equal volume of DEPC-treated sterile water, followed by

153 digestion using TURBO DNA-free (Ambion, Austin, Texas, USA) in accordance with the

154 manufacturer's protocol.

155

156 16S RIBOSOMAL RNA GENE T-RFLP ANALYSIS, CLONE LIBRARY CONSTRUCTION AND Q-PCR

157 ANALYSIS

158   For Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis, PCR

159 amplification of 16S rRNA genes from DNA was carried out with the primers FAM 63F (5′

160 CAGGCCTAACACATGGCAAGTC ′3) (Marchesi *et al.,* 1998) and 518R (5′

161 CGTATTACCGCGGCTGCTCG ′3) (Lane 1991). 50 μl reactions contained 0.4 μM forward

162 and reverse primer, 0.1 mM dNTPs, 2.5U *Taq* polymerase, 5 μl of the reaction buffer

163 supplied with the enzyme (Qiagen, Crawley, UK), and 1 μl of 10^{-1} dilution of DNA template.

164 PCR amplification was carried out in an ABI 2720 Thermo-cycler (Applied Biosystems,

165 Warrington, UK) as follows; 95 °C for 2 min then 30 cycles of 95 °C for 45 sec, 55 °C for 1

166 min and 72 °C for 30 sec and a final extension step of 72 °C for 10 min.

167 Amplified 16S rRNA genes were purified by using a Qiagen PCR purification kit (Qiagen,

168 Crawley, UK) according to the manufacturer's protocol and subsequently independently

169 digested with AluI and CfoI (Roche Diagnostics, Basel, Switzerland) at 37ºC for 3 hours. 5 μl

170 of each digest was desalted; glycogen (20 mg ml^{-1}) (Thermo Scientific, Waltham,

171 Massachusetts, USA) was added to a final concentration of 0.1 μg/ml and ethanol precipitated

172 with 1/10 volume of 3M sodium acetate (pH 5.2) and 2 volumes of ice-cold ethanol. 0.5 μl of

173 the desalted digestion reaction was added to 9.5 μl of deionised formamide and 0.5 μl ROX-

174 labelled Genescan 500 internal size standard (both Applied Biosystems, Warrington, UK).

175 Samples were denatured at 94 ºC for 5 min, cooled on ice and separated on an ABI 3700

176 (Applied Biosystems, Warrington, UK) using a 10 second injection and a 8.5 kV separation

177 voltage.

178 Quantitative PCR was used to quantify 16S rRNA genes in sediments using an ABI

179 Prism 7000 detection system as described by Smith *et al*. (2006) using the primers 1369F and

180 1492R and the *Taq*Man probe TM1389F (table 1).

181

182 Q-(RT)-PCR OF NITRATE AND NITRITE REDUCATES GENES AND TRANSCRIPTS

183 Nitrate reductase genes (*narG* and *napA*) and nitrite reductase (*nirS* and *nrfA*) genes

184 and transcripts were quantified from triplicate sediment samples from each site using Q-(RT)-

185 PCR *Taq*Man assays as described in Smith *et al.,* (2007 & 2006). Briefly, *Taq*Man primer and

186 probe sets targeting two *narG* (*narG*-1 & 2), three *napA* (*napA*-1, 2 & 3), three *nirS* (*nirS*-ef,

187 *nirS*-m, *nirS*-n) and a single *nrfA* phylotype were targeted as genetic markers of nitrate

188 reduction, denitrification and DNRA, respectively. *nirK* was targeted but it was not detected

189 along the estuary at any of the time points in question (data not shown). For transcript

190 quantification, *narG*-1 and *nirS*-ef gene transcripts were targeted at the EH and ME sites only

191 based on results of our earlier study (Dong *et al.,* 2009). Details of primer and probe

192 sequences are provided in table 1. Gene and transcript abundances were calculated from

193 standard curves (table 2).

194

195 STATISTICAL ANALYSES

196 T-RFLP profiles were aligned on the basis of T-RF size in base pairs and the

197 individual peak areas of the T-RFs identified by using T-Align (Smith *et al.,* 2005) based on a

198 0.5-bp moving average, resulting in the generation of datasets of aligned T-RFs that gave

199 individual relative peak areas as a proportion (%) of the overall profile. All T-RFs that

200 contributed less than 1% of the total peak area for a profile were excluded from further

201   analysis. The aligned T-RFs were transformed by log(X+1) to remove any weighting from

202   dominant peaks and analyzed with a Bray-Curtis similarity matrix (Clarke *et al.* 2006) in

203   Primer v6 (Primer-E, Plymouth, United Kingdom). The resultant similarity matrix was

204   analyzed in a two-dimensional multidimensional scaling (MDS) plot.

205   Variation in nitrate reduction rates or gene abundances between sites and within sites

206   at different months were analysed using a one-way ANOVA followed by a post hoc Tukey

207   test (Tukey, 1953) in SPSS v14. Data was log(x + 1) transformed, as necessary. Spearman's

208   rank correlation analysis was performed to investigate correlations between denitrification or

209   DNRA rates and gene abundances of corresponding genetic determinants in SPSS. A Bray-

210   Curtis resemblance matrix (Clarke *et al.,* 2006) of quantitative PCR gene abundances was

211   generated from the log (x+1) transformed data and an Euclidean distance resemblance matrix

212   was constructed from rate process data and nutrient concentrations and used to construct

213   multidimensional scaling plots (MDS). Variation among sites was assessed using ANOSIM a

214   one-way analysis of similarity in Primer-6 (Clarke, 1993). BIO-ENV and LINKTREE were

215   used to link gene abundance patterns with rate process and nutrient data. Seasonal trajectories

216   were added to MDS plots by ordering sampling months numerically using the overlay

217   trajectory function within PRIMER-6. MDS, ANOSIM, BIO-ENV and LINKTREE analysis

218   were carried out in Primer 6 (PRIMER-E Ltd, Plymouth Marine Laboratory, UK).

219

220   **RESULTS**

221   **IN SITU NITRATE CONCENTRATIONS AND WATER TEMPERATURE**

222   Nitrate concentrations in water decreased from the estuary head (EH) to estuary mouth

223   (EM) with an annual mean (±SE) of 399.7 ± 50.0 µM at EH, 98.8 ± 23.4 µM at ME and 43.1

224   ± 10.2 µM at EM. (NO_3^- and NH_4^+ concentrations for individual months are shown in figure

225 1). Water temperatures varied seasonally ranging from 4 ºC to 19 ºC (in February and August

226 respectively).

227

228 **SPATIAL AND TEMPORAL VARIATION IN RATES OF BENTHIC DENITRIFICATION AND DNRA.**

229 Rates of benthic denitrification decreased from the head towards the mouth of the

230 estuary (Figure 1) following the nitrate concentration gradient in the water column. At the

231 estuary head, the mean (± SE) annual rate of denitrification (415.6 ± 131.2 μmol N m^{-2} h^{-1})

232 was significantly higher (ANOVA, $P < 0.05$) than mid-estuary (53.1 ± 16.1 μmol N m^{-2} h^{-1})

233 and the estuary mouth (12.3 ± 8.2 μmol N m^{-2} h^{-1}), while the latter two did not differ

234 significantly from each other (Figure 1; $P = 0.388$). Mean annual rates of DNRA at the EH

235 (679.1 ± 226.9 μmol N m^{-2} h^{-1}) and ME (516.5 ± 230.1 μmol N m^{-2} h^{-1}) did not differ

236 significantly from each other ($P > 0.05$) but were significantly higher (Figure 3, $P < 0.05$)

237 than at EM (106.3 ± 37.2 μmol N m^{-2} h^{-1}). Rates of denitrification and DNRA at all three

238 sites showed significant seasonal variability ($P < 0.05$, Figure 3). At each site, benthic rates of

239 denitrification were greater than DNRA in April and June only. Rates of DNRA were greater

240 than denitrification at all sites in August, October, December and February (Figure 1).

241

242 **SPATIAL AND TEMPORAL VARIATION IN 16S RRNA COMMUNITY STRUCTURE, ABUNDANCE**

243 **AND DIVERSITY**

244 Changes in community structure along the estuary over the year were assessed by 16S

245 rRNA gene T-RFLP analysis. The results of a Bray-Curtis similarity matrix of T-RFLP

246 profiles generated in triplicate from each site along the estuary over the year revealed two

247 distinct clusters (Figure 2), with the EH forming a separate cluster from the lower estuary

248 sites of ME and EM which were more similar to each other than to the EH (Figure 2). This

249 separation were supported by ANOSIM analysis: EH verses ME R = 0.58, P < 0.001, EH

250 verses EM R = 0.913, p < 0.001 and ME verses EM R = 0.218, p < 0.001. At the EH a

251 sequential seasonal shift in community structure was observed as illustrated by the trajectory

252 (Figure 2), but seasonal cycles in community structure were not evident at the other two sites.

253 16S rRNA gene abundances at each site from April 2005 to February 2006 (Figure 3)

254 indicated a significant site and time effect (2 way ANOVA, P <0.001). Gene abundances were

255 highest at the estuary head and significantly higher than in sediments from mid-estuary and

256 the estuary mouth (P <0.001); while there were no significant differences between ME and

257 EM sediments (P = 0.071). Within individual sites, there was only significant variation in 16S

258 rRNA gene copy abundances between months at ME in October (ANOVA P < 0.008,

259 Bonferroni correction).

260

261 **SPATIAL AND TEMPORAL VARIATION IN THE ABUNDANCE OF NITRATE REDUCTASE (*narG***

262 **AND *napA*) AND NITRITE REDUCTASE (*nirS* AND *nrfA*) GENES.**

263 In our previous study of the Colne estuary, a suite of nine *Taq*Man primer and probe

264 sets were designed targeting indigenous nitrate and nitrite reducing phylotypes present (Smith

265 *et al*., 2007). These included two *narG* (*narG*-1 & 2), three *napA* (*napA*-1, 2 & 3), three *nirS*

266 (nirS-e, m and n) and one *nrfA* (nrfA-2) gene targets. Nitrate (Figure 4) and nitrite (Figure 5)

267 reductase gene abundances were greatest at the estuary head and lowest at the estuary mouth

268 ($P < 0.05$) for eight of the nine phylotypes.   The *napA*-3 phylotype was the exception, with

269 no significant difference in gene abundances observed along the estuary ($P < 0.05$).   Within

270 individual sites there was only limited temporal variability in gene abundances for both nitrate

271 and nitrite reductase phylotypes (Figure 4 and 5).

272 Percentage relative abundance of nitrate- and nitrite- reducing functional gene

273 abundances to 16S rRNA gene abundances were calculated for each phylotype (Figure 6). In

274 general the highest relative abundance of nitrate- or nitrite-reducing functional gene

275 abundances to 16S rRNA gene abundances were observed at the estuary head (Figure 6)

276 indicating this site as having not only the most abundant bacterial community but also the

277 highest proportion of nitrate reducers in that community: commensurate with the highest

278 nitrate concentrations along the estuary. Exceptions to this trend were the *narG*-1 and *napA*-3

279 phylotypes, which were greatest at ME and EM sites respectively. For all genes a peak in

280 relative abundance was observed at the EH in February 2006 corresponding to a peak in

281 nitrate and ammonium concentrations (Figure 3).

282

283 **SPATIAL AND TEMPORAL VARIATION IN narG *AND* nirS GENE TRANSCRIPTION.**

284 Trends of transcript abundances reflected those observed at the DNA level - *narG*

285 transcript abundances were highest at the estuary head, while differences in *nirS*-ef transcripts

286 numbers were not observed between sites (Figure 7, $P > 0.05$).

287

288 **INTER-RELATIONSHIPS BETWEEN FUNCTIONAL GENE ABUNDANCES, NITRATE**

289 **CONCENTRATIONS, RATES OF DENITRIFICATION AND DNRA.**

290 An MDS plot of Bray-Curtis square-root transformed mean gene abundances (i.e. four

291 genes; totalling nine phylotypes) at each site from April to February (Figure 8A) shows

292 spatial and temporal variation in the abundance of the nitrate- and nitrite-reductase genes

293 along the estuary. Gene abundances at the EH site site clustered separately from those at ME

294 and EM sites. A one-way ANOSIM indicated a significant difference in the abundance of

295 nitrate- and nitrite-reductases genes at the three sites (R = 0.679, $P < 0.001$). As with the 16S

296 rRNA community analysis (Figure 2), there was evidence of community change over time

297 (seasonality) at the estuary head in nitrate- and nitrite reductase gene abundances as indicated

298 by the trajectory on Figure 8A. Relationships between gene abundances, denitrification and

299 DNRA rates and nitrate and ammonia concentrations were explored using BIO-ENV and

300   LINKTREE in PRIMER-6 (Clarke and Ainsworth, 1993). BIO-ENV identified nitrate (R=

301   0.639, *p < 0.001*) as the single measured abiotic variable that best explained the clustering of

302   nitrate and nitrite reductase gene abundances along the estuary. To further explore the

303   observed clustering of nitrate and nitrite reductase gene abundances, a LINKTREE non-

304   parametric analysis was conducted in PRIMER6 (Figure 8B), to link the gene abundance

305   clusters to the range of nutrient concentrations or rates of denitrification and DNRA. 3 distinct

306   splits labelled 1, 2 & 3, formed in the LINKTREE dendrogram. The first split was observed

307   between the EH and the lower estuary sites and was defined by NO_3^- concentrations > 202

308   μM. All other sites, fell into split 2 where NO_3^- concentrations were <146 μM. The next split

309   separated the EM site in August from the rest based on the lowest observed nitrate

310   concentration. Split 3, divided EM sites in April, August and October based on denitrification

311   rates >71 μmol N m^2 h^{-1} with remaining sites characterised by rates of denitrification below

312   <53 μmol N m^2 h^{-1}.

313   Spearman's correlation co-efficient analysis was preformed to examine correlations

314   between rates of denitrification or DNRA and the abundance of their corresponding genetic

315   determinants (Table 3). For denitrification significant weak to strong (correlations absolute

316   value of r range between 0.40 to 0.79) were observed between the rates of denitrification and

317   corresponding abundances of *nirS*-n and *nirS-m* phylotypes. *nirS-n*, *m* and *e* primer and probe

318   sets target *nirS* phyotypes first recovered as mRNA *nirS* gene sequences from sediments at

319   the head of the Colne estuary similar to *nirS* from gamma and alpha proteobacteria (Nogales

320   *et al.,* 2000, Smith *et al.,* 2007). Weak to moderate, but not significant correlations were

321   observed between denitrification and *nirS-e* and DNRA and *nrfA* gene abundances (Table 3).

322   The *nirS*-e primer and probe set target *nirS* gene sequences first retrieved from the mid-

323   estuary site of the Colne as mRNA and phylogenetically group with gamma proteobacteria

324   (Nogales *et al.,* 2000, Smith *et al.,* 2007). The *nrfA* primer set targeted *nrfA* phyotypes

325 retrieved from the Colne estuary head site that phylogentically group with epilson-

326 proteobacteria (Smith *et al.,* 2007).

327

328 **DISCUSSION**

329   In this study, we report spatial and temporal dynamics in the activity of benthic nitrate

330 reduction coupled to quantification of nitrate reducing functional genes and transcripts along

331 an estuarine gradient. Denitrification, DNRA and corresponding nitrate and nitrite functional

332 gene abundances decreased along the estuary, following the nitrate gradient in the water

333 column as previously observed in the Colne estuary (Ogilvie *et al.,* 1997, Dong *et al.,* 2009)

334 and the Thames (Trimmer *et al.,* 2000). Denitrification at the estuary head greatly exceeded

335 that of the other sites. At the estuary head, rates of denitrification were highest in June,

336 whereas in the middle of the estuary and at the mouth rates peaked in October. Lowest rates

337 of denitrification at all three sites were observed in December. Our earlier studies of the Colne

338 (Dong *et al.,* 2000) and the Great Ouse (Trimmer *et al.,* 1998) had shown highest

339 denitrification rates during late spring/summer and lowest denitrification activity during the

340 winter.

341   The separation of the estuary head from sites lower down the estuary was not as

342 pronounced when it came to DNRA. Rates of DNRA at the estuary head and middle sites

343 were similar, and DNRA at the middle estuary site was, in fact, greater than at the estuary

344 head in four out of six months (October and February). Seasonal variability was also observed

345 for DNRA, with the highest rates in late summer and early autumn, and lowest rates in spring

346 (Figure 1). Giblin *et al.,* (2010) measured denitrification and DNRA in the Parker River

347 estuary for 13 years at a single upstream site (salinity between 0 and 18 ppt) between 1993

348 and 2006. Large seasonal and inter-annual variation in denitrification and DNRA was

349 reported, primarily driven by salinity not temperature. Rates of DNRA in the Parker River

350 Estuary were highest in late summer (August) and lowest in early spring (March). Spatially,

351 denitrification in the Parker River exhibited the opposite trend to the Colne estuary, as it was

352 inversely related to the salinity gradient with a peak in denitrification in late spring in this

353 estuary. In the Norsminde Fjord, Denmark, Jorgensen (1989) reported two maxima in

354 denitrification, the first in early spring and the second in autumn. A peak in DNRA was

355 observed in late summer, attributed to the more highly reduced sediments within the estuary

356 at this time. The results from the Colne data are in agreement with modelling studies (Kelly-

357 Gerreyn *et al.,* 2001), and data from tropical estuaries (Dong *et al.,* 2011) that suggested

358 DNRA tends to become increasingly important at higher environmental temperatures.

359   Denitrification has in the past been considered the dominant nitrate reduction pathway

360 in coastal and marine sediments and DNRA less important, if considered at all (for a review

361 see Giblin *et al.,* 2013, Burgin and Hamilton 2007). Our previous single time-point study of

362 denitrification, DNRA and anammox, showed that more nitrate was removed from the system

363 via denitrification than was reduced to ammonia by DNRA (Dong *et al.,* 2009). Anammox

364 was detected only at the estuary head and accounted for 30% of the N_2 formation. In this

365 study anammox was not measured, due to logistical limitations, instead focusing on nitrate

366 reduction pathways of denitrification and DNRA. Over the annual time period, denitrification

367 rates exceeded those of DNRA only in the late spring/early summer (April and June) at all

368 three sites along the estuary. For the remainder of the year, at the time points measured, rates

369 of DNRA exceeded those of denitrification indicating that more nitrate was being reduced and

370 converted to ammonia than was being lost from the system in gaseous forms via

371 denitrification. This further highlights the importance of not relying on single time point

372 studies to understand the dynamics of the nitrogen cycle in dynamic estuarine systems.

373   Denitrification and DNRA compete for nitrate and carbon within sediments. The

374 availability of nitrate and organic carbon are key factors controlling rates of benthic

denitrification (Cornwell *et al.*, 1999, Dong *et al.,* 2000, Fulweiler and Heiss 2014). Previous studies of the Colne estuary have indicated that denitrification at the estuary head is carbon limited, while denitrification mid-estuary and at the estuary mouth is nitrate limited (Papaspyrou *et al.,* 2014). However, the ratio of electron donor to acceptor can influence the pathway and fate of nitrate. DNRA has a higher affinity for nitrate than denitrification and may be favoured in nitrate-limited, carbon-rich environments (King and Nedwell 1985, Bergin and Hamilton 2007, Kraft *et al.,* 2014). This is due to the requirement of only 5 electrons to reduce nitrate in denitrification verses the 8 required for DNRA (Teidje, 1988). DNRA may therefore outcompete denitrification in nitrate-limited environments where these organisms gain more energy from DNRA than denitrifiers can from denitrification. Indeed nitrate concentrations at the mid-estuary site were much lower than at the estuary head and in the months where DNRA was the dominant process it was 5 to 2120 times greater than denitrification at this site (Alresford). Recent studies in similar environments have shown that DNRA is often the prominent nitrate reduction pathway. For examples, Giblin *et al.,* 2013, showed DNRA was greater than denitrification in 30% of 55 coastal sediments sites examined. Similarly, Song *et al.,* (2014), in a single time point study of benthic DNRA in the New River estuary, North Carolina, USA showed it was responsible for 44 to 74% of nitrate reduction and reported that DNRA rates were greater than denitrification (Lisa *et al.,* 2014).

The results of our study have highlighted, for the first time, the importance of DNRA as a significant pathway for benthic sediment nitrate reduction in the Colne estuary. The rates of benthic DNRA of nitrate from the water column ($DNRA_w$) in the whole estuary was estimated as 11.48 Mmol N yr^{-1} derived by multiplying the mean annual rates of DNRA at the estuary head (679 μmol N m^{-2} h^{-1}), mid-estuary (517 μmol N m^{-2} h^{-1}) and estuary mouth (106.7 μmol N m^{-2} h^{-1}) by the total area of sediment (which is defined as the 72% of the area totally immersed at spring tide; Ogilvie *et al.,* 1997) in the sector of the river centred around

400 each site (Dong *et al.,* 2000). Using the mass balance approach, by subtracting oxidized

401 inorganic nitrogen (NO_3^- + NO_2^-) removal by denitrification supported by nitrate from water

402 column (5.09 Mmol N yr^{-1}) from the total sediment uptake of oxidized inorganic nitrogen

403 (16.28 Mmol N yr^{-1}), a very similar DNRA rate of 11.19 Mmol N yr^{-1} was obtained (Thornton

404 *et al.,* 2007).

405   At the molecular level, the abundances of gene and transcript molecular markers for

406 nitrate reduction (*nar*G & *nap*A), denitrification (*nirS*) and DNRA (*nrf*A) generally showed a

407 consistent overall spatial trend of declining abundances from the estuary head to the estuary

408 mouth (Figures 4, 5 & 6) as observed in the rate process data, supporting and extending our

409 previous studies along the Colne estuary at single time points (Smith *et al.,* 2007, Dong *et al.,*

410 2009). In contrast to our observations in the hypernutrified Colne, *nirS* and *nirK* gene

411 abundances along the lower nutrient Fitzroy estuary, Australia showed no statistical

412 difference between sites, despite some observed variability in the net rates of denitrification

413 along the estuary (Abell *et al.,* 2010). The one exception to this trend of decreasing gene

414 abundances along the estuary was the *napA*-3 phylotype (alphaproteobacteria) where gene

415 abundances throughout the 12 month sampling period remained constant along the estuary

416 gradient (Figure 4), suggesting a different selective mechanism and different ecological

417 significance for this *nap* phylotype than for the other two phylotypes (both

418 gammaproteobacteria). While we have determined the distributions of these different

419 functional genes and their phylotypes along the estuary, one of the key questions remaining to

420 be elucidated is the different functions of these phylotypes, which can probably only be

421 clarified by controlled laboratory-based studies. Richardson *et al.,* (2001) suggested that NAR

422 and NAP provide adaptations to different nitrate environments, the former facilitating nitrate

423 reduction in reduced, high nitrate environments whereas NAP is adaptive to effective nitrate

424 scavenging in lower nitrate and less reduced environments. The distributions that we

425 measured along the Colne estuary would suggest that these different *napA* phylotypes,

426 particularly *napA-3* generally support this hypothesis as total bacterial numbers decline along

427 the estuary but *napA* numbers remain high indicating that they are relatively more important

428 at lower nitrate concentrations than *narG*.

429   While gene and transcript abundances generally decreased along the estuary gradients,

430 as observed in the rate process data, finer scale correlations between rates and gene/transcript

431 abundances on temporal scales were not observed. Some studies have shown good agreement

432 between activity measurements and gene and/or transcript abundances e.g. in studies of

433 archaeal nitrification (Wuchter *et al.,* 2006). However there is evidence in the literature to

434 indicate the direct measurement of functional genes at DNA and even mRNA levels can be

435 uncoupled to activity measurements, indicating that substantial post-transcriptional, protein

436 assembly and/or environmental factors ultimately control activity. For example, Ikeda et al.,

437 (2009) examined the roles of NAR and NAP in nitrate reduction in *Pseudomonas sp.* and

438 found that nitrate reductase activity and *napA* or *narG* gene transcription were not necessarily

439 positively correlated, leading them to conclude that there were subsequent post-translational

440 modifications even in pure culture. In soils, Liu *et al.,* (2010) demonstrated the dramatic

441 effect increasing pH had on reducing denitrification activity, yet this trend was not

442 reciprocated in *nirS* and *nosZ* gene and transcript abundance, leading them to conclude that

443 reduction in pH affected denitrification after transcription. In the highly nutrified Colne

444 estuary, the high nitrate concentrations in the water are drastically reduced within the surface

445 sediment by rapid nitrate reduction in the sub-oxic zone (the surface oxic layer of sediment is

446 usually <2-3mm depth (Robinson *et al.,* 1998). Consequently, benthic nitrate reducers are

447 operating at very low pore water nitrate concentrations where rates of nitrate reduction

448 approximate to first order kinetics; well below any nitrate-saturating concentrations when

449 nitrate reduction might correlate with genetic potentials. The correspondence between rate

450 processes and gene abundances/transcripts is likely to be closer the nearer *in situ* substrate

451 concentrations are to saturating concentrations of substrates. Furthermore other physical-

452 chemical factors such as temperature or pH may control key enzyme activity, and *in situ* rates,

453 without necessarily directly affecting transcription. Further and future studies will focus on

454 determining the links between environmental conditions, nitrate and nitrite reducing

455 communities and rates of nitrate reduction and controlling factors influencing the fate of

456 nitrate within estuarine sediments.

457

458

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625

626   **TITLES AND LEGENDS TO FIGURES**

627   **Figure 1.** Temporal and spatial variation in top: Nitrate and ammonium concentrations and

628   bottom: Rates (+/- SE, n=5) of (A) Denitrification and (B) DNRA in sediments along the

629   Colne estuary sampled from April 2005 to February 2006. For each process, significant

630   differences in the overall process rates between sites along the estuary are indicated by

631   different Greek letters above the letters above coloured lines (black = estuary head (EH), red

632   = mid-estuary (ME) and blue = estuary mouth, EM))

633

634   **Figure 2**. Non-metric Multi-Dimensional Scaling plot of total community T-RFLP profiles

635   from three sites along the estuary sampled at two-monthly intervals from April 2005 to

636   February 2006. Each month is represented in biological triplicates and coded according to

637   site. Insert map showing sample location along the Colne estuary. Black triangles represent

638   the estuary head (EH), gray circles mid-estuary (ME) and white squares the estuary mouth

639   (EM). A = April, J = June, Au = August, O = October, D = December, F = February 2006.

640

641   **Figure 3.** Spatial and temporal variation in 16S rRNA gene copy abundances in sediments

642   from the Colne estuary from April 2005 to February 2006. Standard errors (n = 3) are shown.

643   Greek letters above coloured lines (black = estuary head (EH), red = mid-estuary (ME) and

644   blue = estuary mouth (ME)) indicate statistical differences in the overall gene abundances

645   across the year between sites (p< 0.001)

646

647   **Figure 4.** Variation in abundance (gene copies g^{-1} sediment +/-SE, n=3) of nitrate reductase

648   genes (*narG* and *napA*) in sediments along the Colne estuary sampled from April 2005 to

649   February 2006. For each phylotype, significant differences in annual gene abundances

650   between sites along the estuary are indicated by different Greek letters above coloured lines

651   (black = estuary head (EH), red = mid-estuary (ME) and blue = estuary mouth (EM))

652

653   **Figure 5.** Variation in abundance (gene copies g^{-1} sediment +/- SE, n=3) of nitrite reductase

654   genes (*nirS* and *nrfA*) in sediments along the Colne estuary sampled from April 2005 to

655   February 2006. For each phylotype, significant differences in annual gene abundances

656   between sites along the estuary are indicated by different Greek letters above coloured lines

657   (black = estuary head (EH), red = mid-estuary (ME) and blue = estuary mouth (EM)

658

659   **Figure 6**. Relative abundance (%) of nitrate- and nitrite- reductase phylotype gene

660   abundances to 16S rRNA gene abundances along the estuary over the annual sampling period.

661

662   **Figure 7.** Variation in nitrate (*narG*-2) and nitrite (*nirS*-ef) reductase gene transcript

663   abundances (transcripts g^{-1} sediment +/-SE, n=3) in sediments along the Colne estuary

664   sampled from April 2005 to February 2006. For each phylotype, significant differences in

665   annual transcript abundances between sites along the estuary are indicated by different Greek

666   letters above the coloured lines representing each site (black = estuary head (EH), red = mid-

667   estuary (ME)).

668

669   **Figure 8.**   A: Non-metric multidimensional scaling (MDS) ordination of a Bray-Curtis

670   resemblance matrix calculated from square-root transformed mean Q-PCR nitrate- and nitrite-

671   reductase gene abundances quantified two-monthly intervals from sediments at each site;

672   temporal variation at the estuary head (EH) is illustrated by a time-line trajectory drawn

673   through sampling time points. (B). LINKTREE analysis of all sites and time points to identify

674 environmental variable range (nitrate, ammonia, denitrification or DNRA) driving clustering

675 of nitrate-nitrite reducing community observed in 8A. Three splits (1 -3) and variable range

676 responsible for the divide are shown. %B, an absolute measure of group difference is shown

677 on the x-axis. ANOSIM R value and *p*-values are reported for each split. Months: A = April, J

678 = June, Au = August, O = October, D = December, F = February (2006)

679

680 **Table 1.** Q- (RT)-PCR primer and probe sets used in this study

681 682	**Target (reference)**	**Primer/probe** [a]	**Sequence (5′ - 3′)**	**Amplicon (bp)**	**Annealing °C**
683	16S rRNA	1369F	CGGTGAATACGTTCYCGG	123	56
684	Suzuki *et al.*	1492R	TACGGYTACCTTGTTACGACTT		
685	(2000)	TM1389F	CTTGTACACACCGCCCGTA		
686	*napA*-1	napA-1F	GTYATGGARGAAAAATTCAA	111	55
687	Smith *et al.*	napA-1R	GARCCGAACATGCCRAC		
688	(20007)	napA-1 TM*	AACATGACCTGGAAG		
689	*napA*-2	napA-2F	GAACCKAYGGGYTGTTATG	76	55
690	Smith *et al*	*napA-2R*	TGCATYTCSGCCATRTT		
691	(20007)	*napA*-2 TM*	CTTTGGGGTTCAA		
692	*napA*-3	*napA*-3F	CCCAATGCTCGCCACTG	130	60
693	Smith *et al.,*	*napA*-3R	CATGTTKGAGCCCCACAG		
694	(2007)	*napA*-3 TM*	TGGGTTGTTACGA		
695	*narG*-1	*narG*-1F	GAC TTC CGC ATG TCR AC	69	60
696	Smith *et al.*	*narG*-1R	TTY TCG TAC CAG GTG GC		
697	(2007)	*narG*-1 TM*	TAYTCCGACATCGT		
698	*narG*-2	*narG*-2F	CTCGAYCTGGTGGTYGA	89	55
699	Smith *et al.*	*narG*-2R	TTYTCGTACCAGGTSGC		
700	(2007)	*narG*-2 TM*	AACTTCCGCATGGA		
701	*nrfA*-2	*nrfA*-2F	CACGACAGCAAGACTGCCG	67	60
702	Smith *et al.*	*nrfA*-2R	CCGGCACTTTCGAGCCC		
703	(2007)	*nrfA*-2 TM*	TTGACCGTCGGCA		
704	*nirS*-e	*nirS*-ef-F	CACCCGGAGTTCATCGTC	172	60
705	Smith *et al.*	*nirS*-efR	ACCTTGTTGGACTGGTGGG		

706	(2007)	nirS-ef TM*	TGCTGGTCAACTA		
707	nirS-m	nirS-m-F	GGAAACCTGTTCGTCAAGAC	162	60)
708	Smith et al.	nirS-mR	CSGARTCCTTGGCGACGT		
709	(2007)	nirS-m TM	TCTGGGCCGACGCGCCGATGAAC		
710	nirS-n	nirS-n-F	AAGGAAGTCTGGATYTC	140	55
711	Smith et al.	nirS-nR	CGTTGAACTTRCCGGT		
712	(2007)	nirS-n TM*	ATCCGAAGATSA		

713 Legend: *TM *Taq*Man Minor Groove Binding; TM, *Taq*Man

714
715
716 **Table 2:** Quantitative and reverse transcriptase quantitative -PCR standard curve descriptors
717

	Phylotype	Template	r^2	y-intercept	E (%)	Ct-cutoff
718						
719	16S rRNA	DNA	0.998	35.1	92.6	28.9
720	narG-1	DNA	0.998	41.1	77.6	29.9
721	narG-2	DNA	0.998	43.4	85.4	ND
722	napA-1	DNA	0.994	44.7	95.5	32.3
723	napA-2	DNA	0.999	42.2	86.9	31.3
724	napA-3	DNA	0.997	41.9	89.9	34.1
725	nirS-ef	DNA	0.998	35.0	86.0	35.9
726	nirS-m	DNA	0.996	41.1	86.0	ND
727	nirS-n	DNA	0.998	42.8	82.1	ND
728	nrfA-2	DNA	0.997	39.9	96.0	28.5
729	narG-2	cDNA	0.999	43.3	83.0	ND
730	nirS-ef	cDNA	0.998	43.0	85.0	ND

731
732 *Legend: E* = amplification efficiency, ND= no template control not detected
733
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740 **Table 3:** Spearman's rank correlation co-efficient between rate process measurements and

741 functional gene abundances

Rate Process	gene	r	P	n
743 Denitrification	*nirS*-ef	0.422	0.081	18
744	*nirS-n*	0.562	0.015*	18
745	*nirS-m*	0.608	0.007*	18
746 DNRA	*nrfA*	0.485	0.056	18

747 ** P < 0.05*

Figure 1.TIFF

Nitrate

Ammonium

Denitrification

DNRA

● Estuary Head, EH   ■ Mid Estuary, ME   ▲ Estuary Mouth, EM

Figure 2.JPEG

2D Stress: 0.14

Estuary Head, **EH**

Mid-Estuary, **ME**

Estuary Mouth, **EM**

Colchester

Wivenhoe

River Colne

Brightlingsea

Brightlingsea Reach

N   0   1 km
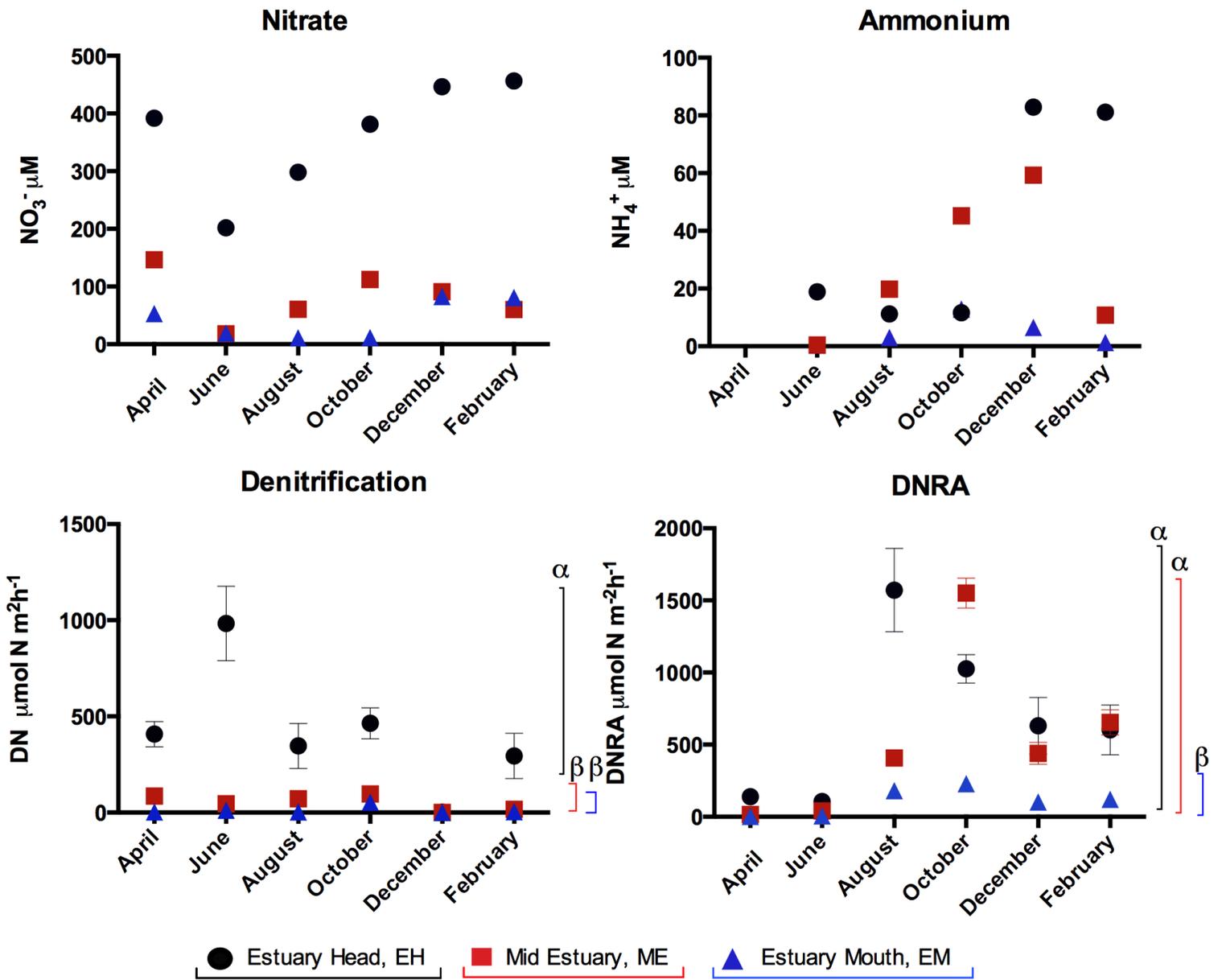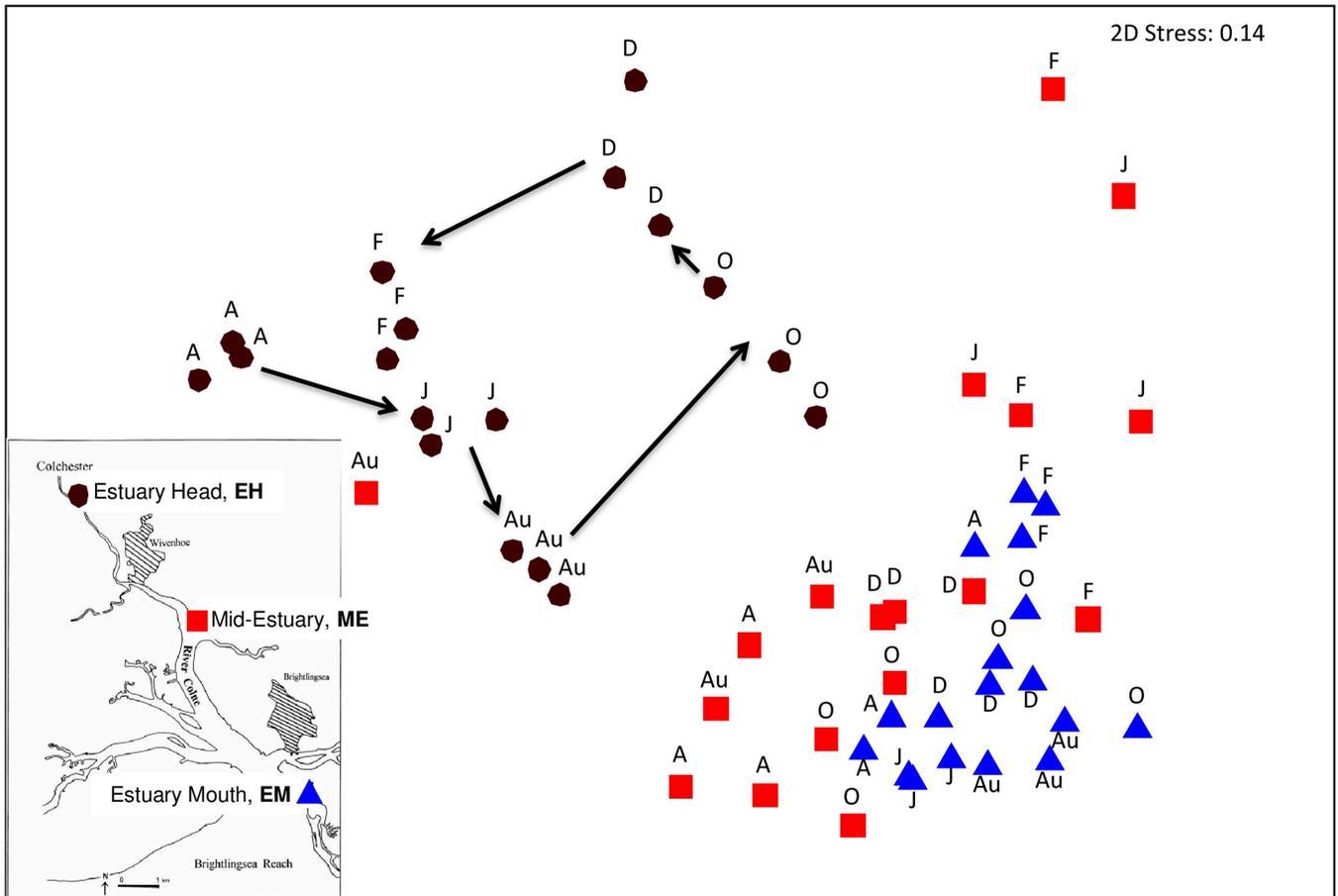
Figure 3.TIFF
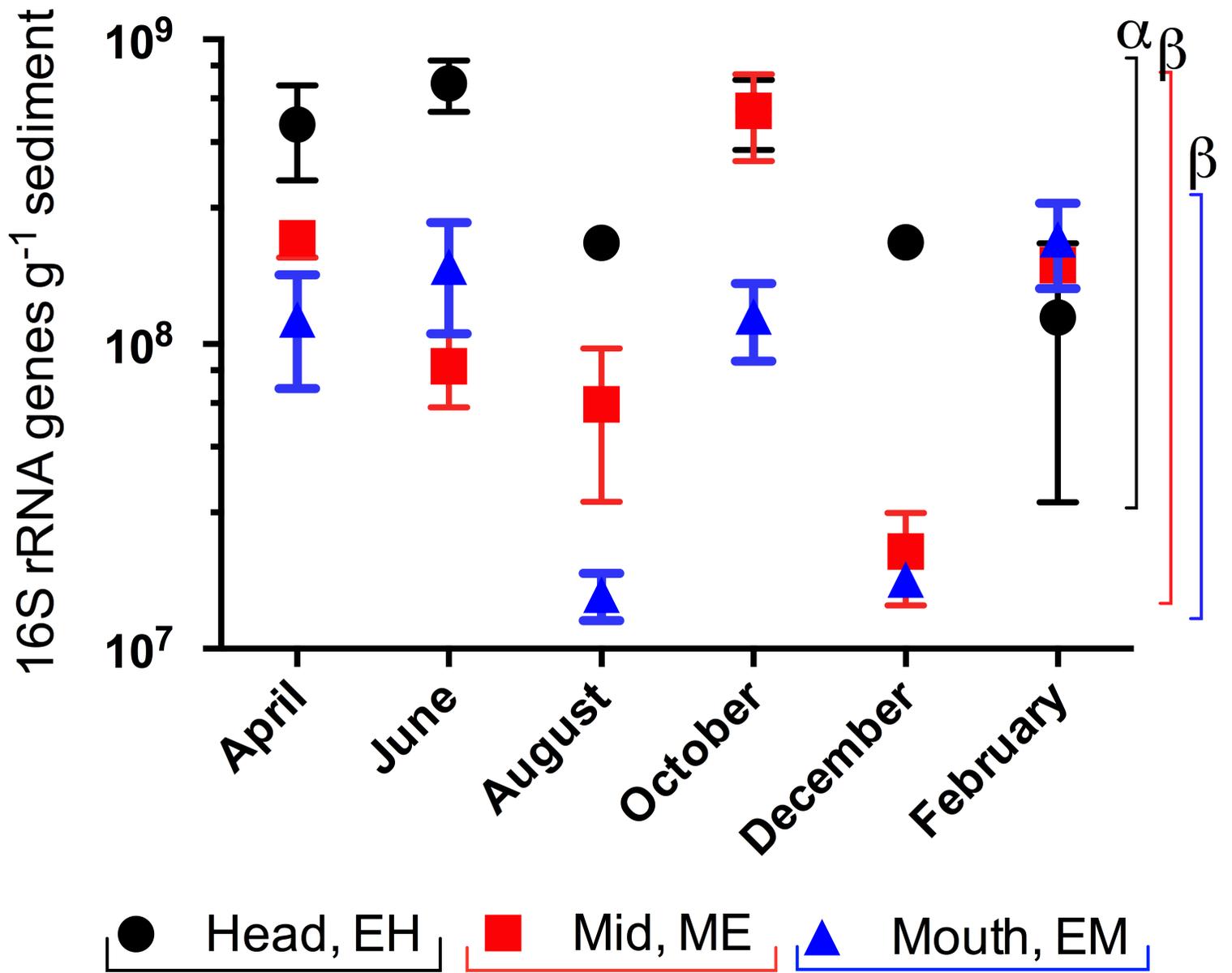
Figure 4.JPEG
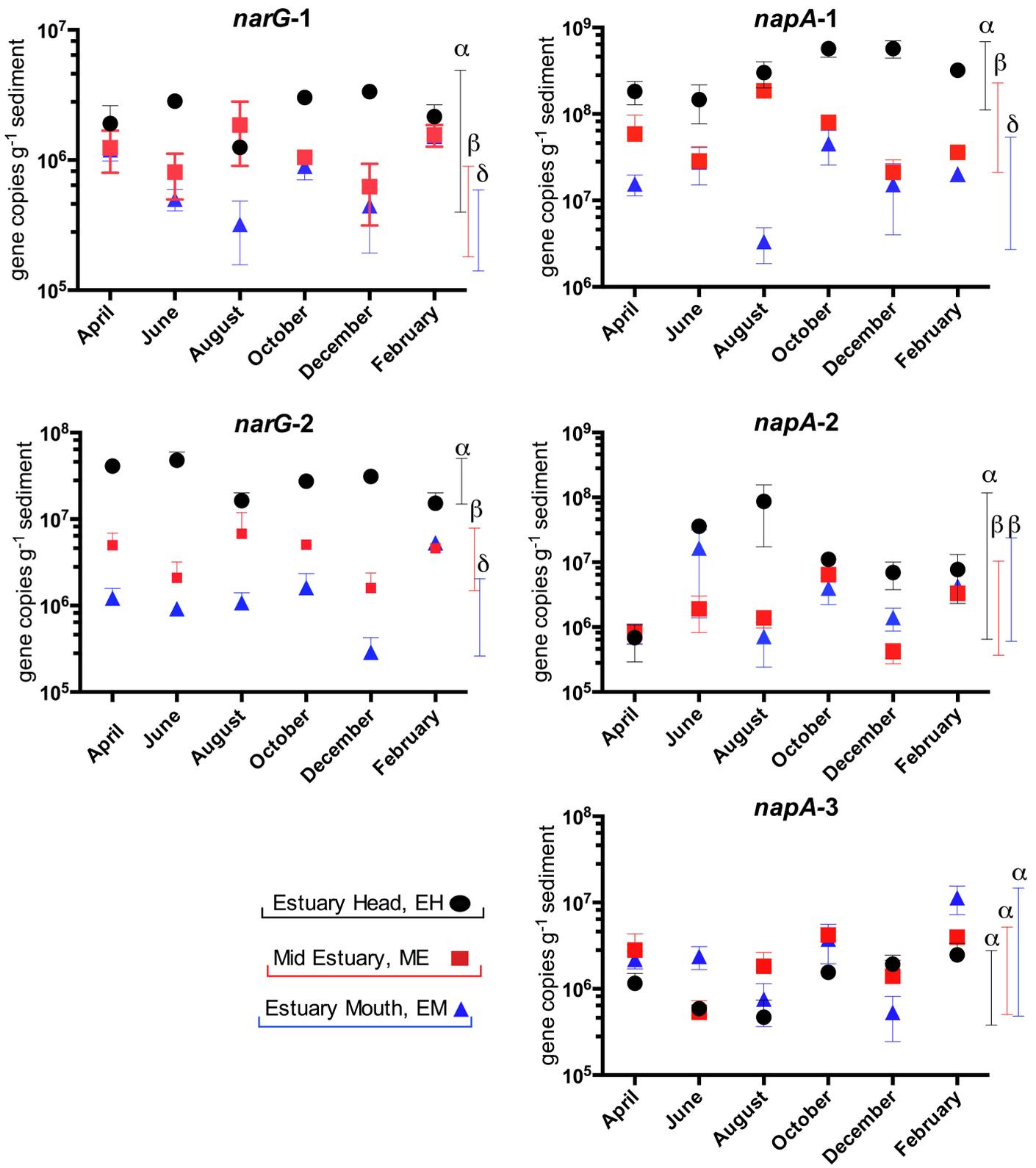
Figure 5.TIFF
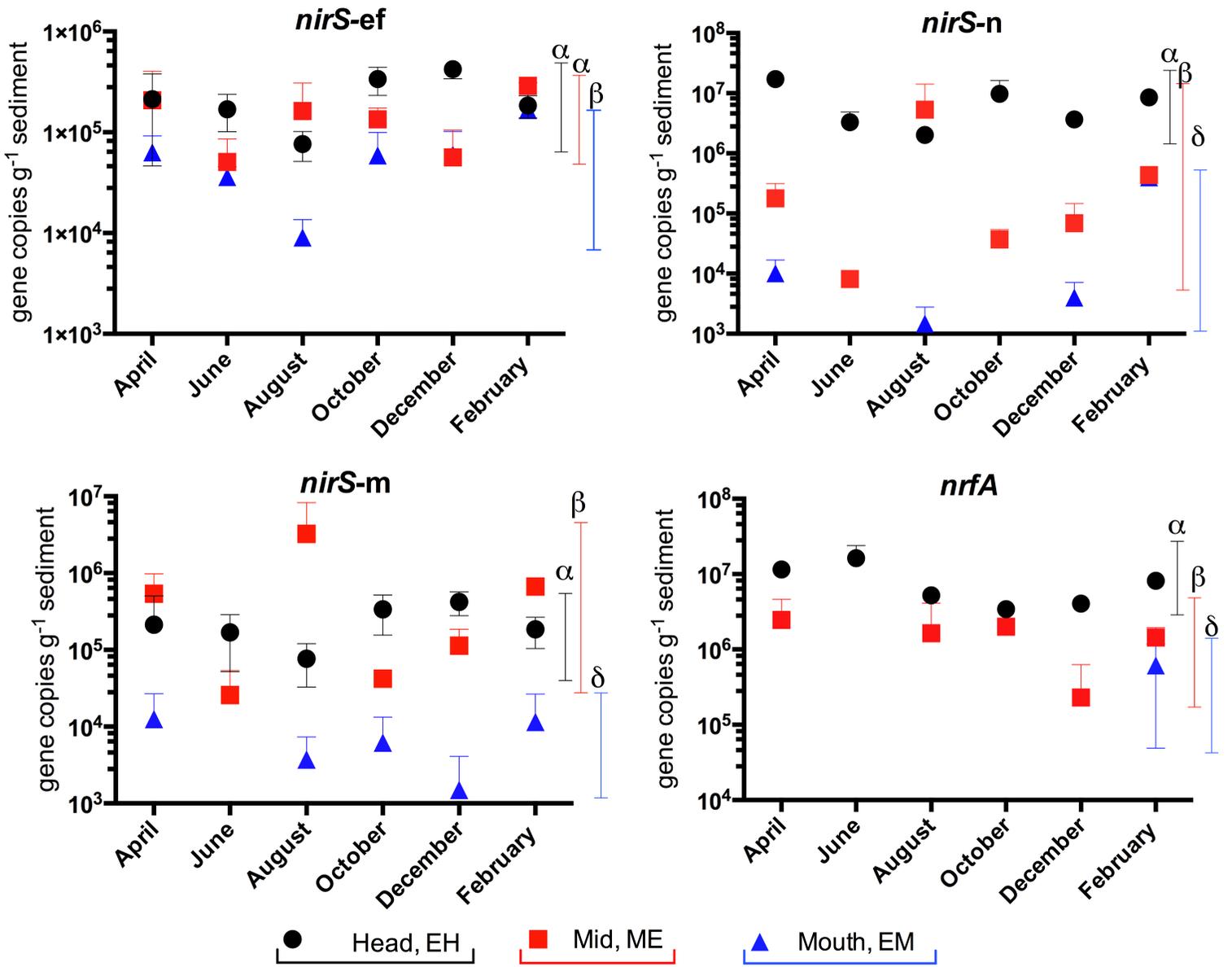
Figure 6.TIFF

Figure 7.TIFF

Figure 8.JPEG