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Effects of multiple dimensions of bacterial diversity on functioning, stability and multifunctionality

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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. The consequences of changes in bacterial diversity in terms of effective number of species, phylogenetic diversity, and functional diversity were studied for (1) bacterial abundance, (2) temporal stability of abundance, (3) nitrogen concentration, and (4) multifunctionality. We observed a richness gradient ranging from 15 to 280 operational taxonomic units (OTUs). Individual relationships between diversity and functioning ranged from negative to positive depending on lake, diversity dimension, and aspect of functioning. Only between phylogenetic diversity and abundance did we find a statistically consistent positive relationship across lakes. A literature review of 24 peer-reviewed studies that used dilution-to-extinction to manipulate bacterial diversity corroborated our findings: about 25% found positive relationships. Combined, these results suggest that bacteria-driven community functioning is relatively resistant to reductions in diversity.

Key words: biodiversity and ecosystem functioning; biodiversity loss; biodiversity metrics; freshwater; functional redundancy; microbial diversity; microcosm; rare biosphere.

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 species loss 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 (Cardinale et al. 2011).

While species richness has been the most widely studied metric of diversity, the sole number of species may be a poor choice as predictor of ecosystem functioning. There are two main reasons for this. First, the abundance of the respective species also matters, i.e., common species may have more influence on functioning than rare ones. Second, species richness may have little bearing on functional trait diversity, and functional diversity may thus be a more relevant metric (Díaz and Cabido 2001). However, capturing the traits relevant for ecosystem functioning is not always straightforward and phylogenetic diversity is potentially a stronger predictor (e.g., Cadotte 2013). The rationale is that overall functional divergence between species may correlate with the time since two species shared a common ancestor (but see Naughton et al. 2015). Hence, abundance-weighted diversity metrics as well as functional and phylogenetic diversity metrics could allow for insights that species richness does not provide. By studying them in concert, we can further our understanding of the causes and consequences of changing diversity.

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. Bacterial systems are orders of magnitude more diverse than their macroecological counterparts, with some estimates ranging as high as 20,000 species/L seawater and 5,000 to 20,000 species/g 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*a*). While Bell et al. (2005) showed that species richness determined community respiration in an assembly experiment ranging from one 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 (e.g., Langenheder et al. 2010, Awasthi et al. 2014).

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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 2013). 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 community abundance) staying rare and dominant species (>1% of the community) staying common (Lindh et al. 2015). Diluting natural microbial communities results in the loss of rare species while retaining the more abundant ones.

Until recently, it has been difficult to accurately quantify bacterial 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. In this study, we used next-generation sequencing technology to quantify three dimensions of diversity (the effective number of species [which takes into account the relative abundance of species], functional diversity, and phylogenetic diversity) for a dilution-to-extinction experiment with four lake communities. We related changes in these diversity metrics to four aspects of ecosystem functioning.

Materials and Methods

Experimental set-up

We collected 40 L of surface water from four lakes in the Gothenburg area in Sweden (Lake 1, 57.67503° N, 11.95283° W; Lake 2, 57.68878° N, 12.03565° W; Lake 3, 57.76656° N, 12.25046° W; Lake 4, 57.82124° N, 12.04036° W). We used 2 L 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™; GE Healthcare, Little Chalfont, UK) and twice through GF/F filters (nominal pore size 0.7 μm) using separate, autoclaved, filter units for each inoculum. From the remaining water we prepared "medium" in three steps: first we prefiltered it through sterile prewashed GF/C and GF/F filters, then we filter sterilized it by gravity filtration through 0.8/0.2 μm membrane filters (AcroPak™; Pall Corportation, Dreieich, Germany), and, finally, the particle-free water was autoclaved (20 min at 120°C) and the pH was readjusted to its original level with HCl and 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 remained in the highest dilution ($1{:}4.5^{10}$). We prepared the dilution gradient in 2-L glass bottles (DURAN© SCHOTT, Lyngby, Denmark), with a starting volume of 1,650 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 ~1,000-L containers, that served as water basins to stabilize temperature (Appendix S1: Photo, Fig. 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 ($T°$ curve, Appendix S1: Fig. S2). We wrapped the bottles in aluminum foil to exclude growth of phototrophic organisms. The experiment started on 1 June 2012 and ran for six weeks until 13 July 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 Corporation, Tuttlingen, Germany) through a BD Q-Syte™ Luer Access Split Septum (BD Biosciences, Stockholm, Sweden) attached via a luer fitting (Watson-Marlow Alitea AB, Stockholm, Sweden), 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% ethanol. 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®, Eugene, Oregon, USA). We used 1.0-μm FluoSpheres (Invitrogen™, Molecular Probes®, Eugene, Oregon, USA) 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 d, after 28 d, and at the end of the experiment after 42 d. The extracted volume was not replaced. For DNA analyses, bacterial cells were collected by vacuum filtration onto a 0.2-μm polycarbonate filter (Supor 200, Pall Corporation) and stored at −80°C until further processing. For the carbon assay, we inoculated Biolog EcoPlates (Biolog Inc., Hayward, California, USA). with 125 μL of 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 minimum of 96 h. Biolog EcoPlates contain

31 distinct carbon sources in triplicates as well a redox dye that turns purple if it is reduced when a given carbon source is metabolized by the community present in the well. Following the color development over time also allows estimation of the rate at which a carbon source is used. We scored a carbon source as positive when two out of three wells reached an optical density of at least 0.2 after subtraction of the median blank from all wells. We subtracted the median blank (as opposed to the mean blank) in order to avoid the influence of outlier readings. 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. 2009) 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 nitrogen ($[NO_3^- + NO_2^-]$ and NH_4^+). The samples were sterile filtered, immediately frozen in liquid nitrogen, and stored at −80°C until analyzed using colorimetric methods (Grasshoff et al. 1999).

Flagellate sampling

To control for possible contamination by eukaryotic flagellates, we sampled 10 mL of culture at the end of the experiment and fixed it with glutaraldehyde (2.5% final concentration). Subsequently, 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, Carlsbad, California, 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 (Illumina Inc., San Diego, California, US), using paired-end 300 base pair (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 is available at http://dx.doi.org/10.5281/zenodo.55294.

Analysis of the sequencing data

The Illumina sequences were preprocessed 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 cut-off 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). We found that 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 with default parameters (http://drive5.com/utax). The final OTU table was manually purged of non-bacterial sequences and 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. 2010*a*) in QIIME (Caporaso et al. 2010*b*) and the tree was constructed using the fasttree algorithm (Price et al. 2009). The heat map presented in Fig. 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 our Supplementary Material.

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 a predictor variable. In order to account for uneven sampling intensity (sequencing depth), we calculated the effective number of species and phylogenetic diversity based on a rarefied OTU table, subsampled for 10,000 reads per sample. One sample had only spurious reads and was excluded. The remaining 47 (out of 144) samples that had less than 10,000 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 justified (Appendix S1: Fig. S3).

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. 2016). 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 cell number ~ time to each growth curve. Stability was then calculated on the residuals extracted from the linear model.

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 using mixed effect models (in the lme4 package [Bates et al. 2015]). Diversity was included as a fixed factor and lake as random factor, allowing both the intercept and the slope of the relationship to vary with lake. We estimated the standardized effect size by scaling all variables (by subtracting the mean and dividing by the standard deviation) before regressing them. The effect size can thus be interpreted as fractions of change in standard deviation units of y for a change of 1 standard deviation in x. For the regression of multifunctionality against diversity, only diversity was standardized. This changes the interpretation to the more intuitive "change in number of functions over threshold for a change of 1 standard deviation in x." We calculated the associated P value by comparing the full model to a null model including only the random factor, using Kenward-Roger approximations of denominator degrees of freedom.

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 searched the literature cited by these articles for further relevant studies. This resulted in a total of 26 articles, all but one published between 2001 and 2015. Three of the papers were excluded: one did not present statistical evidence, the second presented experiments that were conducted on agar plates, and the third incubated the bacterial dilution without any regrowth phase.

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, including potential degradation measured as extracellular enzyme activity; (4) resistance; (5) resilience; (6) stability, measured as the temporal stability of a given ecosystem function; (7) nitrogen cycling, which 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; and (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, nonsignificant, 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 96 diversity–function relationships. A detailed description of the literature review methods is provided in Appendix S1: Tables S1 and S2 and in Appendix S2.

Results

Diversity metrics

We observed bacterial growth in all lakes and dilutions, including the sterile controls (Appendix S1: Fig. 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 ranged from 15 to 438 per sample in the un-rarefied data set and from 15 to around 280 in the rarefied data set, with a median of 40 OTUs. This is in line with what has been reported for other pelagic lake communities (Peter et al. 2014). Diversity (log-transformed) decreased linearly with dilution in all lakes, for both the effective number of species and phylogenetic diversity (Fig. 1). The effective number of species ranged from 1.25 to 32 (Fig. 1, top row), and phylogenetic diversity ranged from 1.13 to 13.5 (Fig. 1 middle row), indicating that the least diverse samples were dominated by a single species (Fig. 2). Effective number of species and phylogenetic diversity were highly correlated ($r^2 > 0.9$ for all lakes; Appendix S1: Fig. 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 ($r^2 < 0.15$, $P > 0.12$; Appendix S1: Fig. 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, 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 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, filter-sterilized, 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. Only the relationship between phylogenetic diversity and maximum cell abundance was significant according to the estimated P value (effect size = 0.45, P value = 0.032; Fig. 3). For all other relationships, there were either (1) consistent but weak relationships or (2) highly variable relationships among lakes (Fig. 3).

Literature review

In total, we found 24 studies (23 from the literature plus ours), of which 14 worked with soil communities and 10 with aquatic communities (Fig. 4, Table 1). Many experiments measured multiple response variables, and the number of diversity–function relationships ($n = 96$) is thus higher than the number of studies. Results are highly variable, with negative, null, and positive relationships. Flat relationships are by far the most common, accounting for ~54% of all relationships, whereas negative relationships are the least common (~10%). Positive results make up 29% of all relationships. The remaining 7% are 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, respectively.

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. Only between phylogenetic diversity and abundance did we find a statistically consistent positive relationship across lakes (Fig. 3), and only if the P values were not adjusted for multiple comparisons. These findings are largely in agreement with results from previous published dilution-to-extinction experiments, as shown in our literature review (Fig. 4).

There are several potential explanations for the absence of a general 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*). A second explanation is that our bacterial communities include a high level of redundancy, meaning that many species are equally

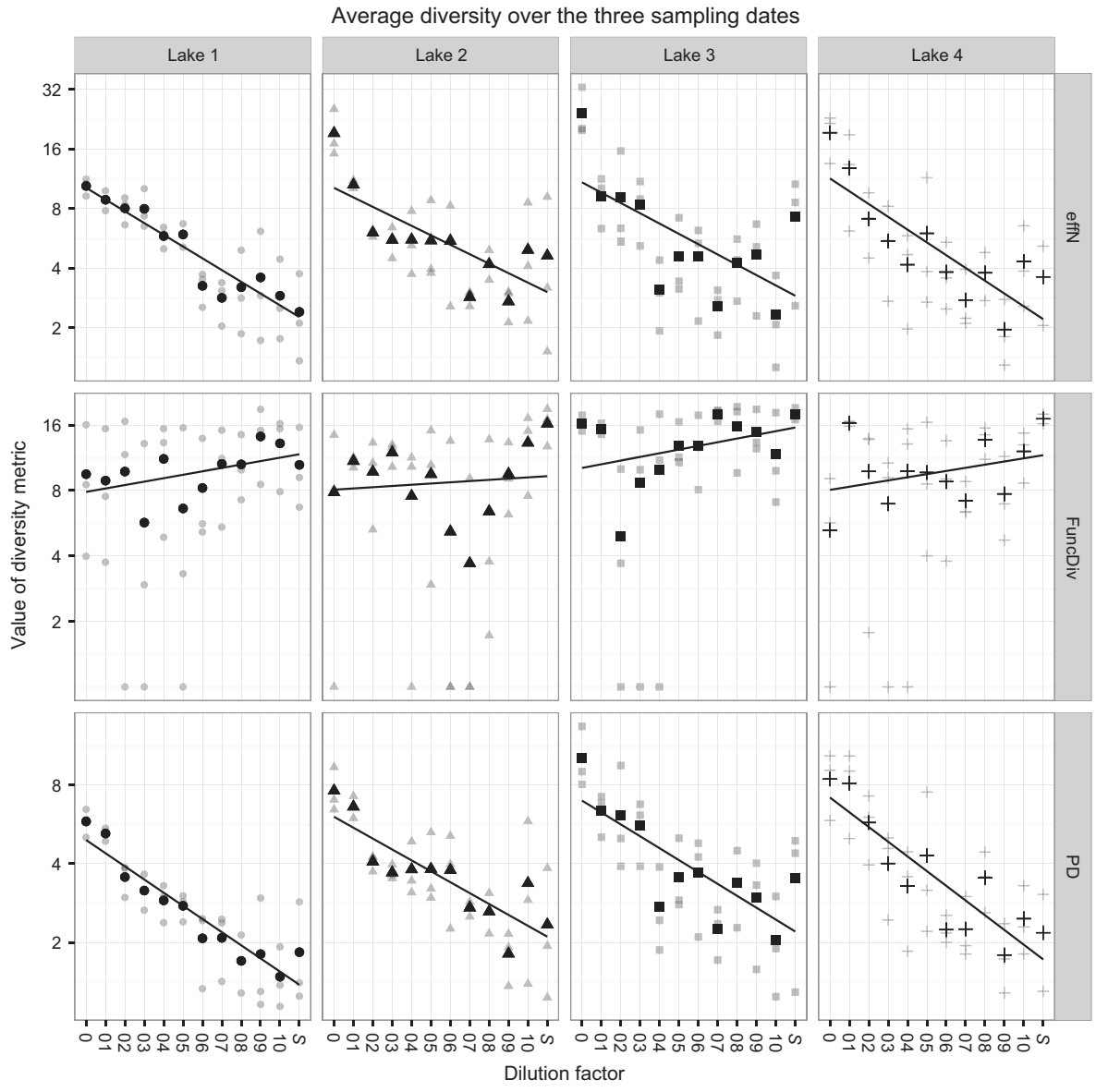

FIG. 1. Realized diversity as a function of the dilution factor for each lake. The dilution axis represents the exponent of the dilution factor, with 0 being the undiluted treatment and 10 the $1{:}4.5^{10}$ diluted treatment. "*S*" labels the sterile control that has been included in the experiment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

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. Functional diversity was a poor predictor of functioning and correlated weakly with species diversity ($r^2 < 0.15$, $P > 0.12$; Appendix S1: Fig. 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

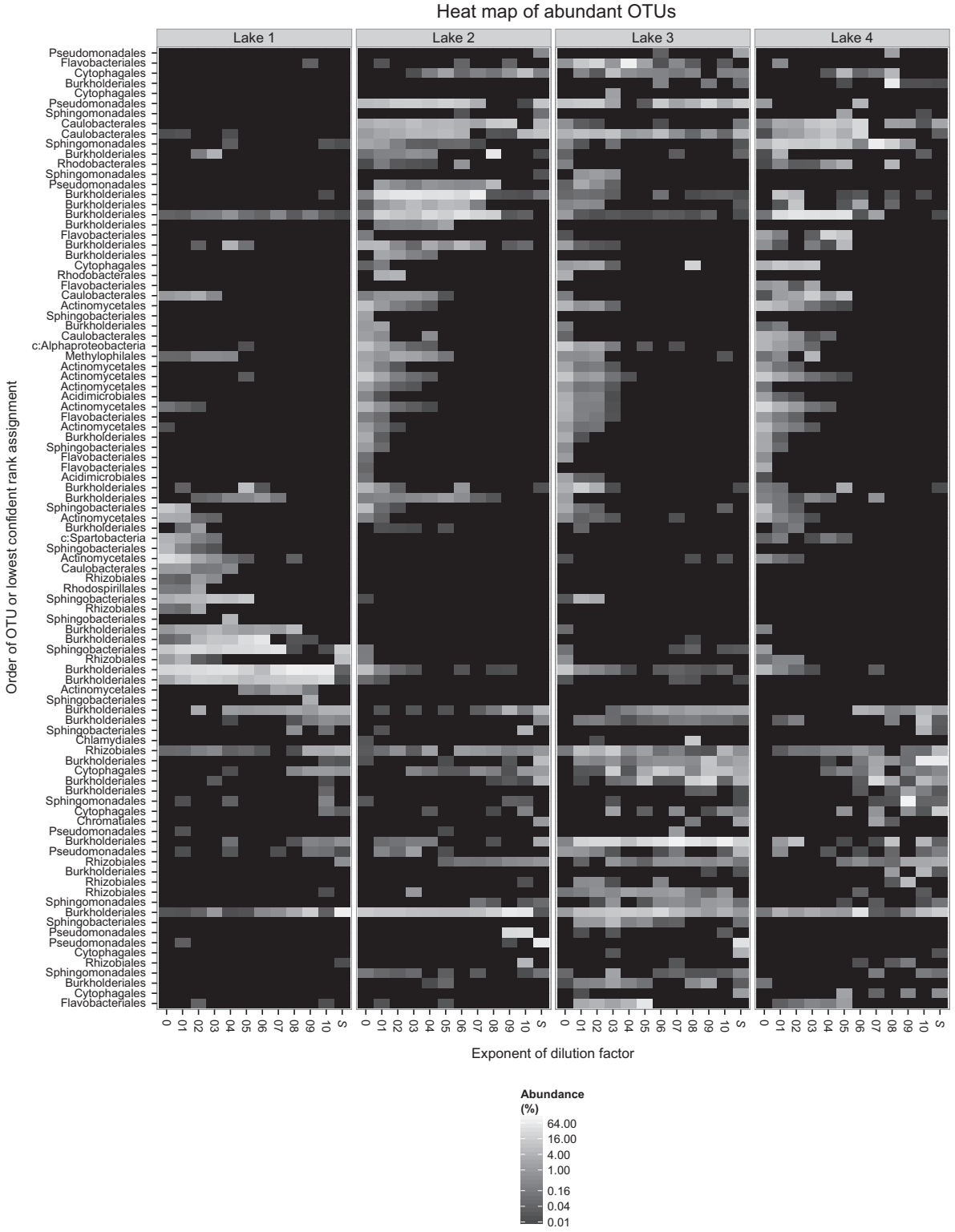

FIG. 2. Heat map of the dominant operational taxonomic units (OTUs) for each lake. Each row represents different OTUs and the order assignment of each OTU is given on the *y*-axis. If the confidence of the order assignment was lower than 90%, the class assignment is given, preceded by "c". Columns show dilution treatments as in Fig. 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.

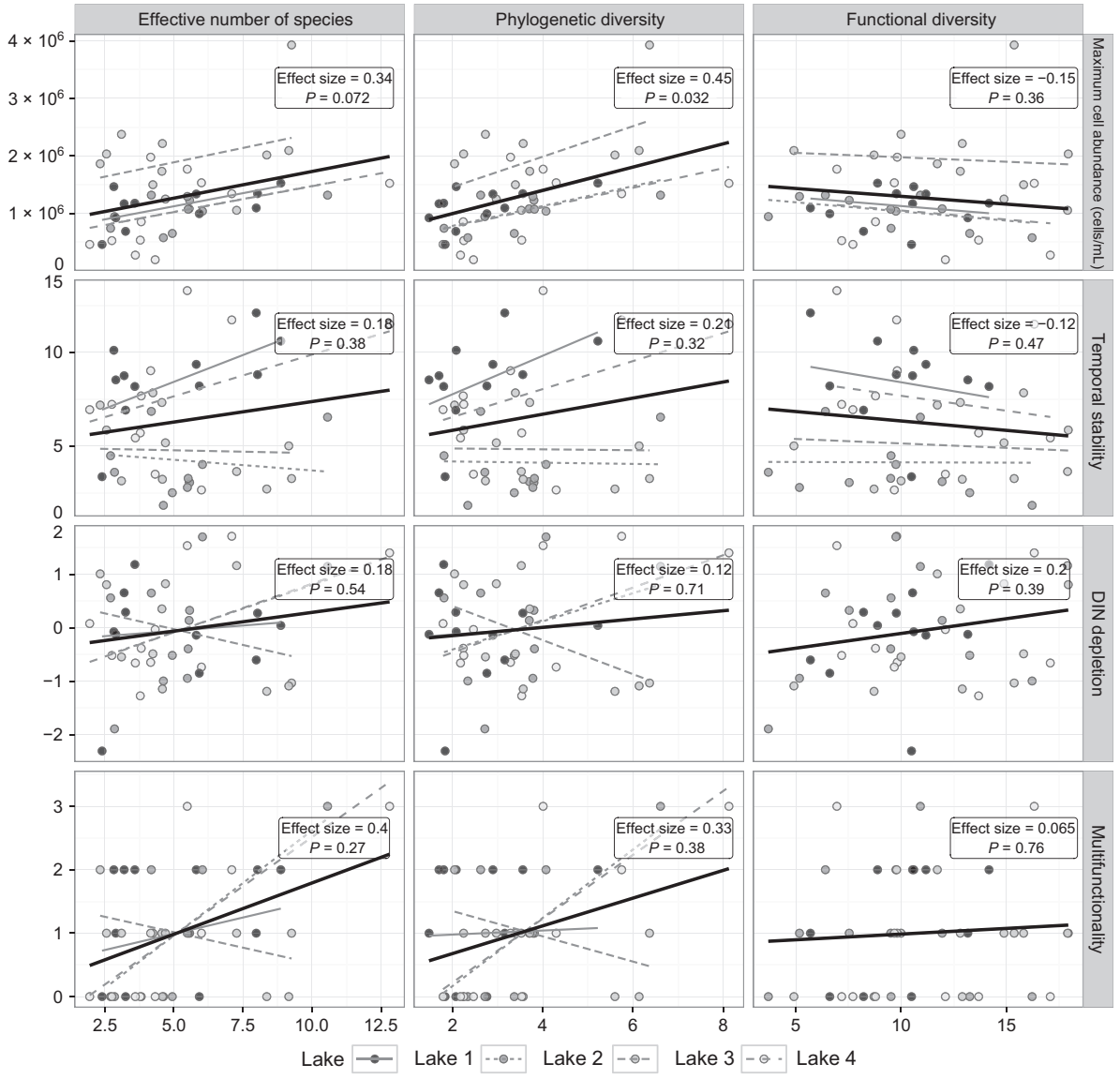

FIG. 3. Maximum cell abundance, stability of cell abundance, depletion of dissolved inorganic nitrogen (DIN), and multifunctionality as functions of the three dimensions of diversity. Diversity is averaged over the three sampling dates. Estimated effect size and *P* value for each model are included. For multifunctionality, the effect size represents the change in fraction of functions (not standard deviations) for a change of 1 standard deviation in the diversity metric. The units of the ecosystem functions are as follows: maximum cell abundance in cells/mL, temporal stability as 1/coefficient of variance, DIN depletion as −1 × scaled DIN concentration in μmol/L, and multifunctionality as number of functions maintained over a threshold of 75% of the maximum function value.

divergence between species may correlate with the time since two species shared a common ancestor. The more functionally 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 2009). 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 (Duffy et al. 2003, Gamfeldt et al. 2008), which is supported by a recent meta-analysis on 94 experiments (Lefcheck et al.

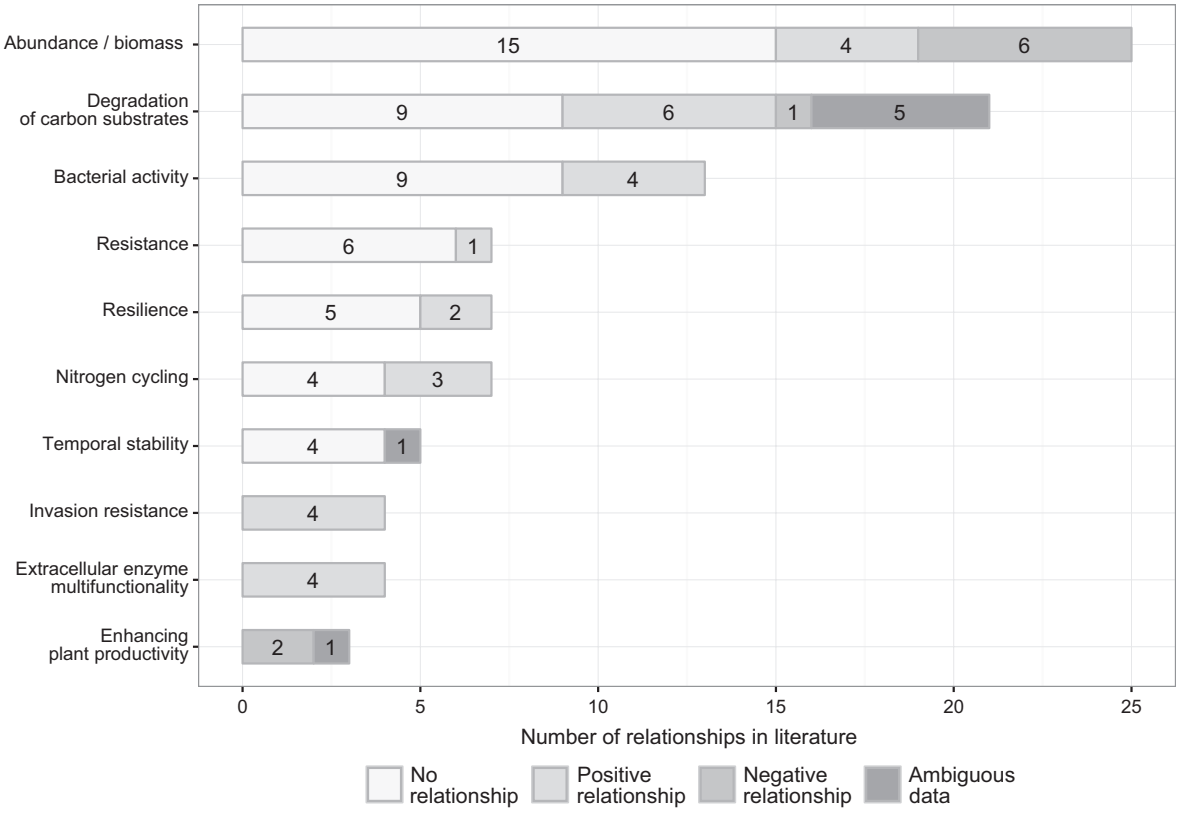

FIG. 4. Summary of the literature review. Each of the 92 diversity–function relationships identified in the literature were categorized as either significantly positive, significantly negative, no significant linear relationship, or ambiguous. The number of relationships for each ecosystem function category is shown.

2015). We found little evidence for this expectation (Fig. 3), suggesting that bacteria are relatively multifunctional. It should be noted though that our multifunctionality metric was based on only three 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 example, the necessary regrowth phase after the initial dilution can have unintended consequences. First, 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 (Fig. 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 (Fig. 2). Despite a median species richness of 40 (in the rarefied data set), the median effective number of species in our experiment was only 5.4. Furthermore, dilution not only manipulates diversity, but also abundance, thereby possibly introducing a confounding factor. Therefore, bacterial experimental communities are often allowed to regrow to initial densities in dilution-to-extinction studies. Yet, intrinsic properties of depauperate communities (lower number of species and absence of species with certain traits) may prevent diluted communities from recovering to pre-dilution densities. Such intrinsic properties are real and not simply artifacts of dilution (Peter et al. 2011*a*). In our experiment, the degree to which diluted communities recovered to original abundances differed among lakes: while Lake 1 showed no overall positive growth trend after the regrowth phase in any dilution treatment, Lake 3 showed a positive growth trend in all treatments starting from dilution 5. To explore to what extent regrowth was likely limited by regrowth time or intrinsic properties of the depauperate communities, we modeled the regrowth potential based on the maximum observed community growth rates with a logistic growth model and the following parameterization:

r_i, the maximum growth rate observed between two consecutive measurements during the first 14 d (the "regrowth phase") in community i;

Table 1. Summary of the articles included in the literature review (see also Fig. 4).

Reference	System	Regrowth (d)	Dilution steps (1:10)	Diversity–ecosystem functioning
Baumann et al. (2013)	soil	42	0, 3, 5	degradation, ambiguous
Bouvier et al. (2012)	aquatic	2	0, 3, 5	activity, positive relationship; biomass, positive relationship; stability, ambiguous
Dimitriu et al. (2010)	soil	152	1, 3, 5, 8	activity, 3 × positive relationship and 1 × no relationship; degradation, 3 × ambiguous and 1 × negative
Franklin and Mills (2006)	aquatic	9	0–6	biomass, no relationship; degradation, ambiguous
Franklin et al. (2001)	aquatic	9	0–6	biomass, negative relationship; degradation, positive relationship
Griffiths et al. (2001)	soil	270	0, 2, 4, 6	activity, no relationship; biomass, negative relationship; resistance, 2 × no relationship; resilience, 2 × no relationship; nitrification, no relationship
Griffiths et al. (2004)	soil	240	2, 4, 6, 8	activity, no relationship; biomass, no relationship; resistance, positive relationship, no relationship; resilience, 2 × positive relationship; nitrification, positive relationship
Hernandez-Raquet et al. (2013)	aquatic	–	1–8 (1, 3, 5, 8)	biomass, positive relationship; degradation, positive relationship
Hol et al. (2010)	soil	210	2, 4, 6	biomass, no relationship; enhancing plant productivity, 2 × negative relationship
Hol et al. (2015)	soil	224	2, 4, 6	biomass, no relationship; N mineralization, no relationship; enhancing plant productivity, ambiguous
Mallon et al. (2015)	soil	79	1, 3, 6	biomass, no relationship; degradation, positive relationship; invasion resistance, positive relationship
Matos et al. (2005)	aquatic	14	0, 4 and gnotobiotic (0, 2–5, gnotobiotic)	biomass, 2 × no relationship; degradation 2 × positive relationship; invasion resistance, positive relationship
Peter et al. (2011*a*)	aquatic	13–15	0–4	activity, no relationship; biomass, positive relationship; degradation, no relationship
Peter et al. (2011*b*)	aquatic	28–42	0, 2, 5	activity, 2 × no relationship; degradation, 2 × no relationship; enzyme multifunctionality, 2 × positive relationship
Philippot et al. (2013)	soil	42	0, 3, 5	biomass, 2 × no relationship; denitrification 2 × positive relationship
Roger et al. (this article)	aquatic	14	0, 0.65, 1.31, 1.96, 2.61, 3.27, 3.92, 4.57, 5.23, 5.88, 6.53	biomass, 4 × no relationship; degradation, 4 × no relationship; stability, 4 × no relationship
Szabó et al. (2007)	aquatic	14	1–8 and 10	biomass, 3 × negative relationship; degradation, no relationship
Tardy et al. (2014)	soil	42	0, 3, 5	activity, no relationship; biomass, no relationship; resistance, 2 × no relationship; resilience, 2 × no relationship
Van Elsas et al. (2012)	soil	30	0, 1, 3, 6	invasion resistance, positive relationship
Vivant et al. (2013)	soil	32	0, 2, 4	invasion resistance, positive relationship
Wertz et al. (2006)	soil	133	1:10, 1–8	activity, no relationship; biomass, no relationship; degradation, no relationship; nitrification, no relationship; denitrification, no relationship

(Continued)

Table 1. Continued.

Reference	System	Regrowth (d)	Dilution steps (1:10)	Diversity–ecosystem functioning
Wertz et al. (2007)	soil	240	1, 3, 4, 5, 6, 8 (1,3,4,5)	biomass positive relationship; resistance, no relationship; resilience, no relationship; denitrification, no relationship; nitrite oxidation, no relationship
Yang et al. (2014)	soil	240	1–14	degradation, positive relationship; activity, no relationship; biomass, negative relationship
Ylla et al. (2013)	aquatic	7	0–5	enzyme multifunctionality, 2 × positive relationship

Note: Dilution steps are given as the exponents of the dilution factors with base 10; *e.g.* 2 corresponds to a $1{:}10^2$ dilution.
If one ecosystem function was measured more then once in the same article the number of times each type of relationship is found is given.
A dash (-) indicates that the information could not be found in the original article.

K_j, the average of the three highest maximum cell numbers observed in any culture (excluding dilution 0) for each Lake *j*; and
$N0_i$, the cell number at the first of the 2 d between which the maximum growth rate has been observed ($T0_i$).

The model was started at $T0$ (time zero). This simple model shows that all but one culture (Lake 4, Sterile control) had the potential to reach the theoretical carrying capacity during the six weeks of the experiment (Appendix S1: Fig. S6). We therefore attribute differences in biomass among dilution treatments mainly to changes in diversity and/or species composition.

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 interactions among bacterial species (e.g., metabolic dependencies [Valentine and 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.

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Supporting Information

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Data Availability

The full code, including all the raw data is available online (http://dx.doi.org/10.5281/zenodo.55294).