Multiple dimensions of bacterial diversity unrelated to functioning, stability and multifunctionality

Fabian Roger[1], Stefan Bertilsson[2], Silke Langenheder[3], Omneya Ahmed Osman[4], and Lars Gamfeldt[5]

[1,5]**Department of Marine Sciences, University of Gothenburg, Box 461, SE-40530 Gothenburg, Sweden**
[2,3,4]**Department of Ecology and Genetics, Limnology, and Science for Life Laboratory, Uppsala University, Uppsala, Sweden**

ABSTRACT

Bacteria are essential for many ecosystem services but our understanding of factors controlling their functioning is incomplete. While biodiversity has been identified as an important driver of ecosystem processes in macrobiotic communities, we know much less about bacterial communities. Due to the high diversity of bacterial communities, high functional redundancy is commonly proposed as explanation for a lack of clear effects of diversity. The generality of this claim has, however, been questioned. We present the results of an outdoor dilution-to-extinction experiment with four lake bacterial communities. We found no general effects of bacterial diversity in terms of effective number of species, phylogenetic diversity or functional diversity on (i) bacterial abundance, (ii) temporal stability of abundance, (iii) nitrogen concentration, or (iv) multifunctionality. A literature review of 21 peer-reviewed studies that used dilution-to-extinction to manipulate bacterial diversity corroborated our findings: only about 25% found positive relationships. Combined, these results suggest that bacterial communities are able to uphold multifunctional ecosystems even at extensive reductions in diversity.

Keywords: biodiversity and ecosystem functioning, rare biosphere, biodiversity loss, bacterial diversity, functional redundancy, microcosm, freshwater

INTRODUCTION

Theory predicts that diverse communities can use resources more efficiently and produce more biomass than less diverse communities (Naeem et al., 2009). Indeed, it has been shown across hundreds of experiments that losing species generally results in impaired ecosystem functioning (Cardinale et al., 2011; Gamfeldt et al., 2015). There is, however, great variation among experiments. For example, while resource complementarity prevails in some studies, in others it has been demonstrated that species overlap in their use of the available resource space.

The bulk of experiments on biodiversity and ecosystem functioning have focused on large eukaryotic species, mainly plants, algae and animals. We know much less about the role of bacterial diversity in defining their functioning. Bacterial systems are orders of magnitude more diverse than their macroecological counterparts, with some estimates ranging as high as 20000 species per liter of seawater and 5000 to 20000 species per gram of soil (Sogin et al., 2006; Roesch et al., 2007). Given this tremendous diversity, it is an active matter of debate if biodiversity matters for ecosystem functioning in such systems (Peter et al., 2011). While Bell et al. (2005) showed that species richness determined community respiration in an assembly experiment ranging from 1 to 72 species, this impressive effort still lies at the low end of

richness estimates for natural communities. The bulk of experiments that assembled bacteria into communities of varying diversity worked with far lower richness levels (Awasthi et al., 2014; Langenheder et al., 2010).

One way to manipulate natural levels of bacterial diversity is with a dilution-to-extinction approach (Salonius, 1981). The majority of taxa in most ecosystems follow a skewed abundance distribution with a few common and many rare species (Pedrós-Alió, 2012; Magurran, 2004). Indeed, for Baltic Sea bacteria it has been shown that the proportion of populations within a community is stable, with rare species (¡ 0.1% of the community) staying rare and dominant species (¿ 1% of the community) staying common (Lindh et al., 2015). Diluting natural microbial communities simulates losing the rare species while retaining the more abundant ones.

Until recently, it has been difficult to accurately quantify realized diversity. Most dilution studies used either the dilution factor as proxy for diversity, or coarse molecular techniques that are only able to capture the presence/absence of the most common species. Yet, while species richness has been the most widely studied metric of diversity, the number of species may be a poor predictor of ecosystem functioning. The abundance of the respective species also matters (Hillebrand et al., 2008), as does their relatedness (Cadotte et al., 2009). Hence, abundance-weighted diversity metrics as well as functional and phylogenetic diversity metrics are necessary to further our understanding of the causes and consequences of changing diversity (but see Naughton et al. (2015)).

In this study we present the results from a dilution-to-extinction experiment with four different lake communities. We used next-generation sequencing technology to quantify three dimensions of diversity: the effective number of species, phylogenetic diversity, and functional diversity. Even though we observed a bacterial richness gradient ranging from 15 to 230 operational taxonomic units, we found no significant relationship with functioning. This was true regardless of lake community, diversity metric, focal ecosystem function, or multifunctionality.

MATERIAL AND METHODS

Experimental set-up

We collected 40 liter of surface water from four lakes in the Gothenburg area in Sweden (Lake 1: 57.675 03°N, 11.952 83°E Lake 2: 57.688 78°N, 12.035 65°E; Lake 3: 57.766 56°N, 12.250 46°E; Lake 4: 57.821 24°N, 12.040 36°E).We used 2 liter of water from each lake to prepare four inocula containing only bacteria, archaea and viruses. All microeukaryotes larger than 0.8 µm were excluded by subsequently filtering the water through GF/C filters (nominal pore size 1.2 µm, Whatman^{TM}, GE) and twice through GF/F filters (nominal pore size 0.8 µm) using separate, autoclaved, filter units for each inoculum. From the remaining water we prepared "medium" in three steps: first we pre-filtered it through sterile pre-washed GF/C and GF/F filters secondly we filter-sterilized it by gravity filtration through 0.8/0.2 µm membrane filters (Acropak^{TM} 1500, Pall Corporation) and thirdly, the particle-free water was autoclaved (20 min at 120°C) and the pH was re-adjusted to its original level with HCl/NaOH.

From each of the four inocula, we prepared a 10-step dilution gradient, yielding 11 diversity levels. We chose a dilution factor of 1 : 4.5, such that approximately 1 cell ml^{-1} remained in the highest dilution (1 : 4.5^{10}). We prepared the dilution gradient in 2-litre glass bottles (Duran®, Schott AG,), with a starting volume of 1650 ml. One bottle per lake with only autoclaved medium was kept as sterile control. We treated the sterile controls identically to the experimental units throughout the experiment. The bottles were placed outdoors, in two 1000 liter containers, that served as water-basins to stabilize temperature (Photo, Figure S1). The volume of the water basins was sufficiently large to buffer peak air temperatures and to mimic the natural temperature fluctuation of a shallow lake (Temperature curve, Figure S2). We wrapped the

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

bottles in aluminum foil to exclude growth of phototrophic organisms. The experiment started on June 1st 2012 and ran for six weeks until July 13th 2012.

Biomass sampling and medium exchange

We sampled 5 ml water every second day for bacterial abundances. The samples were immediately fixed in borax-buffered and sterile-filtered formaldehyde (2% final concentration) and stored at −80°C for later analysis. While sampling, we replaced 4% of the medium with freshly autoclaved medium from the respective lake. The sampling and medium exchange was done with sterile syringes (Norm-ject®, Henke Sass Wolf) through a BD Q-Syte™ membrane (BD Biosciences) attached via a luer-fitting (Watson-Marlow Pumps) which in turn was screwed into the bottle lid. This allowed for repeated needle-free sterile sampling. Before each sampling the Q-Syte membrane was rinsed with 70% ethylene. Between each sampling, we sterilized the syringes in 2% hydrochloric acid overnight and rinsed them with milli-q water.

Flow cytometry

For the determination of cell abundance, we counted the 1 ml of sample using a BD FACSCalibur flow cytometer (BD Biosciences). Prior to counting, bacterial cells were stained with SYBR® Green I nucleic acid stain (molecular probes®, life technologies™). We used 1.0 µm FluoSpheres® (Invitrogen, molecular probes®) as internal standard. The FluoSphere solution was sonicated between each use and the concentration was checked with Trucount™ absolute counting beads (BD Biosciences) for every 48 samples.

DNA and carbon assay sampling

At three occasions, we sampled 150 ml water for DNA-based microbial community analysis and 15 ml for the carbon utilization assays. The sampling was scheduled after a regrowth phase of 14 days, after 28 days and at the end of the experiment after 42 days. The extracted volume was not replaced. For DNA analyses, bacterial cells were collected by vacuum filtration onto a 0.2 µm polycarbonate filters (Supor® - 200, Pall cooperation) and stored at −80°C until further processing. For the carbon assay, we inoculated Biolog EcoPlates™ (Biolog Inc.) with 125 µL sample in each well. We incubated the EcoPlates at room temperature in the dark, and measured optical density at 700 nm with a plate reader approximately every 12 h for a min of 96 hours. Biolog EcoPlates™ contain 31 distinct carbon sources in triplicates as well a color dye that turns purple if a given carbon source is metabolized by the community present in the well. Following the color development over time allows both, to estimate if, and at which rate, a carbon source is used. We scored a carbon source as positive when 2 out of 3 wells reached an optical density of at least 0.2 after subtraction of the median blank from all wells. Additionally, we modeled the color development in each well that we scored as positive with a modified gompertz model and took the modeled "growth rate" r as uptake rate. We did the curve fitting with the nlsLM function from the minpack.lm package (Elzhov et al., 2015) in R (R Core Team, 2015).

Inorganic nutrients

At the end of the experiment we collected a 10 ml sample from each bottle to measure remaining dissolved inorganic nutrients ($[NO_3^- + NO_2^-]$, NH_4^+ and PO_4^-). The samples were sterile-filtered, immediately frozen in liquid nitrogen and stored at −80°C until analyzed using colorimetric methods (Grasshoff et al., 2009).

Flagellate sampling

To control for possible contamination by eukaryotic flagellates, we sampled 10 ml culture at the end of the experiment and fixed it with glutaraldehyde (2.5% final concentration). Subsequently

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

we froze the samples in liquid nitrogen and stored them at −80°C until further processing. Eight samples that showed growth dynamics that could indicate flagellate grazing were visually checked using an epifluorescence microscope after staining with DAPI (4-6-diamidino-2-phenylindole) and no flagellates were detected.

DNA extraction and sequencing

We extracted Microbial DNA from filters using the Power soil DNA isolation Kit (MO BIO Laboratories Inc, CA, USA) and checked the quality by gel electrophoresis (1% agarose). Bacterial 16S rRNA genes were amplified using non-barcoded PCR primers, Bakt_341F and Bakt_805R following the "two-step PCR" protocol described in Sinclair et al. (2015). Amplicon sequencing was carried out by the SNP/SEQ SciLifeLab facility hosted by Uppsala University, following the protocol described in the same paper. The sequencing technology was Illumina MiSeq, using paired-end 300 bp read lengths.

Data analysis

All analyses and graphics were performed in R (R Core Team 2015) unless otherwise noted. The full code, including all the raw data except the sequencing data is provided in Appendix 2, which can be accessed at `https://github.com/FabianRoger/Roger_et_al_Supplementary`.

Analysis of the sequencing data

The Illumina sequences were pre-processed and quality filtered as described in Sinclair et al. (2015). The merged and quality filtered reads were further processed with USEARCH (Edgar, 2010) and clustered at 97% identity cutoff with the centroid sequence, using the option of excluding global singletons from the clustering step. The resulting operational taxonomic units (OTUs) were chimera checked with UCHIME against the rdp gold reference database (Cole et al., 2013) downloaded 2014-10-22). 90.5% of the joined reads could successfully be mapped to an OTU, 9.5% of the reads, composed by singletons that didn't map to any OTUs as well as chimeric sequences, were discarded. A taxonomic annotation was assigned to each resulting OTU with UTAX (public communication, `http://drive5.com/utax`) with default parameters. The final OTU table was manually purged of non-bacterial sequences as well as from sequences that had a lower than 50% likelihood to be genuine bacterial sequences as predicted by the UTAX algorithm. For the construction of a phylogenetic tree, the centroid sequences were aligned with PyNAST (Caporaso et al., 2010a) in QIIME (Caporaso et al., 2010b) and the tree was constructed using the fasttree algorithm (Price et al., 2009). The heat map presented in Figure 2 was created with the phyloseq package (McMurdie and Holmes, 2013) using the approach from Rajaram and Oono (2010). All steps are described in detail in Appendix 2, Processing_of_Seqeuncing_Data.html

Dimensions of diversity

Three aspects of diversity were explored: the effective number of OTUs (hereafter referred to as the effective number of species), phylogenetic diversity and functional diversity. The diversity metrics were calculated at three time points and the average diversity over time was taken as predictor variables. In order to account for uneven sampling intensity (sequencing depth) we calculated effective number of species and phylogenetic diversity based on a rarefied OTU table, subsampled for 10000 reads per sample. One sample had only spurious reads and was excluded. The remaining 47 (out of 144) samples that had less than 10000 reads were kept as is. A sensitivity analysis of the diversity estimation to rarefaction showed that the chosen metrics were largely insensitive to the sequencing depth so that an exclusion of the samples was not

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

justified (Figure S3, see also Analysis_of_OTU_Data.html, Appendix 2). The effective number of species (of order 1, based on Shannon diversity) weights all species by their proportional abundance (Jost, 2006). It is called "effective number" as it is the number equivalent of the species richness of an equally diverse assemblage where all species are equally abundant.

We calculated the phylogenetic diversity sensu Chao et al. (2010) in the implementation of Marcon and Hérault (2015). It is based on the concept of effective number of species and is the equivalent of the richness of an assemblage where all species are equally abundant and completely unrelated to each other.

Functional diversity was calculated in a similar matter, using the data from the carbon assay. Each carbon source was taken as community trait, and the uptake rate of the carbon source was taken as trait value. We weighted all carbon sources by their uptake rate and calculated the "effective number of metabolized" carbon sources equivalent to the calculation of the effective number of species using the diversity function in the vegan package (Oksanen et al., 2015). Given the 31 carbon sources tested, our metric of effective functional diversity could range between 0 (no carbon source metabolized) and 31 (all carbon sources are metabolized equally fast).

Response variables

We studied three ecosystem properties individually and jointly (i.e. multifunctionality). The individual properties were maximum bacterial cell abundance, temporal stability of cell abundance and the concentration of dissolved inorganic nitrogen.

Maximum cell abundance was calculated as the average maximum cell number from the five highest values measured for each sample during the course of the experiment. We defined the temporal stability of the cell abundance as the inverse of the coefficient of variance over time. Beforehand, we excluded the regrowth phase of the experiment (day 1-12) and removed the long-term temporal trend of the growth curves by fitting a linear model of the form $Cellnumber \sim time$ to each growth curve . Stability was then calculated on the residuals extracted from the linear model. (Bacterial_Cell_Counts.html , Appendix 2)

The concentration of dissolved inorganic nitrogen (DIN) was calculated as the sum of the three measured components: NO_2^-, NO_3^- and NH_4^+. In order for this response variable to represent higher nutrient depletion and not remaining DIN concentrations, we standardized the DIN concentrations by their mean and standard deviation and changed the sign of the standardized variable by multiplying it with -1.

Multifunctionality was calculated as the number of the three properties that were sustained above 75% of the maximum measured function value (Gamfeldt et al., 2008), where the maximum value was calculated as the average of the two highest measured values.

Statistical models

We regressed each of the three measured ecosystem properties and the multifunctionality index against each of the three diversity metrics. We calculated regressions separately for each lake. Since a linear relationship may not always be expected we also analyzed the data with non-parametric Spearman rank correlations.

Literature review

We conducted a qualitative literature overview of the bacterial diversity-ecosystem functioning literature that used natural bacterial communities and a dilution-to-extinction approach to create a diversity gradient. We searched for relevant articles on Google scholar with the search string ["dilution to extinction" AND "bacterial diversity" OR "microbial diversity" AND "community function" OR "ecosystem function"]. This search resulted in 12 articles that met our criteria. We

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

searched the literature cited by these articles for further relevant studies. This resulted in a total of 22 articles, all but one published between 2001 and 2015. Two of the papers were excluded: one did not present statistical evidence and the other presented experiments that were conducted on agar plates, which we judged as not comparable.

We grouped the response variables into 10 categories ("ecosystem functions"): (1) Abundance or biomass; (2) Activity, measured either as respiration or the uptake rate of isotope-labeled amino acids and nucleic acids; (3) Degradation of carbon sources; (4) Resistance; (5) resilience; (6) Stability, measured as the temporal stability of a given ecosystem function; (7) Nitrogen cycling. Includes denitrification, potential nitrification, nitrate accumulation, nitrite oxidation, and arginine ammonification; (8) Enzyme multifunctionality, measured as the capacity to sustain the simultaneous activity of a set of extracellular enzymes at certain threshold levels; (9) Invasion resistance, measured as the ability of an invader to survive in the host community; (10) Enhancing plant productivity, measured as the effect of soil bacterial diversity on plants.

For each article, we looked at the relationship between manipulated diversity and ecosystem functioning. A relationship counted as significant if the p-value was below 0.05. We categorized the relationships into four categories: positive, negative, non significant and ambiguous. The last category was applied if two different response variables were presented that measured the same function according to our definition and the results didn't agree. If a study presented several separate experiments or treatments we counted each experimental treatment separately, unless the authors made the choice to pool the data before the analysis in which case we took the results as presented by the authors. In total we counted 82 diversity-functioning relationships.

A detailed description of the literature review methods is provided in Appendix 3 and additional data for each study are listed in Table S1.

RESULTS

Diversity metrics

We observed bacterial growth in all lakes and dilutions, including the sterile controls (Figure S4). This indicates that contamination occurred in the experiment. As we assessed realized diversity, and as the contaminant bacteria stem from the same environment as the samples, we don't regard the contamination as a major problem. We included the sterile controls as additional diversity levels in our analyses. Total bacterial richness across all samples and time points was 967 OTUs, clustered with an OTU radius of minimum 3% identity. The OTU richness per sample ranged from 15 to 438 per sample in the un-rarefied dataset, and from 15 to 282 in the rarefied dataset with a median of 45 OTUs. Log diversity decreased linearly with dilution in all lakes, for both the effective number of species and phylogenetic diversity (Figure 1). The effective number of species ranged from 1.25 to 32.8 (Figure 1, top row), and phylogenetic diversity ranged from 1.13 to 13.5 (Figure 1 middle row), indicating that the least diverse samples were dominated by a single species (Figure 2). Effective number of species and phylogenetic diversity were highly correlated ($r^2 > 0.9$ for all lakes, Figure S5) showing that the average relatedness among the species in each sample was similar. This is coherent with random species loss across the phylogenetic tree during dilution.

Functional diversity was uncorrelated to both the effective number of species and phylogenetic diversity in all lakes ($r^2 < 0.15$, $p > 0.12$, Figure S5). It ranged from 1 to 19 effective number of metabolized carbon sources and showed no trend with dilution. While all diversity metrics increased slightly over time, mean functional diversity increased the most pronounced, from 5.4 at the first sampling to 15.2 at the last sampling. Overall, the realized diversity gradient proved that we successfully manipulated species diversity as well as phylogenetic diversity and functional diversity, resulting in communities that differed in diversity by a factor ranging from

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

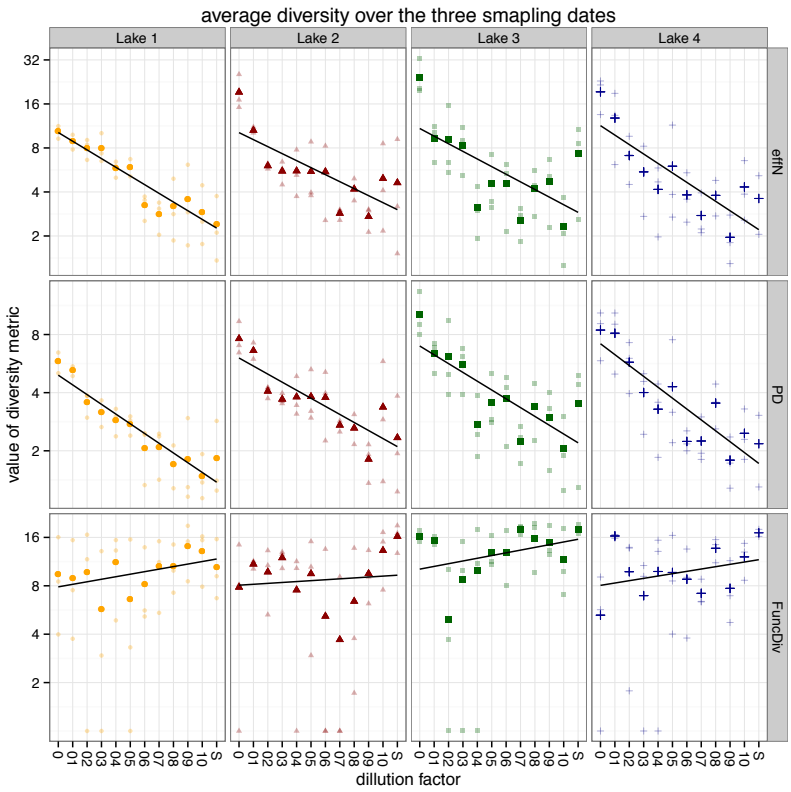

Figure 1. Realized diversity as a function of the dilution factor for each lake. The dilution axis represents the exponent of the dilution factor with 0 being the undiluted treatment and 10 the $1 : 4.5^{10}$ diluted treatment. "S" labels the sterile control that has been included in the experiment as additional treatment. effN = effective number of species, PD = phylogenetic diversity, FuncDiv = functional diversity.

around 10 to 25.

Effects of diversity

We excluded the undiluted treatment from all analyses, as we judged it not comparable to the diluted treatments in our experiment. All diluted treatments started off with at least 80% medium which was pH-adjusted, sterile filtered and autoclaved repeatedly. Hence we were unable to disentangle a possible "medium effect" from the effects of diversity in the undiluted treatments. Furthermore, in the undiluted treatment, diversity, maximum biomass and stability measurements may all be biased by the detection of species that remained present throughout the experiment but did not grow under culture conditions and hence did not contribute to ecosystem functioning. None of the four response variables were consistently and positively related to any of the three diversity metrics in the four lakes (Figure 3). The r^2 was generally low, ranging from 0 to 0.65 with a median of 0.13.

Literature review

In total we found 21 studies (including ours), of which 11 worked with soil communities and 10 with aquatic communities (Figure 4, Table S1). Many experiments measured multiple response variables, and the number of diversity-function relationships is thus higher than the

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

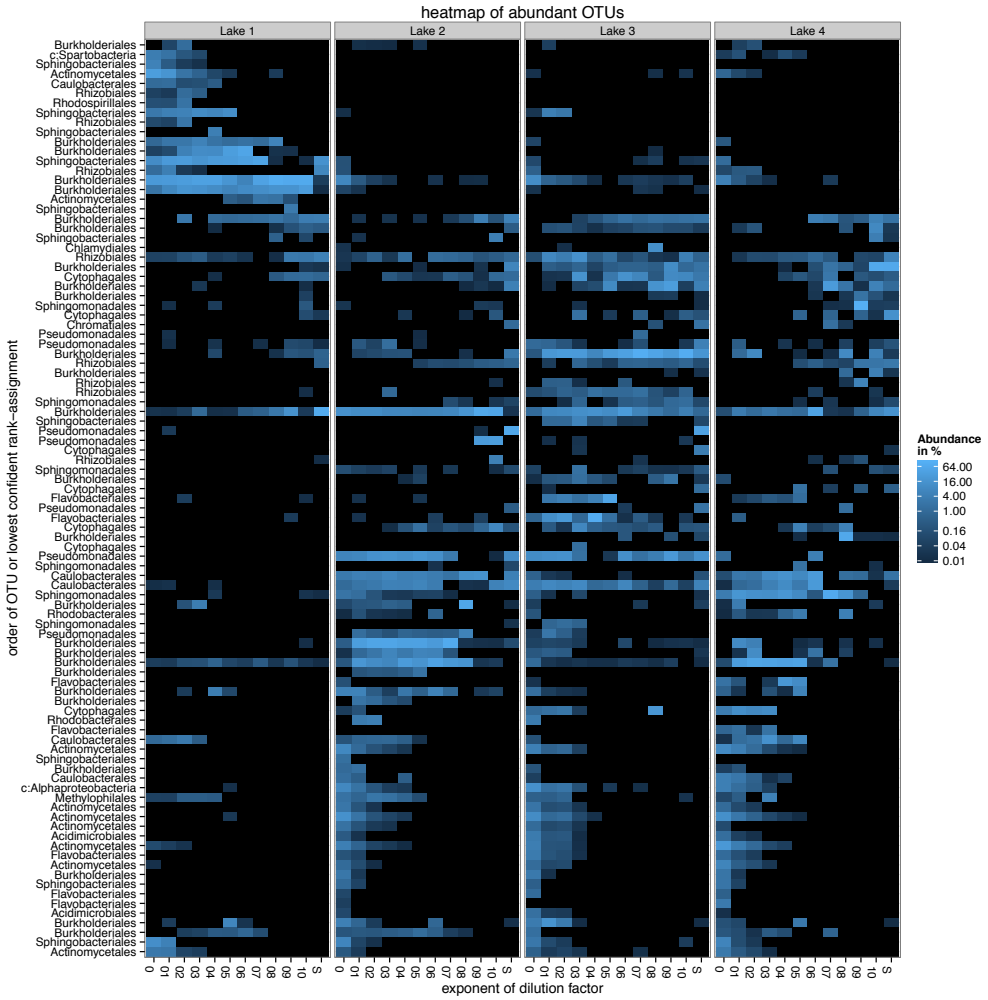

Figure 2. Heat map of the dominant OTUs for each lake. Each row represents different OTUs and the order assignment of each OTU is given on the y-axis. If the confidence of the order assignment was lower than 90%, the class assignment is given, preceded by "c". Columns show dilution treatments as in Figure 1. An OTU was defined as dominant if it represented $>= 1\%$ of the relative abundance in at least one sample. Abundances are averaged over the three sampling times.

number of studies. Results are highly variable, with negative, null, and positive relationships. Flat relationships are by far the most common, accounting for $\sim$ 60% of all relationships, whereas negative relationships are the least common (8%). Positive results make up 27% of all relationships. The remaining 5% where ambiguous. The only response variables with a consistent pattern are invasion resistance and the activity of extracellular enzymes (enzyme multifunctionality), both of which are negatively affected by diversity loss. However, the sample size is only three and two studies respectively.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

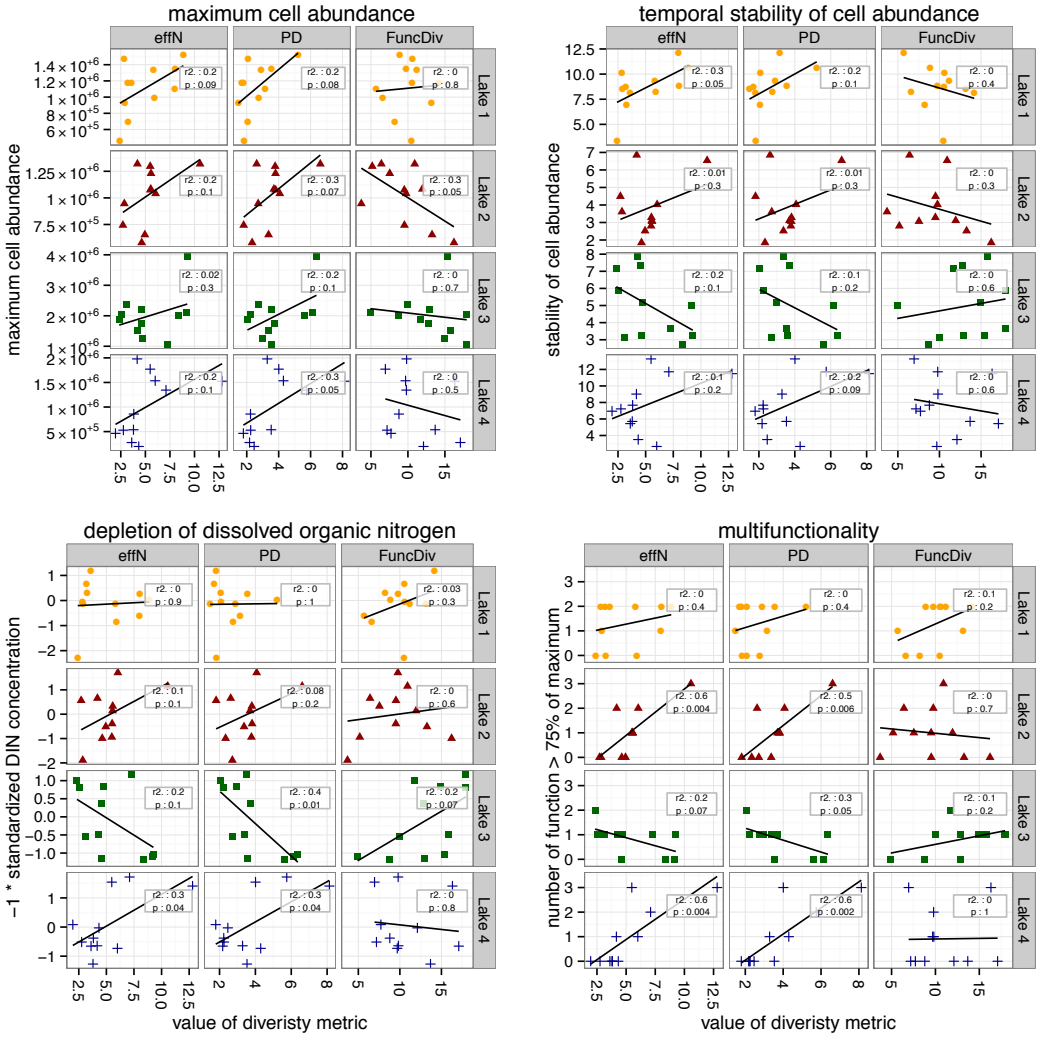

Figure 3. Cell abundance, stability of cell abundance, DIN concentration and multifunctionality as functions of the three dimensions of diversity. Diversity is averaged over the three sampling dates. R-square and p-value for each linear model are given in each panel. effN = effective number of species, PD = phylogenetic diversity, FuncDiv = functional diversity.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

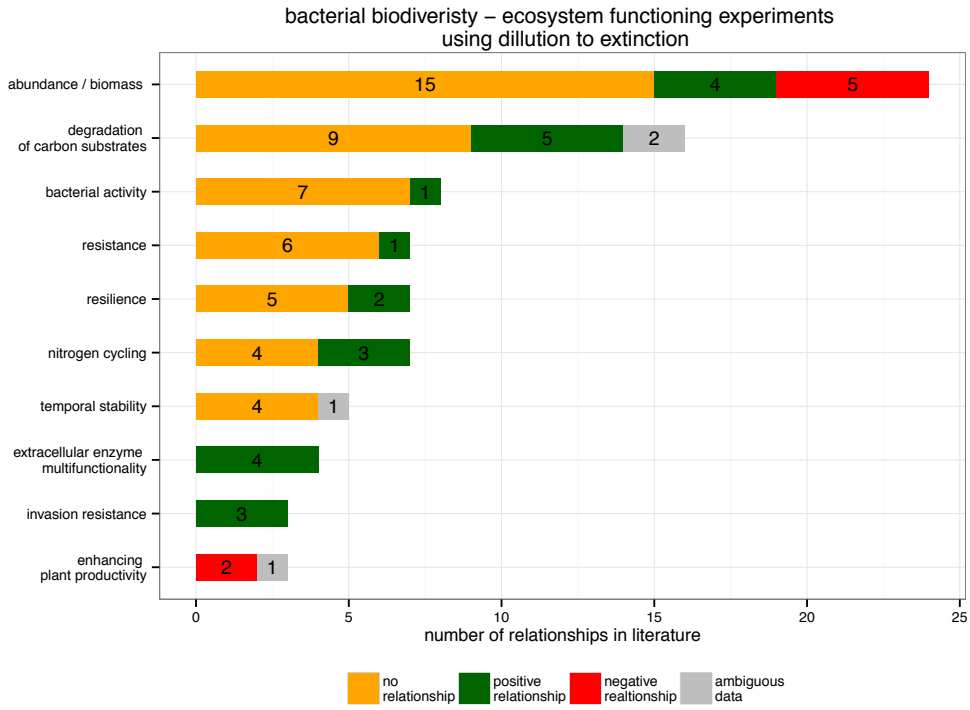

Figure 4. Cell abundance, stability of cell abundance, DIN concentration and multifunctionality as functions of the three dimensions of diversity. Diversity is averaged over the three sampling dates. R-square and p-value for each linear model are given in each panel. effN = effective number of species, PD = phylogenetic diversity, FuncDiv = functional diversity.

DISCUSSION

We found no evidence of a general positive diversity effect on ecosystem functioning in our experiment. This was true regardless of lake community, diversity metric, or response variable of choice. Relationships varied from significantly positive (17 % of the 48 relationships at $alpha = 0.05$), to non-significant (77 %), to significantly negative (6 %). Spearman rank correlations supported these results (Figure S6). If the p-values were adjusted for multiple comparisons using the Holm-Bonferroni method, none of the relationships remained significant. Regardless of the statistical model of choice, bacterial diversity was a weak driver of functioning. These findings are largely in agreement with results from previous published dilution-to-extinction experiments, as shown in our literature review (Figure 4).

There are several potential explanations for the absence of a positive relationship between diversity and functioning. First, a few species may be responsible for most of the functioning, regardless of the diversity of the community as a whole. This was previously suggested in a similarly designed dilution-to extinction study where polymer degradation and overall growth of lake bacteria were studied (Peter et al., 2011). A second explanation is that our bacterial communities include a high level of redundancy, meaning that many species are equally efficient in using the same resources and turning these into biomass. It matters marginally which exact species is dominating any particular community (see also Langenheder et al. (2005)).

High redundancy among species is supported by our measure of functional diversity. Func-

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

tional diversity was a poor predictor of functioning and correlated weakly with species diversity ($r^2 < 0.15$, $p > 0.12$, see Figure S5). It may be that the use of the carbon sources has little bearing on the traits that actually matter for bacterial biomass production and nitrogen uptake. If, on the other hand, the 31 carbon sources reflect functional diversity more broadly, bacterial communities are indeed redundant in terms of resource acquisition. It is plausible that our study, and other experiments performed hitherto, have yet to incorporate the relevant levels of functioning and environmental heterogeneity. In a homogeneous environment, only a subset of all species traits will be relevant, which will result in many species becoming functionally redundant. Phylogenetic diversity can potentially be a stronger predictor of functioning than both species and functional diversity since it can be related to traits captured by neither of the two. The rationale is that overall functional divergence between species may correlate with the time since two species shared a common ancestor. The more functional unique a species is, the more it contributes to overall ecosystem functioning and the higher the chance for complementarity. Indeed, previous studies have shown that phylogenetic diversity can be a stronger predictor of primary producer productivity than either species richness or functional diversity (e.g. Cadotte (2013)). In contrast, we found no effect of phylogenetic diversity. This is line with recent evidence showing that plant phylogenetic diversity generally explains little of the variation of the functioning in grasslands (Venail et al., 2015). The 16 grassland biodiversity experiments examined by Venail et al. (2015) had an overall high correlation between species and phylogenetic diversity ($r^2 = 0.90$). Likewise, in our study, the effective number of species and phylogenetic diversity were highly correlated ($r^2 > 0.85$).

Biodiversity has been proposed to be more important for multifunctionality than for single functions (Emmett Duffy et al., 2003; Gamfeldt et al., 2008), which is supported by a recent meta-analysis on 94 experiments (Lefcheck et al., 2015). We found little evidence for this expectation (Figure 3), suggesting that bacteria are relatively multifunctional. It should be noted though that our multifunctionality metric was based on only 3 variables, which is at the low end for a multifunctionality assessment.

Dilution-to-extinction experiments provide many advantages compared to assembly experiments. First, they work with natural communities as a starting point and thus ensure that all bacteria interacting in the community also interact in nature. Second, they have the virtue of creating a diversity gradient that includes realistically high levels of bacterial diversity. Finally, they include all species found in the original communities and not only the tiny fraction of species that can be cultivated in vitro. The approach, however, also has disadvantages. For instance, dilution does not only manipulate diversity, but also abundance. Therefore, bacterial experimental communities are often allowed to regrow to initial densities in dilution-to-extinction studies. This regrowth phase has two unintended consequences. The first is that it favors opportunistic species. This can be seen by the dominance of species belonging to the class of betaproteobacteria at all dilution levels in our experiment (Figure 2); a class that has been described as generally "fast-growing and nutrient-loving" (Newton et al., 2011). The second consequence, at least in our experiment, is that the communities that established after the re-growth phase generally had low evenness. Despite high species richness (median of 35 in the rarefied dataset), the median effective number of species was a modest 5.4.

In summary, there is to date little experimental evidence for natural levels of bacterial diversity influencing ecosystem functioning. This may indeed reflect real ecosystems, with high microbial functional redundancy. Consistently, a recent long-term experimental selection study showed no difference in growth characteristics, or community composition, of a freshwater bacterial community regardless of the type or combination of amino acid substrates included in the growth medium (Canelhas et al., 2016). Yet, there exist a wealth of published examples of positive interaction among bacterial species (e.g. metabolic dependencies (Valentine and

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

Reeburgh, 2000) and commensalism (Ueda et al., 2004)). Furthermore, many species can only be cultured in co-cultures with other species (Stewart, 2012). However, and as our study suggests, it appears that these positive interactions are not strong enough to affect processes at the level of whole communities.

It should also be noted that studying diversity effects at the level of bacterial communities is equivalent to examining diversity effects at the level of whole macrobiotic communities – something that has rarely been attempted. Hence, diversity effects might be more likely to be found in subsets of the bacterial communities much in the same way as they have been found frequently in grassland experiment while they may be more elusive in whole prairie ecosystems. More experimental work is sorely needed if we are to gain a more thorough understanding of how bacterial diversity mediates ecosystem functioning.

ACKNOWLEDGEMENTS

This research was supported by grants from the Helge Ax:son Johnsons Stiftelse and the Wilhelm och Martina Lundgrens Vetenskapsfond 1. We would like to thank Friederike Roger for help with building the experimental set-up and Maria Karlberg to help out with the sampling.

REFERENCES

Awasthi, A., Singh, M., Soni, S. K., Singh, R., and Kalra, A. (2014). Biodiversity acts as insurance of productivity of bacterial communities under abiotic perturbations. *The ISME Journal*, 8(12):2445–2452.

Bell, T., Newman, J. A., Silverman, B. W., Turner, S. L., and Lilley, A. K. (2005). The contribution of species richness and composition to bacterial services. *Nature*, 436(7054):1157–1160.

Cadotte, M. W. (2013). Experimental evidence that evolutionarily diverse assemblages result in higher productivity. *Proceedings of the National Academy of Sciences*, 110(22):8996–9000.

Cadotte, M. W., Cavender-Bares, J., Tilman, D., and Oakley, T. H. (2009). Using phylogenetic, functional and trait diversity to understand patterns of plant community productivity. *PLoS ONE*, 4(5):e5695.

Canelhas, M. R., Eiler, A., and Bertilsson, S. (2016). Are freshwater bacterioplankton indifferent to variable types of amino acid substrates? *FEMS Microbiology Ecology*, page fiw005.

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*, 26(2):266–267.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7(5):335–336.

Cardinale, B. J., Matulich, K. L., Hooper, D. U., Byrnes, J. E., Duffy, E., Gamfeldt, L., Balvanera, P., O'Connor, M. I., and Gonzalez, A. (2011). The functional role of producer diversity in ecosystems. *American Journal of Botany*, 98(3):1–21.

Chao, A., Chiu, C.-H., and Jost, L. (2010). Phylogenetic diversity measures based on Hill numbers. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 365(1558):3599–3609.

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Brown, C. T., Porras-

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

Alfaro, A., Kuske, C. R., and Tiedje, J. M. (2013). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research*, 42(D1):D633–D642.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, 26(19):2460–2461.

Elzhov, T. V., Mullen, K. M., Spiess, A.-N., and Bolker, B. (2015). *minpack.lm: R Interface to the Levenberg-Marquardt Nonlinear Least-Squares Algorithm Found in MINPACK, Plus Support for Bounds*. R package version 1.1-9.

Emmett Duffy, J., Paul Richardson, J., and Canuel, E. A. (2003). Grazer diversity effects on ecosystem functioning in seagrass beds. *Ecology Letters*, 6(7):637–645.

Gamfeldt, L., Hillebrand, H., and Jonsson, P. R. (2008). Multiple functions increase the importance of biodiversity for overall ecosystem functioning. *Ecology*, 89(5):1223–1231.

Gamfeldt, L., Lefcheck, J. S., Byrnes, J. E. K., Cardinale, B. J., Duffy, J. E., and Griffin, J. N. (2015). Marine biodiversity and ecosystem functioning: what's known and what's next? *Oikos*, 124(3):252–265.

Grasshoff, K., Kremling, K., and Ehrhardt, M. (2009). *Methods of seawater analysis*. John Wiley & Sons.

Hillebrand, H., Bennett, D. M., and Cadotte, M. W. (2008). Consequences of dominance: a review of evenness effects on local and regional ecosystem processes. *Ecology*, 89:1510–1520.

Jost, L. (2006). Entropy and diversity. *Oikos*, 113(2):363–375.

Langenheder, S., Bulling, M. T., Solan, M., and Prosser, J. I. (2010). Bacterial Biodiversity-Ecosystem Functioning Relations Are Modified by Environmental Complexity. *PLoS ONE*, 5(5):e10834.

Langenheder, S., Lindström, E. S., and Tranvik, L. J. (2005). Weak coupling between community composition and functioning of aquatic bacteria. *Limnology and Oceanography*, 50(3):957–967.

Lefcheck, J. S., Byrnes, J. E. K., Isbell, F., Gamfeldt, L., Griffin, J. N., Eisenhauer, N., Hensel, M. J. S., Hector, A., Cardinale, B. J., and Duffy, J. E. (2015). Biodiversity enhances ecosystem multifunctionality across trophic levels and habitats. *Nature Communications*, 6:6936.

Lindh, M. V., Sjöstedt, J., Andersson, A. F., Baltar, F., Hugerth, L. W., Lundin, D., Muthusamy, S., Legrand, C., and Pinhassi, J. (2015). Disentangling seasonal bacterioplankton population dynamics by high-frequency sampling. *Environmental Microbiology*, 17(7):2459–2476.

Magurran, A. E. (2004). *Measuring biological diversity*. Blackwell Publishing, Oxford, United Kingdom.

Marcon, E. and Hérault, B. (2015). Decomposing phylodiversity. *Methods in Ecology and Evolution*, 6(3):333–339.

McMurdie, P. J. and Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE*, 8(4):e61217.

Naeem, S., Bunker, D. E., Hector, A., Loreau, M., and Perrings, C., editors (2009). *Biodiversity, ecosystem functioning, and human wellbeing - an ecological and economic perspective*. Oxford University Press, United Kingdom.

Naughton, H. R., Alexandrou, M. A., Oakley, T. H., and Cardinale, B. J. (2015). Phylogenetic distance does not predict competition in green algal communities. *Ecosphere*, 6(7):art116.

Newton, R. J., Jones, S. E., Eiler, A., McMahon, K. D., and Bertilsson, S. (2011). A guide to the natural history of freshwater lake bacteria. *Microbiology and molecular biology reviews*, 75(1):14–49.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., and Wagner, H. (2015). *vegan: Community Ecology Package*. R package version 2.2-1.

Pedrós-Alió, C. (2012). The Rare Bacterial Biosphere. *Annual Review of Marine Sciences*,

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016

4(1):449–466.

Peter, H., Beier, S., Bertilsson, S., Lindström, E. S., Langenheder, S., and Tranvik, L. J. (2011). Function-specific response to depletion of microbial diversity. *The ISME Journal*, 5(2):351–361.

Price, M. N., Dehal, P. S., and Arkin, A. P. (2009). FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Molecular biology and evolution*, 26(7):1641–1650.

R Core Team (2015). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rajaram, S. and Oono, Y. (2010). NeatMap - non-clustering heat map alternatives in R. *BMC Bioinformatics*, 11:45.

Roesch, L. F. W., Fulthorpe, R. R., Riva, A., Casella, G., Hadwin, A. K. M., Kent, A. D., Daroub, S. H., Camargo, F. A. O., Farmerie, W. G., and Triplett, E. W. (2007). Pyrosequencing enumerates and contrasts soil microbial diversity. *The ISME Journal*, 1(4):283–290.

Salonius, P. O. (1981). Metabolic capabilities of forest soil microbial populations with reduced species diversity. *Soil Biology and Biochemistry*, 13:1–10.

Sinclair, L., Osman, O. A., Bertilsson, S., and Eiler, A. (2015). Microbial community composition and diversity via 16S rRNA gene amplicons: evaluating the illumina platform. *PLoS ONE*, 10(2):e0116955.

Sogin, M. L., Morrison, H. G., Huber, J. A., Mark Welch, D., Huse, S. M., Neal, P. R., Arrieta, J. M., and Herndl, G. J. (2006). Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences of the United States of America*, 103(32):12115–12120.

Stewart, E. J. (2012). Growing unculturable bacteria. *Journal of Bacteriology*, 194(16):4151–4160.

Ueda, K., Yamashita, A., Ishikawa, J., Shimada, M., Watsuji, T.-o., Morimura, K., Ikeda, H., Hattori, M., and Beppu, T. (2004). Genome sequence of *Symbiobacterium thermophilum*, an uncultivable bacterium that depends on microbial commensalism. *Nucleic Acids Research*, 32(16):4937–4944.

Valentine, D. L. and Reeburgh, W. S. (2000). New perspectives on anaerobic methane oxidation. *Environmental Microbiology*, 2(5):477–484.

Venail, P., Gross, K., Oakley, T. H., Narwani, A., Allan, E., Flombaum, P., Isbell, F., Joshi, J., Reich, P. B., Tilman, D., Ruijven, J., and Cardinale, B. J. (2015). Species richness, but not phylogenetic diversity, influences community biomass production and temporal stability in a re-examination of 16 grassland biodiversity studies. *Functional Ecology*, 29:615–626.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1688v1 | CC-BY 4.0 Open Access | rec: 28 Jan 2016, publ: 28 Jan 2016