Can we predict ecosystem functioning using tightly linked functional gene diversity?

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ABSTRACT

Biodiversity generally affects ecosystem processes, but to what extent detailed knowledge about specific aspects of biodiversity helps us understand and predict specific ecosystem functions is not well-known. We hypothesised that information about functional gene abundance and diversity would provide a better way of predicting a particular function catalysed by that gene product, than would a more generic descriptor of species diversity. For our purposes, we used marine benthic nitrogen-fixing bacteria as a model system. We first used published observational data to relate nitrogen fixation to the abundance of the gene *nifH*, which encodes enzymes involved in the fixation of atmospheric nitrogen gas. We then used the fitted model, which explained 37% of the variance, to predict nitrogen fixation in other sediment samples for which nitrogen fixation and *nifH* abundance was determined. The model provided no predictive power for benthic nitrogen fixation on independent data. Additional information on the diversity of the general bacterial community, and the nitrogen fixing community in particular did not improve predictions. It was also not possible to predict nitrogen fixation based on the abundance of particular gene variants or bacterial taxa. Our results demonstrate that process rates can be intrinsically difficult to predict based on community metrics even when the community data and the process are tightly coupled.

INTRODUCTION

Biodiversity is generally assumed to favour ecosystem functioning. This is due to niche complementarity, positive interactions or the higher chance of including high-performing species (Tilman et al., 2014). The qualitative trend has been consistent with that hypothesis across a variety of systems (Schmid et al., 2009; Cardinale et al., 2011; Gamfeldt et al., 2015), including microbial ecosystems (Bell et al., 2005; Philippot et al., 2013; Delgado-Baquerizo et al., 2016). Papers in the field hence frequently include verbal predictions that future loss of diversity will have adverse consequences for ecosystem services and human well being (Cardinale et al., 2012). Quantitative predictions, however, are largely absent or highly speculative (Cardinale et al., 2011; Hooper et al., 2012) and the estimates of the relationship between biodiversity and functioning are often noisy and characterised by large variance around the mean (Maestre et al., 2012; Gamfeldt et al., 2013; Delgado-Baquerizo et al., 2016). Therefore, the ability to make predictions has been called into question (Houlahan et al., 2017).

The field of microbial ecology, too, has called for predictive modeling of ecosystem functioning (Allison, 2012; Wieder et al., 2013; Powell et al., 2015). Microorganisms dominate Earth's biogeochemical cycles.

Being able to accurately model functions, such as carbon sequestration or nitrogen cycling, would thus be valuable. Therefore, the inclusion of microbial community metrics into ecosystem models has been advocated to improve predictions of process rates (Moorhead and Sinsabaugh, 2006; Todd-Brown et al., 2011; Allison, 2012; Reed et al., 2014). However, attempts to include microbial community diversity, structure or size have had mixed success. While some environmental studies report that prediction of process rates could be improved by the inclusion of biotic variables (Powell et al., 2015) or that process rates were strongly correlated to community diversity or size (Hsu and Buckley, 2008; Wessén et al., 2011) others report no link (Graham et al., 2014; Rocca et al., 2015). Bier et al. (2015) reviewed the literature reporting microbial community structure and process rates and found that 75% of the studies that tested for it found a significant link. Roger et al. (2016) reviewed experiments that manipulated bacterial diversity by dilution-to-extinction and reported that only 25% of 92 reported relationships were positive and statistically supported. An analysis of 82 data sets that investigated the contribution of functional gene abundance, diversity or community composition for carbon and nitrogen cycling rates found that overall community metrics improved model fit in only 29% of the cases (Graham et al., 2016). The improvement was small on average as adding microbial community metrics to a model with edaphic factors alone improved the model fit from an adjusted r-square of 0.56 to 0.65. Nevertheless, for obligate processes performed by phylogenetically narrow functional guilds, the model performed better.

In this study, we aimed to first predict and then explain marine benthic nitrogen fixation based on the abundance and diversity of the nitrogen-fixing community, as well as the overall bacterial community. We hypothesised that community metrics based on the functional community performing nitrogen fixation would be a better predictor of fixation rates than the overall bacterial community metrics. Nitrogen fixation, the biological transformation of atmospheric nitrogen gas into bioavailable ammonium, is a crucial ecosystem function and this process is exclusively performed by free-living and symbiotic bacteria and archaea. To detect these diazotrophs, it is established praxis to study the *nifH* gene, which encodes the enzyme dinitrogenase reductase involved in nitrogen fixation.

In a previous study by Andersson et al. (2014), *nifH* abundance explained 37% of the variance in nitrogen fixation in shallow marine sediments (Fig. 1 a), suggesting that the size of the diazotrophic community could be used to predict nitrogen fixation rates. In the present study, we first test a model based on data from Andersson et al. (2014) to predict the observed nitrogen fixation rates in shallow marine sediments published in Alsterberg et al. (2017), using *nifH* abundance. The samples for both studies were collected in the same environments (illuminated sediments in shallow coastal bays along the Swedish west coast), in the same season (summer), using the same protocols for measuring nitrogen fixation. Alsterberg et al. (2017) did not quantify *nifH* gene abundance. Therefore *nifH* abundance was determined for this study from DNA samples from Alsterberg et al. (2017) using largely the same protocols as in Andersson et al. (2014). In a second step, we sequenced the diazotrophic community based on *nifH* and included the general bacterial community, based on 16S rRNA gene sequences, obtained from Alsterberg et al. (2017). With these additional data, we evaluated the relative ability of 1) the general bacterial diversity, 2) the diversity of the diazotrophic community, 3) the abundance of the diazotrophic community, and 4) the habitat type for explaining nitrogen fixation rates. We also analysed whether the abundance of any particular bacterial or diazotroph species correlated with nitrogen fixation rates.

MATERIALS AND METHODS

Study design and sample origin

Process rates and DNA samples were collected in a previous study (Alsterberg et al., 2017). For that study, a total of 112 sediment cores from four different habitat types, hereafter referred to as 'Sandy beach', 'Silty mud', 'Cyanobacterial mats' and '*Ruppia maritima* meadows' were collected from shallow bays in the summer of 2013 on the Swedish west coast. As the original experiment investigated the effects of habitat diversity on ecosystem multifunctionality, the cores were assembled into habitat diversity treatments of 1 to 4 different habitats. Each habitat-diversity sampling unit consisted of 4 sediment cores jointly placed in a larger cylinder were they shared the same overlying water. The final sample arrangement is shown in (Fig. S1). The sediment cores were placed in a greenhouse with a continuous water flow from surface water pumped from an adjacent bay and the experiment was left running for 2 weeks. In the present study we used only DNA extracts and data from the summer sampling in Alsterberg et al. (2017).

As the original experiment was replicated in three seasons, it was not possible to analyse samples from all 112 cores in each season. For that reason, sediment samples from the same habitats within the same large cylinders were combined to a single sample, resulting in a total of 52 samples. From the original 52 samples, 12 samples were excluded. 6 samples are missing and we excluded another 6 samples as they are very likely to have been misassigned to the wrong habitat as consistently revealed by the multidimensional clustering of the phylogenetic marker gene (16S) and the nifh gene as well as two separate functional genes not included in this study (Fig. S2).

Nitrogen fixation

Nitrogen fixation rates were measured with the acetylene reduction assay (Stewart and Fitzgerald, 1967) as modified by (Capone and others, 1993). The details of the measurements are described in Alsterberg et al. (2017) and Andersson et al. (2014). Briefly, four sample of 3 ml homogenized sediment were incubated in gas tight exetainers together with 1.5 ml filtered seawater. The headspace with untreated air (1.3 ml) was enriched with 20% acetylene (C_2H_4) gas (v:v) and the incubation was terminated through the addition of 6.1 mol L^{-1} $ZnCl_2$ after 0, 24, 48 and 72 hours, respectively. Nitrogen fixation was then measured by analysing the concentration of ethylene (C_2H_4) gas in the headspace and converting the ethylene production into atmospheric nitrogen fixation using a ratio of N_2 : ethylene of 1:3 (Capone and others, 1993; Capone et al., 2005). The published nitrogen fixation rates have been recalculated and are now expressed as µmol N_2 g^{-1} sediment (wet weight) d^{-1}.

Analyses of of 16S rRNA and *nifH* gene sequences

DNA extraction was performed in the previous study by Alsterberg et al.. The samples for DNA extraction were taken at the same time as the samples for nitrogen fixation thus assuring that the analysed community was the initial community used in the nitrogen fixation assay. Five g homogenized (and where indicated above pooled) sediment samples were frozen and stored at -20°C. DNA was then extracted from 0.3 g thawed sample, using the FastDNA Spin Kit for Soil (MP Biomedical) as indicated by the manufacturer.

Using the extracted DNA from Alsterberg et al. (2017), we amplified and sequenced *nifH* as marker gene for genetic potential for nitrogen fixation. The V3-V4 region within the 16S rRNA gene as taxonomic marker for bacteria and archaea was already sequenced and data were obtained from our previous study (Alsterberg et al., 2017). Both the 16S rRNA and *nifH* genes were sequenced with Illumina MiSeq technology and 2 x 300 bp paired-end chemistry by Microsynth (Microsynth AG, Switzerland). The details of the primer sequences and PCR conditions are given in Table S1.

While the 16S data were sequenced and published in the previous experiment, they were re-analysed with a slightly different pipeline for this study. The sequences were trimmed using FASTX-Toolkit (`http://hannonlab.cshl.edu/fastx_toolkit`) and merged with PEAR (Zhang et al., 2014) after which we performed quality filtering using USEARCH v8.0 (Edgar, 2010). The criterion for quality filtering was 1 maximum expected error per merged read and a sequence length between 420 and 470 bp. OTU clustering was performed in vsearch (Rognes et al., 2016) at 97% identity. Chimera checking was also done with vsearch de novo chimera checking as well as reference chimera checking against the SILVA reference database (Pruesse et al., 2007) was performed. We dropped global singletons from the analysis after each step. We assigned taxonomy using the `assignTaxonomy` function from DADA2 package (Callahan et al., 2016) in R using the provided SILVA training set as reference. OTUs identified as eukaryotic, mitochondric or originating from chloroplasts were removed. The final data set included 8187 bacterial and archeal OTUs. For the construction of a phylogenetic tree, the representative sequences were aligned globally with the DECIPHER package (Wright, 2015) and the tree was constructed with FastTree (Price et al., 2009) and made ultrametric with PATHd8 (Britton et al., 2007).

The *nifH* sequences were analysed in a similar way as the 16S rRNA reads with an OTU similarity threshold at 97%. Reference chimera checking was performed against the *nifH* ARB database, curated and maintained by the Zehr lab (Ludwig et al., 2004; Heller et al., 2014). The representative sequences were checked for frameshift errors with HMMFRAME (Zhang and Sun, 2011) using an hmmfile generated by hmmbuild based on the full protein alignment extracted from the *nifH* ARB database. Only 14 representative sequences with frameshifts were detected and were excluded. The final set of representative sequences was submitted to a length filtering and we kept only sequences between 384 and 396 bp.

We aligned the representative sequences together with reference sequences from the largest available tree in the ARB *nifH* database. The alignment was performed with a custom python script aligning the reverse translation of the DNA sequences to the reference protein alignment using hmmalign. The primers amplify four *nifH* paralogues known to encode proteins that are not implicated in nitrogen fixation (*bchx*, *bchl*, *frxc* and *nflh* genes; cluster 4 and 5 sequences *sensu* Young (2005)). We retrieved representative sequences for these paralogues from UniProt `http://www.uniprot.org` and joined them to the degapped full set of protein sequences. Finally, we re-aligned all sequences with hmmalign and built a phylogenetic tree based on the protein alignment using FastTree. The phylogeny showed that all sequences retrieved from our samples aligned closely with a reference *nifH* sequence or a reference sequence for *nifH* paralogues. All sequences that clustered with known *nifH* paralogues were pruned. This lead to the exclusion of 53% of all reads belonging to 39% of all OTUs. OTUs excluded from the tree were also removed from the OTU table and all excluded sequences were removed from the full sequence file. Finally all reference sequences were removed from the tree and the tree was made ultrametric with PATHd8. The final table contained 1484 *nifH* OTUs.

Analysis of diversity data

For each set of sequences, the 16S rRNA and *nifH* genes, we calculated the effective number of OTUs sensu (Jost, 2006) of order q = 1. This is equivalent to the exponential of the Shannon diversity and can be interpreted as the number of species in an equally diverse community with evenness = 1. We accounted for uneven sampling depth by applying the estimator developed by Chao et al. (2015) and Marcon and Hérault (2015). However, rarefaction curves for diversity of order q = 1 showed that all samples reached or were close to saturation wherefore it is unlikely that diversity estimates were biased by uneven sampling depth. We also calculated the effective number of phylogenetic unrelated species *sensu* Chao et al. (2010), taking into account the relatedness of the OTU sequences. However, as phylogenetic diversity was strongly correlated to the effective number of species (Pearsons's r 0.78 - 0.94), we only used the former metric in the statistical analysis.

Quantification qPCR of *nifH* genes

The abundances of the nitrogen fixing community were determined by quantifying the *nifH* genes with quantitative real-time PCR (qPCR). Each reaction contained 10 ng template DNA, iQ SYBR Green Supermix (BioRad), 0.1% Bovine Serum Albumin (BSA) and primer concentrations of 0.8 µM in a total volume of 15 µL. The qPCR reactions were run in duplicate using the BioRad CFX Connect Real-Time System. Efficiencies, primer sequences and thermal cycling conditions are listed in Table S1. As standard, we used linearized plasmids containing a *nifH* gene fragment. Prior to quantification, an inhibition test was performed for all samples with a plasmid specific qPCR assay containing the pGEM-T plasmid (Promega) as template in the presence of 10 ng sediment DNA or water. The controls with water were not significantly different from those with sediment DNA, indicating no PCR inhibition.

The *nifH* abundance was corrected by considering the fractions of reads per sample that were identified as *nifH* paralogues.

Statistical analysis

The aim of this study was to attempt to predict or else explain the large variation in nitrogen fixation rates observed in Alsterberg et al. (2017). As reported in that study, part of the variation was explained by habitat diversity. To account for that we fitted a linear model of the form $nitrogen\,fixation \sim habitat\,diversity$, with habitat diversity as discrete variable with four levels (1-4) and extracted the residuals from this model (Fig. S3). The absolute minimum residual value was added to all residuals to shift the values back on the original scale. These values were used as response variable for all subsequent analysis. However, using the original nitrogen fixation rates did not change any of the results qualitatively.

We analysed the data in three steps. In the first step, we took the following model that was fitted to data from a previously published survey (Andersson et al., 2014):

$$log_{10}(nitrogen\,fixation) = 0.96\,log_{10}(nifH\,copies\,g^{-1}\,wetsediment) - 21.07 \quad (1)$$

The range of *nifH* copies observed in Andersson et al. (2014) [$2 \times 10^5 - 5 \times 10^7$ copies g^{-1} sediment] nearly covered the full range of observed copy numbers in this study [$7.2 \times 10^4 - 7.4 \times 10^6$ copies g^{-1}

sediment]. However, as Andersson et al. (2014) did not sequence the *nifH* gene, the *nifH* copy numbers could not be corrected for the fraction of *nifH* genes picked up by the primers but not implicated in nitrogen fixation. We therefore also used the uncorrected *nifH* gene copy numbers obtained in the present study as predictor of nitrogen fixation.

In a second step, we investigated whether the general bacterial diversity as well as the diversity and abundance of the diazotrophic community, could alone or in combination explain more of the observed variation in nitrogen fixation rates. For that, we correlated nitrogen fixation to the effective number of bacterial diversity, effective number of *nifH* types, corrected *nifH* copy number abundance, and to habitat type. The last variable was included, as in the absence of abiotic and other biotic variables, habitat type represents the integrated unmeasured biological, chemical and physical parameters. Finally, we also tried a linear model including all four predictor variables, but without interactions.

In a third step, we explored whether the abundance of any single OTU, either *nifH* or 16S rRNA, correlated with nitrogen fixation rates across samples using the DESeq2 package (Love et al., 2014). The package tests for differential abundance by modeling the response variable (in our case nitrogen fixation) as function of the log2 fold changes in abundance in the count data. The `DESeq` function uses generalized linear models where the counts are modeled using a negative binomial distribution with fitted mean (taking into account the uneven sampling depth) and OTU specific dispersion parameter. We tested two models: 1) for differential abundance correlated to nitrogen fixation rates across habitats, after accounting for habitat differences in OTU abundance and 2) differential abundance correlated to nitrogen fixation within each habitat separately. The false discovery rate was set to 0.05 and we chose the Wald test to assess significance and the Benjamini & Hochberg correction for multiple testing. All statistical analysis were performed in R (R Core Team, 2016).

RESULTS

Prediction of nitrogen fixation rate

The copy number of *nifH* was a reasonably good predictor of nitrogen fixation rates reported by Andersson et al. (2014) (Fig. 1 a). Yet, the model performed poorly when used to predict nitrogen fixation rate on independent data. The observed values for nitrogen fixation fall outside the prediction intervals, and the predictions are systematically biased (Fig. 1 b). Notably, a model where the observed nitrogen fixation rate was simply predicted by the mean observed rate outperformed the predictive model informed by previous data.

Correlation of nitrogen fixation with other community metrics

The effective number of microbial OTUs ranged from 108 - 826. The diversity was different between habitats ($p = 0.0017$) with, 'Cyanobacterial mats' having the lowest median diversity (Div, $n = 8$, Div = 386) followed by '*Ruppia maritima* meadows' ($n = 10$, Div = 392), 'Silty mud' ($n = 11$, Div = 547) and 'Sandy beach' ($n = 10$, Div = 724). The diversity of *nifH* genes ranged from 33 to 266 effective number of OTUs, likewise with differences between the habitat types ($p = 0.0007$). However the order of habitats ranked by diversity was different, with 'Sandy beach' having the lowest median diversity ($n = 10$, Div = 98), followed by 'Cyanobacterial mats' ($n = 8$, Div = 105), '*Ruppia maritima* meadows' ($n = 10$, Div = 151) and 'Silty mud' ($n = 11$, Div = 220). Across habitat, there was a positive, but weak relationship between effective number of *nifH* OTUs and effective number of bacterial OTUs (adj. $R2 = 0.1$, $p = 0.03$).

The nifH gene copy number ranged from $7.2 \times 10^4 - 7.4 \times 10^6$ copies g^{-1} sediment. Yet, 44% ('Silty mud') to 68% ('Sandy beach') of those reads were identified as *nifH* paralogues and after correcting the copy number of *nifH* genes in each sample , the *nifH* copy number ranged from $4 \times 10^4 - 3.2 \times 10^6$ copies g^{-1} sediment. There was no difference in *nifH* abundance across habitat types ($p = 0.3$) and no relationship between *nifH* diversity and abundance (adj. $R2 = 0.05$, $p = 0.08$).

The nitrogen fixation rate was not correlated with either of the described community metrics (Fig. 2). However, nitrogen fixation varied with habitat type ($p = 0.009$), which explained 28% of the variance. A multiple linear model including all four variables as predictors (without interactions) had only weak statistical support ($p = 0.05$). Additionally, an ANOVA with type II sum of squares, to test each main effect over the other main effects, showed that only habitat type had any explanatory power ($p = 0.017$).

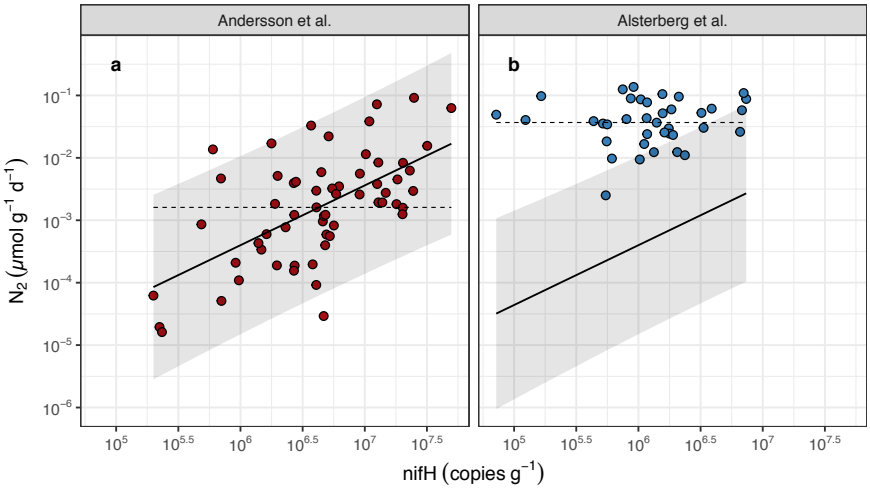

Figure 1. Predicting nitrogen fixation by the abundance of the *nifH* gene in marine shallow water sediments. **a** Data from Andersson et al. (2014). The authors measured nitrogen fixation at 60 sampling sites along the Swedish west coast and quantified the abundance of *nifH* genes with qPCR. The solid line is the best fit model of $log_{10}(nitrogen fixation) \sim log_{10}(nifH\,Abundance)$ (R2 = 0.37, p = 2.9×10^{-7}) and the shaded area shows the 95% prediction interval. The horizontal dashed line indicates the geometric mean. **b** nitrogen fixation data from Alsterberg et al. (2017). We quantified *nifH* abundance for this study. The black solid line is the predicted mean nitrogen fixation using the model **a**, the shaded area represents the 95% prediction interval and the dashed horizontal line shows the geometric mean. The model is a poor fit to the independently collected data and is outperformed by an intercept only model (dashed line).The samples were collected in the same season in the same region and the quantification of nitrogen fixation and *nifH* abundances were determined using the same protocols.

Correlation of nitrogen fixation with specific OTUs

Both the overall bacterial and the diazotrophic communities differed across habitat types (Fig. 3). Yet, after controlling for different mean abundances between habitats, no OTU, neither *nifH* nor 16S rRNA based, correlated with nitrogen fixation rates - with the exception of a single OTU that had lower abundance in samples with higher rates (Fig. 4). When we check for differential abundance within each habitat type, we found one *nifH* OTU that covaried with nitrogen fixation rates in the '*Ruppia maritima*' habitat. Moreover we found 8 16S rRNA OTUs that were related to nitrogen fixation rates; 3 in the 'Cyanobacterial mats', (all negative), 4 in 'Silty mud' (all positive) and one in 'Ruppia maritima'. The adjusted p-values and effect sizes for all OTUs are given in Table S2.

DISCUSSION

Our results show that applying a model with good statistical support to predict nitrogen fixation from *nifH* gene abundances had no predictive power. In fact an intercept-only model outperformed the model informed by the previous data on the new independent dataset. Notably, even though the model fitted to the data of Andersson et al. (2014) had strong statistical support and reasonably high R2, it did not predict nitrogen fixation well in an absolute sense. The predicted values differed from the observed values by 20 to 966% (10^{th} and 90^{th} quantile respectively). Hence, even in the case where the functional relationship would have been the same in both data sets, *nifH* abundance would have been a poor indicator for the absolute nitrogen fixation rate. This highlights the crucial differences between good statistical support, quantified as the probability that the slope is different from 0 (p-value) and the proportion of variance explained by the model (R2), and good predictive ability of a model. We can only claim to have a good mechanistic understanding of the system if the latter is small on independent data (Houlahan et al., 2017). The mismatch of the functional form of the relationship demonstrates the limited frame of inference of any single study, even when performed at relatively large scales and applied to very similar data. The ecological forecast horizon (*sensu* Petchey et al. (2015)) was thus limited, either in space or time, or both.

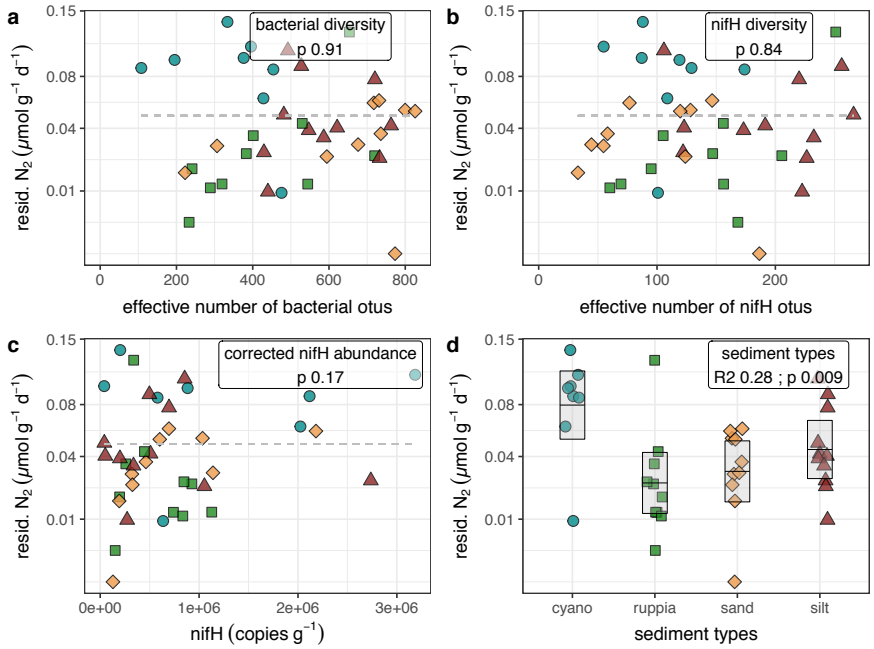

Figure 2. Predicting nitrogen fixation by microbial community metrics. **a)** bacterial and archeal diversity measured as effective number of bacterial OTUs (based on 16S RNA); **b)** diazotroph diversity expressed as effective number of *nifH* OTUs; **c)** abundance of *nifH* copies (copies g^{-1} sediment) after correcting for the fraction of reads encoding *nifH* paralogues not implicated in nitrogen fixation; **d)** by habitat type. Colors represent habitat type: turquoise, cyanobacterial mats; green, *Ruppia maritima* meadows; orange, sandy sediment and red, silty mud. The p-value of linear models of the form $sqrt(nitrogen\ fixation) \sim X$ is given in the inset of each panel. For the only significant relationship (with habitat type) the R2 is also given. The p-values are not adjusted. The dashed lines represent the intercept only models (the mean of nitrogen fixation)

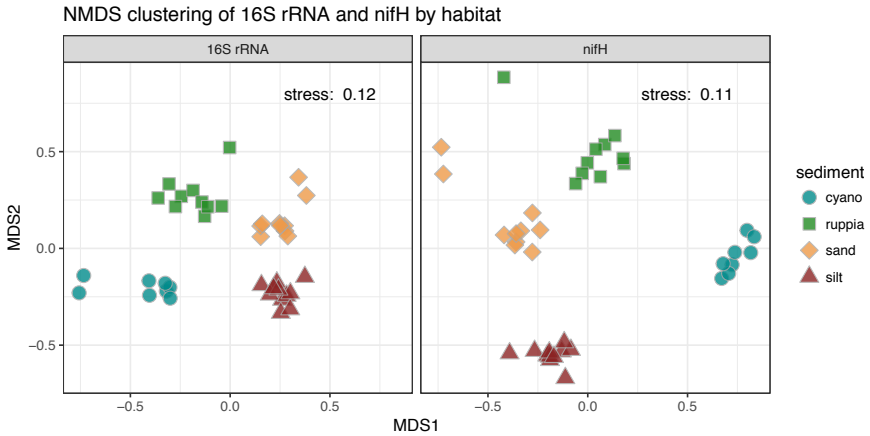

Figure 3. Non-metric multidimensional scaling of the samples based on the OTU-based community composition by gene and habitat. The distance metric is Bray-Curtis dissimilarity. The raw reads have been transformed using the `varianceStabilizingTransformation` function in DESeq2, which transforms the count data to meet approximate homoskedasticity and normalizes for library depth (unequal read numbers in different samples).

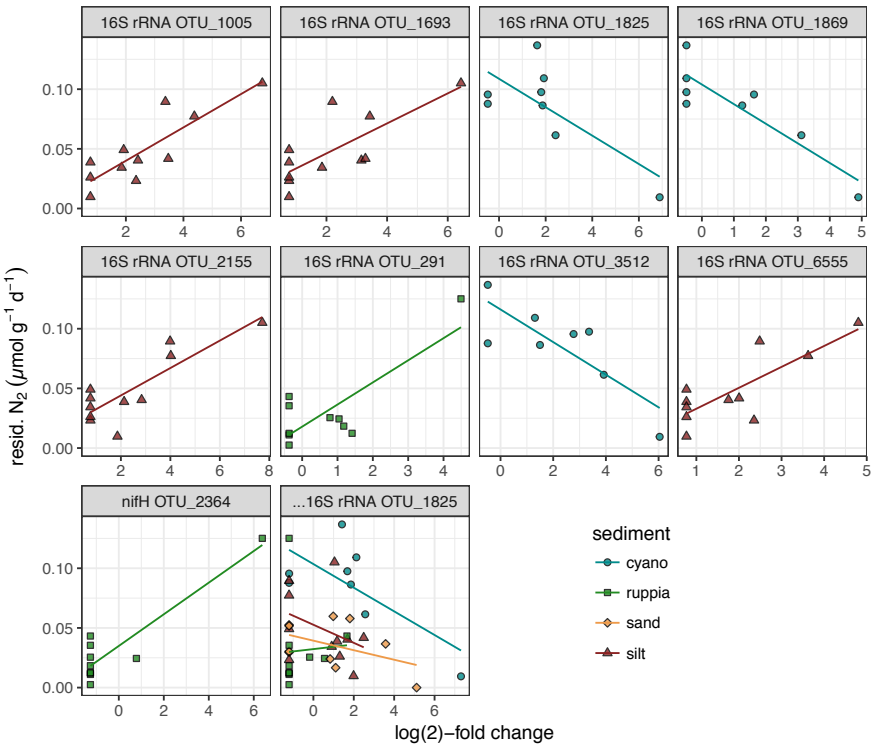

Figure 4. Read abundances of six 16S rRNA and one *nifH* OTU that covaried with nitrogen fixation rates. The abundances on the x-axis are transformed counts by the `varianceStabilizingTransformation` function in DESeq2, which transforms the count data to meet approximate homoscedasticity and normalizes for library depth. The test for association, however, was done using the raw data (glm with model mean-variance relationship) not the transformed data.

Our failure to predict nitrogen fixation rates could be because our independent variables are not directly connected to nitrogen fixation, as only the active protein is truly related to function. The causality chain that connects the detected gene abundance to the realized protein activity is several steps long and each step is influenced by a range of factors that may weaken the direct link. A simple model exercise shows that in a causality chain of length 4, the correlation between the start and the endpoint can be low (Fig. S4). However, in our model system, it is unclear how well each step correlates to the next, *i.e.* from gene to final activity. For example, biological nitrogen fixation is highly regulated at the transcriptional level (Dixon and Kahn, 2004) and not all diazotrophs that are detected are active or alive. The fraction of active cells in the total prokaryotic community in marine sediments has been estimated to be just 40% (Manini and Danovaro, 2006) and it has recently been suggested that 40% of detectable DNA might stem from extracellular DNA, remaining in soils after cell death (Carini et al., 2016). In general, gene abundance is only expected to be tightly linked to the process rate if it is a limiting factor. As Fig. 2 c shows, both the lowest and the highest nitrogen fixation rates occurred in the lowest quantile of measured gene abundances in our samples. Thus, high gene abundance was not a prerequisite for high nitrogen fixation rates. While the highest observed gene abundances coincided with high nitrogen fixation rates, a quantile regression on the 20^{th}% quantile was not significant (data not shown).

Detailed knowledge about both the overall prokaryotic community and the nitrogen-fixing community in particular did not significantly increase our ability to explain or predict the observed nitrogen fixation rates. Instead, habitat type was the only factor that had statistical support in explaining variation in process rates. This could be because habitat type might be correlated with abiotic factors known to regulate nitrogen fixation such as the concentration of dissolved nitrogen, pH, or the concentration of dissolved oxygen (Andersson et al., 2014; Hsu and Buckley, 2008). Diazotroph diversity has been linked to nitrogen fixation rates in some cases (Hsu and Buckley, 2008), but not in others (Rocca et al., 2015). Since species (or biological units *sensu* Krause et al. (2014), i.e. OTUs, genetic varieties, functional groups etc) occupy

different niches, the full community can occupy more of the total niche space than any individual species. The *nifH* gene for example can be separated into three main clusters and 42 subclusters involved in nitrogen fixation (Zehr et al., 2003). These clusters are partly congruent with organismal phylogeny (cluster I), but partly also delineate functional groups. As such, cluster III is found in a range of distantly related bacteria that are dominantly obligatory anaerobes. This suggests niche differentiation between diazotrophs which opens the possibility for niche complementarity. Niche differentiation has also been shown for benthic denitrifying bacteria and archaea under different oxygen regimes (Wittorf et al., 2016). An alternative hypothesis supporting a link between diversity and process rate is that a diverse diazotroph community is more likely to contain high performing species (sampling effect *sensu* Huston (1997)). A sampling effect yields higher functioning if the process that we measure gives a competitive advantage to a species that is high performing with regard to that process (positive selection effect, Loreau (2000)). This could be a reasonable assumption in the case of nitrogen fixation, if bioavailable nitrogen is a limiting resource. Prokaryotic diversity, in turn, is only expected to be connected to nitrogen fixation rates if either diazotroph diversity is closely related to nitrogen fixation and a good proxy for diazotroph diversity or, if the diversity of non-diazotroph prokaryotes favors nitrogen fixation for some reason. However diazotroph diversity was largely uncorrelated to prokaryotic diversity.

The community metrics that we chose to analyse are in principle suitable for prediction on independent data as they are transferrable. For example, we quantified diversity as effective number of species, which takes into account the relative abundance of the single species (Jost, 2006). As effective number it fulfills the doubling property, which demands that the common diversity of two communities that share no species equals the summed diversity of both communities. The effective number of species of order q ≥ 1 can also be reliably estimated on an absolute scale as it is much less sensitive to sampling intensity compared to species richness (Haegeman et al., 2013). Likewise, the abundance of *nifH* genes were quantified on an absolute scale (gene copies g^{-1} sediment). Finally, the abundance of certain OTUs can be compared across studies, if the OTUs are defined coherently. Nevertheless, OTU abundance underlies certain limitations as read abundance data are only semiquantitative (Větrovský and Baldrian, 2013).

In the absence of a relationship between community metrics and community functioning, the influence of certain influential species has often been evoked (Straub and Snyder, 2006; Peter et al., 2011). We tested for species identity effects, but found little evidence for it. Only one bacterial OTU was significantly correlated with nitrogen fixation rates across habitats (Fig. 4). The relationship was negative, precluding a direct effect of the detected OTU on process rates. Within habitats, the abundance of five bacterial OTUs was significantly and positively related to nitrogen fixation rates. Yet, the evidence is purely correlative and the direction of the causality is not clear. High nitrogen fixation might also have favored these OTUs. Only a single diazotroph OTU was correlated with the rate of nitrogen fixation and the relationship seems driven by one influential sample (Fig. 4).

The bacterial and the diazotrophic community differed markedly in community composition among habitats (Fig. 3). Yet in all habitat types, low and high nitrogen fixation rates were observed, and while average nitrogen fixation rates differed between habitats, the variation within habitats was high (Fig. 2 d). In this study we did not attempt to correlate community structure to observed process rates. Doing so asks the question of whether communities that are more alike have more similar functioning but not which community composition is related to what functioning. Therefore the results, even if significant, cannot be used to predict process rates on independent data.

CONCLUSION

There is a clear biological link between functional genes and the processes they encode. Yet, neither the abundance nor the diversity of the *nifH* genes explained variation in nitrogen fixation rates and habitat type was the only factor that had statistical support. A range of factors could obscure the link between community metrics of diazotrophs and the corresponding process rates, including both biological and technical limitations. This shows that it remains challenging to use diversity and other community metrics as predictors for process rates, even in a system where the causality chain is known. Following Houlahan et al. (2017), this highlights the fact that our understanding of when and where community metrics are important for nitrogen fixation is limited. While we provide a *for instance* with this study, the point is

general: Unless we can show that prior knowledge of community metrics informs our expectation of process rates or ecosystem functioning, the link remains elusive and speculative.

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