Resource Quantity Affects Benthic Microbial Community Structure and Growth Efficiency in a Temperate Intertidal Mudflat

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Abstract
Estuaries cover <1% of marine habitats, but the carbon dioxide (CO2) effluxes from these net heterotrophic systems contribute significantly to the global carbon cycle. Anthropogenic eutrophication of estuarine waterways increases the supply of labile substrates to the underlying sediments. How such changes affect the form and functioning of the resident microbial communities remains unclear. We employed a carbon-13 pulse-chase experiment to investigate how a temperate estuarine benthic microbial community at 6.5 °C responded to additions of marine diatom-derived organic carbon equivalent to 4.16, 41.60 and 416.00 mmol C m⁻². The quantities of carbon mineralized and incorporated into bacterial biomass both increased significantly, albeit differentially, with resource supply. This resulted in bacterial growth efficiency increasing from 0.40±0.02 to 0.55±0.04 as substrates became more available. The proportions of diatom-derived carbon incorporated into individual microbial membrane fatty acids also varied with resource supply. Future increases in labile organic substrate supply have the potential to increase both the proportion of organic carbon being retained within the benthic compartment of estuaries and also the absolute quantity of CO2 outgassing from these environments.

Introduction
Estuaries are net heterotrophic systems [1,2] and represent a significant source of CO2 to the atmosphere: Regional-scale estimates suggest that European estuaries represent 5–10% of Western Europe’s anthropogenic CO2 emissions [3]. The exact contribution of estuaries to the global atmospheric CO2 emissions remains contentious, but estimates range between 0.25–0.45 Pg C y⁻¹ [2,4]. Bacteria play a pivotal role in the mineralization of organic matter along estuaries, with CO2 emissions from these ecosystems originating principally from bacterial respiration [2,5]. They are also fundamental for mediating changes in the nitrogen cycle [6], and thereby influence the availability of key nutrients for primary producers. It follows that understanding the processes controlling microbial mineralization in estuarine waterways is a prerequisite for predicting the future role of this habitat in global elemental cycles and thus climate regulation. Estuarine sediments can efficiently bury organic matter, sequestering both carbon and nitrogen from the atmosphere [1,7,8]. A deeper appreciation of estuarine biogeochemistry is therefore also required if we are to forecast how the production and storage of organic matter in coastal ecosystems will be affected by further anthropogenic change.

Agricultural practices and human wastewaters both contribute significantly to the eutrophication of rivers and coastal systems [9,10], resulting in a greater supply of labile organic substrates to the seabed. Work conducted on bacterioplankton communities has demonstrated that the rates and efficiencies with which they grow are both positively related to substrate availability [11–13]. A positive relationship between benthic estuarine mineralization rates and carbon input also exists when multiple locations are considered [1]. Far less is known about how increased organic matter supply affects benthic microbial community structure and function within a single estuary. Indeed, estimates of estuarine benthic bacterial growth efficiency (BGE) and the factors controlling it are scarce [14]. We used a carbon-13 (¹³C) tracer study to explore the hypothesis that substrate quantity affects the composition and short-term metabolic response of the benthic bacterial community in a temperate estuary. Increasing quantities of ¹³C-labelled diatoms were added to hand-collected sediment cores, allowing us to quantify the amounts of diatom-derived carbon that were mineralized and incorporated into benthic bacterial biomass during the experiment. Our results demonstrate that carbon mineralization, bacterial biomass production and BGE are all coupled to the supply of labile substrates, illustrating that resource quantity plays a key role in controlling the short-term fate of organic matter in temperate estuarine sediments.

Materials and Methods
Study Site and Sediment Collection
The experiment was conducted on natural whole-sediment communities retrieved from the tidal mudflats in the lower reach...
of the Ythan Estuary, Aberdeenshire, Scotland, UK (57°20.085′N, 02°0.206′W). All necessary permissions for work on the Ythan and Forvie National Nature Reserve were obtained from Scottish Natural Heritage. The sediments at the experimental location have a mean particle diameter of 50 μm [15] and contained 1.5% organic carbon by dry weight in the upper 1 cm. A total of 12 Perspex cores (10 cm ID x 600 mm length) were inserted 12 cm into the sediments by hand at low tide and the resulting material retrieved. All sediment cores were transferred to a temperature controlled laboratory, set at the in situ temperature of 6.5°C, within 30 minutes of collection. Each core subsequently received 3.8 L of UV-sterilized, 10 μm filtered seawater previously collected from the estuary at high tide (≈35 psu).

Experimental Setup

The experiment consisted of four treatments, with three replicates of each: Control (no substrate addition), low-, medium-, and high-quantity of organic material. Sediments in the latter three treatments received the equivalent of 4.16, 41.60 and 416.00 mmol organic carbon m⁻² respectively in the form of a ¹³C-labelled marine diatom, Chaetoceros calcitrans (49.4±0.3 atom % ¹³C). These quantities of carbon were chosen to fall below and above 11.42 mmol organic carbon m⁻², the mean daily amount of phytoplankton estimated to be deposited on Ythan sediments [16]; the three levels of organic enrichment are representative of the mean daily quantities of carbon received by oligotrophic, mesotrophic and hypertrophic estuaries respectively [17]. The use of ¹³C-labelled C. calcitrans enabled us to trace the fate of the constituent carbon into dissolved inorganic carbon (DIC¹³C) and bacterial biomass in a quantitative manner. Specific details of algal culture techniques and biochemistry of the C. calcitrans are presented elsewhere [18]. Immediately prior to experimentation, the algal substrates were suspended in 10 ml of seawater and gently pipetted directly onto the sediment surface to ensure homogenous distribution. All cores were subsequently sealed with lids to prevent gas exchange and incubated in darkness. Water samples from each core, collected via lid ports immediately after lids were rotated immediately prior to each sampling interval to a head space. Stirrer-bars that passed through the lids via an o-ring seal were gently depressed into the sediments by hand at low tide and the resulting material retrieved. All sediment cores were transferred to a temperature controlled laboratory, set at the in situ temperature of 6.5°C, within 30 minutes of collection. Each core subsequently received 3.8 L of UV-sterilized, 10 μm filtered seawater previously collected from the estuary at high tide (≈35 psu).

Sample Processing

Samples for determination of oxygen concentrations were transferred into 10 ml Winkler bottles, fixed and subsequently analysed using an automated Winkler titration system (785 DMP Titrino, Metrohm U.K.). Concentrations of NH₄-N and TOx-N were determined using an automated segmented flow analyser (Bran & Luebbe QuAAtro SFA, SEAL Analytical Ltd., U.K.). Aliquots of water for the analysis of DIC and DI¹³C ammonium-nitrogen (NH₄-N) and total oxidised nitrogen (TOx-N; NO₂⁻+NO₃⁻). Core lids were gently depressed into the cores as the water samples were drawn to avoid the production of a head space. Stirrer-bars that passed through the lids via an o-ring seal were rotated immediately prior to each sampling interval without disturbing the sediment surface to ensure that the water was well homogenized. Sediments were extruded at the end of the experiment and the upper 1 cm was retained and stored at −80°C for subsequent quantification of diatom carbon uptake into bacterial biomass.

Mean amplitude of five replicate sample peaks was used to calculate DIC concentration from a calibration curve derived from an appropriate range of sodium carbonate standard solutions. Purified phospholipid fatty acids (PLFAs) extracted from freeze-dried sediment samples [20,21] were derivitized to yield fatty acid methyl esters (FAMEs). The concentrations and carbon isotope ratios of individual FAMEs were measured using a GC Trace Ultra with combustion column attached via a GC Combustion III to a Delta V Advantage IRMS (all Thermo Finnigan, Germany). Individual PLFAs were quantified by combining the area of their mass peaks, m/z = 44, 43 & 46, after background subtraction, and comparison with a known internal standard (19:0) added to each sample [22]. Bacterial carbon uptake was calculated from label incorporation into the bacterial biomarker PLFAs i15:0, a15:0 and i16:0 [23], assuming these represent 10% of total bacterial PLFAs and 0.056 gC PLFA/gC biomass [24]. All calculations relating to the uptake of ¹³C were made using well-established equations [23]. Data are expressed as the total uptake and mineralization of added diatom-derived carbon (¹³C +¹³C). Bacterial growth efficiency (BGE) was estimated as: IB/(IB+RR), where IB and RR are the quantities of diatom-derived carbon incorporated into bacterial biomass and respired over the duration of the experiment respectively. The resulting estimates are considered to be minimum estimates as a proportion of the quantified respiration may have been attributable to metazoan organisms (see Discussion).

Statistical Analyses

All statistical analyses were conducted in the ‘R’ programming environment [25] using the ‘nlme’ and ‘MASS’ packages [26,27]. Repeated seawater sampling from each core necessitated that all of the resulting benthic flux data were analysed using linear mixed-effects (LME) models that included core identity as a random effect [28]. Variance covariate terms were also incorporated in the random structure of the models in instances of unequal variances. The fixed structures of the statistical models initially incorporated time and treatment and an interaction between these terms. Backwards model selection, based on the likelihood ratio test using maximum likelihood estimation, was employed to determine the fixed structures of the optimal models (OMs) [28,29]. Restricted maximum likelihood estimation was used to generate model parameter estimates. All OMs were validated to check that the underlying assumptions were met: Normality of residuals was examined by plotting theoretical quantiles versus standardized residuals (Q-Q plots); homogeneity of variance was assessed by plotting residual versus fitted values; independence was verified by plotting residuals versus each covariate [28]. Estimated values± standard errors (se) are presented.

Bacterial carbon uptake and BGE data were box-cox transformed to attain homogeneity of variance prior to analysis using one-way analysis of variance (ANOVA). Post-hoc multiple comparisons were achieved using Tukey’s honest significant difference tests. Treatment effects on the proportional uptake of diatom-derived carbon into individual PLFAs, a relative indication of the PLFAs and 0.056 gC PLFA/gC biomass [24]. All calculations relating to the uptake of ¹³C were made using well-established equations [23]. Data are expressed as the total uptake and mineralization of added diatom-derived carbon (¹³C +¹³C). Bacterial growth efficiency (BGE) was estimated as: IB/(IB+RR), where IB and RR are the quantities of diatom-derived carbon incorporated into bacterial biomass and respired over the duration of the experiment respectively. The resulting estimates are considered to be minimum estimates as a proportion of the quantified respiration may have been attributable to metazoan organisms (see Discussion).

Results

Benthic Fluxes

Concentrations of NH₄-N and TOx-N were inversely related (Figs. 1A and 1B): NH₄-N increased at a rate of 1.71 mmol
±0.11 m\(^2\) d\(^{-1}\) (L.Ratio = 105.44, df\(_1\), p<0.001; Table S1) and TOx-N decreased at a rate of 1.27±0.15 mmol m\(^{-2}\) d\(^{-1}\) (L.Ratio = 50.90, df\(_1\), p<0.001; Table S2). These rates were not affected by the quantities of added diatoms (Time x Treatment interactions; L.Ratios<4.5, df\(_3\), p>0.21 in both cases). Oxygen concentrations declined significantly over time (Fig. 1C; L.Ratio = 146.91, df\(_1\), p<0.001; Table S3) but drawdown rates were not affected by the quantity of added diatoms (Time x Treatment interaction; L.Ratio = 5.09, df\(_3\), p = 0.165); oxygen was consumed at a rate of 33.45±1.43 mmol m\(^{-2}\) d\(^{-1}\). In contrast, the rate at which diatom-derived carbon was mineralized increased significantly with increasing quantities of added material (Fig. 1D; Time x Treatment interaction; L.Ratio = 146.73, df\(_3\), p<0.001; Table S4); mineralization rates in the low, medium and high treatments are presented in Table 1.

### Microbial Carbon Processing

The quantities of diatom-derived carbon incorporated into bacterial biomass at the end of the experiment increased significantly with the quantity of material added (Table 1; F = 436.45, df\(_2\),6, p<0.001). Treatment effects on BGE were also apparent (Table 1; F = 10.25, df\(_2\),6, p = 0.012). The proportional uptake of diatom-derived carbon into individual fatty acids differed by treatment (Fig. 2). The microbial community in the low treatment discriminated on the first principal component (PC1), with positive loadings of 10-Me18:0, i16:0 and i16:1 and negative loadings of 15:0, i15:0 and i17:0. Communities in the medium and high treatments discriminated on the second principal component (PC2); the former was characterised by increased carbon uptake into 17:1(n-8)c, 17:0, 19:1(n-8), ai17:0 and 12-Me16:0 and decreased uptake into 17:0cy and 19:1(n-6). The inverse pattern was observed in the high treatment.

### Discussion

Our measured fluxes of NH\(_4\)-N, TOx-N and oxygen (Fig. 1A, B and C) are in good agreement with earlier observations from other temperate, intertidal mudflat sediments [32–36]. The null effect of resource supply on these fluxes (p>0.16 in all cases; Fig. 1) is consistent with previous benthic enrichment studies in which relatively small quantities of organic carbon have been added.

### Table 1. The effect of resource-quantity on estuarine benthic carbon budgets.

| Resource quantity | Low  | Medium | High |
|-------------------|------|--------|------|
| Mineralization    | 0.143±0.05 (3.4) | 1.149±0.06 (2.8) | 6.589±0.50 (1.6) |
| Bacterial uptake  | 0.096±0.01 (2.3) | 1.314±0.12 (3.2) | 8.201±1.51 (2.0) |
| BGE               | 0.40±0.02 | 0.53±0.02 | 0.55±0.04 |

Mineralization and uptake units are mmol C m\(^{-2}\) d\(^{-1}\). Estimated bacterial growth efficiencies (BGE) are expressed as proportions±SEM. Values in parentheses represent the percentage of total carbon added.

doi:10.1371/journal.pone.0038582.t001
estimated values in estuarine and deep-sea sediments [14,37,38] and agree closely with the value of 0.5 observed for a natural marine bacterioplankton community growing on diatom aggregates [47,49]; they also correspond with an apparent plateau in BGE of ~0.5 observed across a range of eutrophic pelagic systems [48]. Benthic meiofauna, particularly nematodes, contribute significantly to carbon mineralization in estuarine sediments [1,49]. We did not quantify meiofaunal contributions to mineralization processes, therefore our values of BGE may be considered to be minimal estimates. However, a growing number of studies report negligible mineralization and uptake of diatom-derived carbon by meiofauna during short-term tracer incubation studies [37,50–52]. This suggests that they are not directly involved in the catabolism of detrital material, at least within the time-scale of the present study. Close agreement between the BGEs presented herein and previous estimates suggests that the meiofaunal contribution to carbon mineralization in our experiments was low. This interpretation is further supported by other work which indicates that estuarine detrital carbon cycling is predominated by bacteria whereas the constituent meiofauna feed selectively on living, autochthonous microphytobenthos [53]. Our observations indicate that the growth of the bacterial community in our experimental sediments was directly regulated by resource availability, as previously reported for bacterioplankton communities [11–13]. The positive relationship between BGE and resource supply reflects a progressive uncoupling between bacterial biomass production and respiration. This finding is consistent with theory and previous observations; a greater proportion of assimilated resources must be allocated to meet basal demands for biomass maintenance (as opposed to growth) when resources are scarce [12,13,54].

The relative uptake of tracer carbon into the different PLFAs examined in our study changed between the low, medium and high substrate additions (Fig. 2), likely reflecting a range of complex and interacting processes. Previous work using terminal restriction fragment length polymorphism analysis found no effect of resource supply on the prokaryotic community composition of tidal creek sediments [55]. Similarly, there were no appreciable changes in the proportional uptake of 13C into different PLFAs in a deep-sea sediment community when exposed to two different quantities of 13C-enriched diatoms [46]. Differences in the relative distribution of 13C-labelling between the treatments in our experiment may therefore reflect a resource-dependent change in the balance between catabolism and anabolism of individual PLFAs within the active component of the bacterial community; the relative abundance of certain PLFAs are known to be affected by external stressors [56,57] and can change in response to the substrates used for biosynthesis [58]. However, the most dominant factors discriminating between the low-, medium- and high-treatments, 10Me-18:0, 17:1(n-8) and 17:0cy respectively, are typical of sulfate-reducing bacteria [56,59]. This group of organisms is responsible for approximately 50% of all carbon degradation in shallow water sediments [60]. They grow under anaerobic conditions but are capable of aerobic carbon mineralization [61,62]. We therefore suggest that the observed changes in 13C uptake into individual PLFAs predominantly reflects a progressive shift towards a sulfate-reducing microbial community as substrate supply increased [56]. However, the present data do not allow us to conclusively differentiate between the suggested explanations. The isotope-based PLFA technique is a powerful and sensitive method for discerning carbon uptake in natural microbial communities. The effectiveness of this approach is, however, tempered by an inability to differentiate between a true shift in the microbial community structure and metabolic changes...
within the same community owing to the poor specificity of individual biomarker PLFAs. Irrespective of the underlying mechanism, substrate-induced shifts in the synthesis of individual compounds has implications for the energetic and nutritional value of estuarine sediments to the communities of deposit feeding animals that inhabit them.

In conclusion, resource quantity had a profound effect on the rates of carbon mineralization and uptake into specific PLFAs in a temperate estuarine sediment microbial community. Processes that increase the supply of labile resources to this environment will result in a greater proportion of the organic carbon being retained in the benthic food web due to increased bacterial growth efficiency. Nevertheless, the absolute quantities of CO₂ resulting from microbial mineralization will increase with the input of labile organic matter, at least over the range investigated herein. More work is needed to refine our understanding of the longer-term impacts of resource availability on microbial community structure and functioning and the implications for stocks of previously sequestered carbon in estuarine sediments.

Supporting Information

Table S1 Model output from the NH₄-N concentration data analysis. The optimal model (OM) was a LME model that incorporated core identity as a random effect (L. ratio = 14.230, df1, pcorr<0.001) and allowed the residual spread to increase exponentially over time (L. ratio = 44.507, df1, p<0.001):

\[
NH₄N_{ij} = \text{Intercept} + Time_{ij} + a_i + e_{ij}
\]

\[
a_i \sim N(0, \sigma^2_{core})
\]

\[
e_{ij} \sim N(0, \sigma^2 \times e^{2b \times Time_{ij}})
\]

where \(a_i\) is a random intercept and the index \(i\) refers to the core identity \((i = 1, \ldots, 12)\), and \(j\) to the observations within each core \((j = 1, \ldots, 7)\). Random effect \((a)\), variance function \((b)\), correlation coefficients of observations made within each core [intra-class correlation] \((\text{b})\) and fixed effects \((d)\). *Note the intercept (baseline) is the control treatment. (DOC)

Table S2 Model output from the TOx-N concentration data analysis. The optimal model (OM) was a LME model that incorporated core identity as a random effect (L. ratio = 15.366, df1, p<0.001) and allowed the residual spread to increase exponentially over time (L. ratio = 179.335, df3, p<0.001):

\[
TOxN_{ij} = \text{Intercept} + Time_{ij} + a_i + e_{ij}
\]

\[
a_i \sim N(0, \sigma^2_{core})
\]

\[
e_{ij} \sim N(0, \sigma^2 \times e^{2b \times Time_{ij}})
\]

where \(a_i\) is a random intercept and the index \(i\) refers to the core identity \((i = 1, \ldots, 9)\), and \(j\) to the observations within each core \((j = 1, \ldots, 3)\). Random effect \((a)\), variance function \((b)\), correlation coefficients of observations made within each core [intra-class correlation] \((\text{b})\) and fixed effects \((d)\). *Note the intercept (baseline) is the control treatment. (DOC)

Table S3 Model output from the oxygen concentration data analysis. The optimal model (OM) was a LME model that incorporated core identity as a random effect (L. ratio = 19.467, df1, p<0.001):

\[
Oxygen_{ij} = \text{Intercept} + Time_{ij} + a_i + e_{ij}
\]

\[
a_i \sim N(0, \sigma^2_{core})
\]

\[
e_{ij} \sim N(0, \sigma^2 \times e^{2b \times Time_{ij}})
\]

where \(a_i\) is a random intercept and the index \(i\) refers to the core identity \((i = 1, \ldots, 12)\), and \(j\) to the observations within each core \((j = 1, \ldots, 7)\). Random effect \((a)\), correlation coefficients of observations made within each core [intra-class correlation] \((\text{b})\) and fixed effects \((d)\). *Note the intercept (baseline) is the control treatment. (DOC)

Table S4 Model output from the DIC concentration data analysis. The optimal model (OM) was a LME model that incorporated core identity as a random effect (L. ratio = 48.237, df1, p<0.001) and allowed the residual spread to increase exponentially over time and to vary by treatment (L. ratio = 179.335, df3, p<0.001):

\[
Carbon_{ij} = \text{Intercept} + Time_{ij} + Treatment_{ij} + Time_{ij} \times Treatment_{ij}
\]

\[
treatment_{ij} + a_i \sim N(0, \sigma^2_{core})
\]

\[
e_{ij} \sim N(0, \sigma^2 \times e^{2b \times Time_{ij}})
\]

where \(a_i\) is a random intercept and the index \(i\) refers to the core identity \((i = 1, \ldots, 9)\), \(j\) to the observations within each core \((j = 1, \ldots, 6)\) and \(k\) to the treatment \((k = 1, \ldots, 3)\). Random effect \((a)\), variance function \((b)\), correlation coefficients of observations made within each variance grouping [intra-class correlation] \((\text{b})\) and fixed effects \((d)\). *Note the intercept (baseline) is the low diatom-addition treatment. (DOC)

Acknowledgments

M. Rose is gratefully acknowledged for assistance with nutrient analyses. We thank everyone that commented on this article prior to publication.

Author Contributions

Conceived and designed the experiments: DM. Performed the experiments: DM. Analyzed the data: DM AZ. Contributed reagents/materials/analysis tools: DM BT. Wrote the paper: DM BT AZ.

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