environmental microbiology

Environmental Microbiology (2014) doi:10.1111/1462-2920.12429

The functional and taxonomic richness of wastewater treatment plant microbial communities are associated with each other and with ambient nitrogen and carbon availability

David R. Johnson,[1,2]* Tae Kwon Lee,[3] Joonhong Park,[3] Kathrin Fenner[1,4] and Damian E. Helbling[2,5]

[1]*Department of Environmental Systems Science, ETH Zürich, Zürich, Switzerland.*

Departments of [2]*Environmental Microbiology and* [4]*Environmental Chemistry, Eawag, Dübendorf, Switzerland.*

[3]*School of Civil and Environmental Engineering, Yonsei University, Seoul, Korea.*

[5]*School of Civil and Environmental Engineering, Cornell University, Ithaca, NY, USA.*

Summary

The number of functional traits of a wastewater treatment plant (WWTP) microbial community (i.e. functional richness) is thought to be an important determinant of its overall functional performance, but the ecological factors that determine functional richness remain unclear. The number of taxa within a community (i.e. taxonomic richness) is one ecological factor that might be important. Communities that contain more taxa are more likely to have more functional traits, and a positive association is therefore expected between functional and taxonomic richness. Empirical tests for this positive association among WWTP communities, however, are lacking. We address this knowledge gap by measuring the functional and taxonomic richness of 10 independent WWTP communities. We demonstrate that functional and taxonomic richness are positively associated with each other. We further demonstrate that functional and taxonomic richness are negatively associated with the effluent NH_4-N and BOD_5 concentrations. This led us to hypothesize that correlated variation in functional and taxonomic richness is likely related to variation in ambient nitrogen and carbon availability. We finally demonstrate that this hypothesis is consistent with the functional and taxonomic attributes of the WWTP communities. Together, our results improve our basic understanding of the ecology and functioning of WWTP communities.

Introduction

Wastewater treatment plant (WWTP) communities are employed to convert a wide variety of organic substrates into CO_2 and biomass (Rittmann and McCarty, 2001), biodegrade many thousands of pollutants (Schwarzenbach *et al.*, 2006), and withstand perturbations to their environment (Briones and Raskin, 2003). All of these performance objectives are thought to depend on functional richness (i.e. the number of functional traits of a community) (Fernandez *et al.*, 2000; Hashsham *et al.*, 2000; Briones and Raskin, 2003; Cook *et al.*, 2006; Gentile *et al.*, 2007; Wittebolle *et al.*, 2008; Werner *et al.*, 2011; Yang *et al.*, 2011; Hernandez-Raquet *et al.*, 2013). The ecological factors that determine functional richness, however, are often unclear. Taxonomic richness (i.e. the number of taxa within a community) is one ecological factor that might be important. Communities with more taxa are more likely to have more functional traits, and a positive association is therefore expected between functional and taxonomic richness (Chapin *et al.*, 1997; Naeem and Wright, 2003; Cadotte *et al.*, 2011). Empirical tests for this positive association among WWTP communities, however, are lacking or are not supported by statistical analyses, thus representing an important gap in our knowledge about the ecology and functioning of WWTP communities.

Although there have not been any rigorous empirical tests for positive associations between functional and taxonomic richness among WWTP communities, there have been tests among other types of microbial communities. Collectively, however, these tests have generated inconsistent outcomes. In one study, a positive association was observed among undisturbed soil microbial communities but not among disturbed soil microbial communities (Yergeau *et al.*, 2012). In another study, a positive association was observed among a diverse set of

Received 11 October, 2013; revised 30 December, 2013; accepted 11 February, 2014. *For correspondence. E-mail david.johnson@env.ethz.ch; Tel. (+41) 58 765 5520; Fax (+41) 58 765 5802.

vegetative and non-vegetative soil microbial communities but not among a less diverse set of only vegetative soil microbial communities (Fierer *et al*., 2012). Finally, positive associations were observed among some types of aquatic microbial communities (Gilbert *et al*., 2010; Bryant *et al*., 2012) but were not observed by our own analyses of published datasets for other types of aquatic microbial communities (Spearman rank correlation tests between gene-based measures of functional and taxonomic richness, two-sided $P > 0.9$) (Parnell *et al*., 2010; Beazley *et al*., 2012). These inconsistent outcomes raise the following question: Why are positive associations observed among some types of microbial communities but not among others? In other words, what are the likely causes of correlated variation in functional and taxonomic richness among different microbial communities?

There are several plausible explanations for why a positive association between functional and taxonomic richness might not be observed. One explanation is that functional redundancy might be sufficiently pervasive to mask the expected positive association. If most taxa within a community have functional traits that are largely redundant with those of other taxa, then taxonomic richness would predominantly affect functional redundancy rather than functional richness. Another explanation is that inaccuracies in functional richness measurements might cause false negative outcomes. One inaccuracy is the systematic underestimation of functional richness. Consider that many different types of functional traits distinguish different taxa and contribute towards their overall functionality, including catabolic, anabolic, motility, regulation, cell signalling, environmental sensing, resistance and stress response traits. If functional richness measurements only encompass a few types of functional traits, such as catabolic traits, then important differences between different taxa might be missed, which would make it more difficult to observe the expected positive association. Another inaccuracy is the inclusion of non-expressed traits into functional richness measurements, which likely occurs when using gene-based measures of functional richness. Non-expressed traits might lead to ecologically and biologically misleading measurements of functional richness, which would also make it more difficult to observe the expected positive association. Metatranscriptome sequencing could help overcome both of these inaccuracies by generating functional richness measurements that encompass many different types of functional traits while excluding non-expressed traits, thus enabling more robust tests for positive associations between functional and taxonomic richness (Gilbert *et al*., 2010).

In this study, we used metatranscriptome sequencing to answer the following two unresolved questions about the ecology and functioning of WWTP communities. First, are functional and taxonomic richness positively associated with each other among different WWTP communities? Second, what are the likely causes of correlated variation in functional and taxonomic richness? To address these questions, we measured the functional and taxonomic richness of 10 independent WWTP communities from mRNA and 16S rRNA amplicon sequence reads respectively. We then tested whether the functional and taxonomic richness measurements are associated with each other. We next tested whether the functional and taxonomic richness measurements are associated with specific operational and environmental metrics of the WWTPs. Finally, we postulated a hypothesis about the likely relationships between the functional and taxonomic richness measurements, and the operational and environmental metrics of the WWTPs, and tested whether this hypothesis is consistent with the functional and taxonomic attributes of the WWTP communities.

Results

We first tested whether functional and taxonomic richness are positively associated with each other among different WWTP communities. We sequenced mRNAs and 16S rRNA amplicons from 10 independent WWTP communities, assigned the mRNA sequence reads to SEED subsystems functional annotations or level 3 functional categories (Overbeek *et al*., 2005; Meyer *et al*., 2008), and assigned the 16S rRNA amplicon sequence reads to operational taxonomic units (OTUs). We then measured the observed functional and taxonomic richness of each WWTP community as the observed numbers of unique functional annotations or level 3 functional categories and OTUs respectively (Table S1). We found that the observed functional and taxonomic richness measurements are significantly and positively associated with each other, regardless of whether we used functional annotations or level 3 functional categories (Fig. 1; Spearman rank correlation coefficients ≥ 0.67, two-sided $P < 0.05$). We further tested whether ACE extrapolated functional and taxonomic richness measurements are also positively associated with each other (Table S1) and found that this was also the case (Fig. S1; Spearman rank correlation coefficients ≥ 0.67, two-sided $P < 0.05$). Thus, WWTP communities that contain more taxa are indeed more likely to have more functional traits. Based on the consistency in the data, we used observed functional and taxonomic richness measurements and unique functional annotations for all further analyses (unless specified otherwise).

We next tested whether the functional and taxonomic richness measurements are associated with specific operational and environmental metrics of the WWTPs (Table S2). We found that both the functional and taxo-

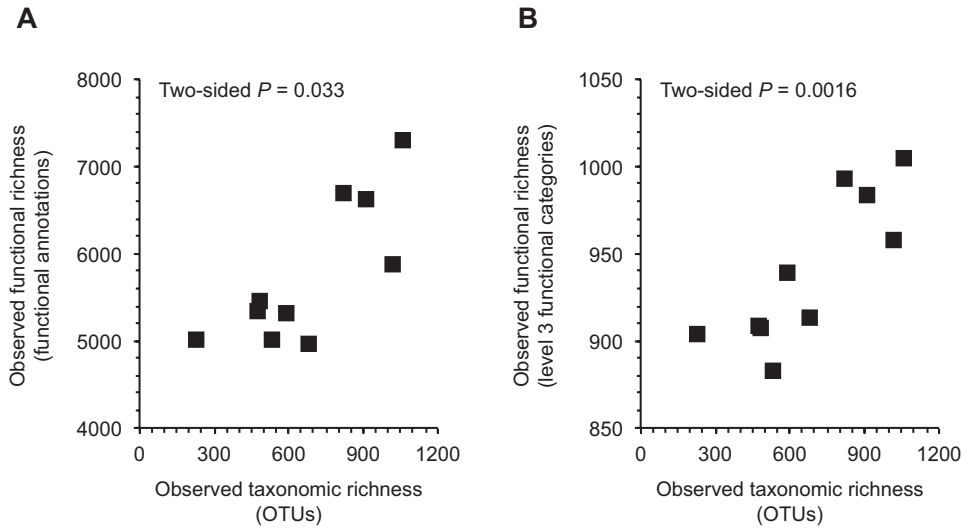

Fig. 1. Positive associations between the observed functional and taxonomic richness measurements of 10 independent WWTP communities (Spearman rank correlation coefficients ≥ 0.67, two-sided $P < 0.05$). The functional richness measurements are the observed numbers of (A) unique SEED subsystems functional annotations or (B) unique SEED subsystems level 3 functional categories. The taxonomic richness measurements are the observed numbers of unique OTUs.

nomic richness measurements are significantly and negatively associated with the effluent ammonia-nitrogen (NH_4-N) concentrations (Spearman rank correlation coefficients < –0.84, two-sided $P < 0.005$) and with the effluent 5-day biological oxygen demand (BOD_5) concentrations (Spearman rank correlation coefficients < –0.75, two-sided $P < 0.05$) (Fig. 2). In contrast, the functional and taxonomic richness measurements are not significantly associated with any of the other operational or environmental metrics of the WWTPs (Fig. 2). Thus, because effluent NH_4-N and BOD_5 concentrations approximate to ambient NH_4-N and BOD_5 concentrations in completely mixed reactors, our results lead to the hypothesis that correlated variation in functional and taxonomic richness is likely related to variation in ambient nitrogen and carbon availability.

We then reasoned that if correlated variation in functional and taxonomic richness is indeed related to variation in ambient nitrogen and carbon availability, then the functional annotations and OTUs that are responsible for the observed variation in the functional and taxonomic richness measurements should not be random assemblages. Instead, they should have additional attributes that are also associated with ambient nitrogen and carbon availability. To test this, we partitioned the functional annotations and OTUs of each WWTP community into two

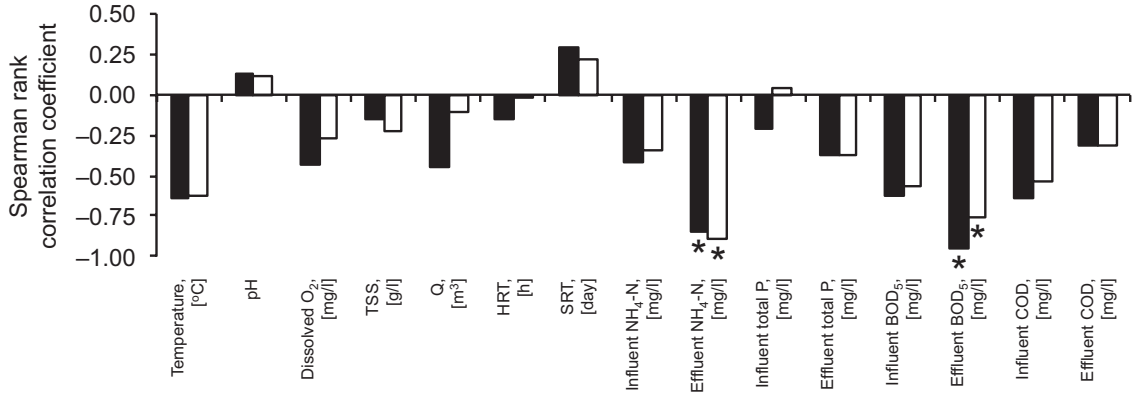

Fig. 2. Spearman rank correlation coefficients for the associations between the functional (black bars) or taxonomic (white bars) richness measurements of the WWTP communities and the measured operational and environmental metrics of the WWTPs (*two-sided $P < 0.05$). The functional and taxonomic richness measurements are the observed numbers of unique SEED subsystems functional annotations and OTUs respectively. TSS, total suspended solids; Q, total daily flow rate; HRT, hydraulic retention time; SRT, solids retention time; NH_4-N, ammonia-nitrogen; BOD_5, 5-day biological oxygen; COD, chemical oxygen demand.

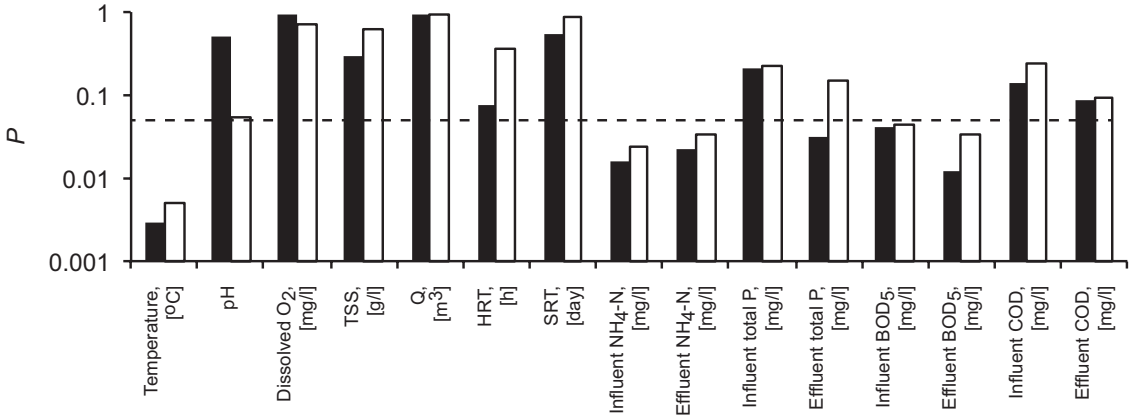

Fig. 3. Permutation tests for the associations between the NMDS ordinations of the non-shared SEED subsystems functional annotations (black bars) or OTUs (white bars) of the WWTP communities and the measured operational and environmental metrics of the WWTPs (horizontal dashed line; $P = 0.05$). TSS, total suspended solids; Q, total daily flow rate; HRT, hydraulic retention time; SRT, solids retention time; NH_4-N, ammonia-nitrogen; BOD_5, 5-day biological oxygen; COD, chemical oxygen demand.

parts. The shared functional annotations and OTUs are those that were observed among all 10 WWTP communities, and they therefore do not contribute to the observed variation in the functional and taxonomic richness measurements. The non-shared functional annotations and OTUs are those that were not observed among all 10 WWTP communities (i.e. they were observed in nine or fewer WWTP communities), and they are therefore solely responsible for the observed variation in the functional and taxonomic richness measurements (Fig. 1). Because only the non-shared functional annotations and OTUs are responsible for the observed variations in the functional and taxonomic richness measurements, we analysed the non-shared functional annotations and OTUs in isolation from the shared functional annotations and OTUs. We provide complete lists of the abundance values and the shared and non-shared designations for every functional annotation and OTU in Tables S3 and S4 respectively.

We first reasoned that if our hypothesis is correct, then differences in the compositions of the non-shared functional annotations and OTUs across the different WWTP communities (i.e. patterns in beta-diversity) should also be associated with ambient nitrogen and carbon availability. To test this, we grouped the non-shared functional annotations into level 2 functional categories as described by Fierer and colleagues (2012). This reduced the number of non-detection events and provided more conservative estimates of differences between the different WWTP communities. We then used non-metric multidimensional scaling (NMDS) (Minchin, 1987) to identify operational and environmental metrics of the WWTPs that associate with the compositions of the non-shared functional annotations and OTUs. As expected, we observed statistically significant associations with the effluent NH_4-N and BOD_5 concentrations (Fig. 3; permutation test, $P < 0.05$). We also observed significant associations with the temperature and influent NH_4-N and BOD_5 concentrations (Fig. 3), but these metrics do not associate with functional or taxonomic richness (Fig. 2). We next used constrained correspondence analysis (CCA) to quantify how much of the variation in the non-shared functional annotations and OTUs could be explained by the effluent NH_4-N and BOD_5 concentrations. We found that 52% ($P < 0.05$) and 49% ($P < 0.05$) of the variation in the non-shared functional annotations and OTUs, respectively, could be explained by the effluent NH_4-N and BOD_5 concentrations. This was true regardless of whether we analysed the non-shared functional annotations and OTUs alone or included the less variable shared functional annotations and OTUs into the analysis. Together, these results demonstrate that differences in the compositions of the non-shared functional annotations and OTUs are indeed associated with ambient nitrogen and carbon availability.

We additionally used partial CCA to test whether the effluent NH_4-N and BOD_5 concentrations explain different aspects of the variation in the non-shared functional annotations and OTUs. We found that the effluent NH_4-N and BOD_5 concentrations do not explain different aspects of the variation ($P > 0.05$). This was expected given that the oxidation of NH_4 contributes to BOD_5, and the effluent NH_4-N and BOD_5 concentrations are therefore not completely independent measurements. To test their independence, we performed pairwise association tests between all of the operational and environmental metrics of the WWTPs (Table S5). We found that the effluent NH_4-N and BOD_5 concentrations are indeed significantly associated with each other (Spearman rank correlation coefficient > 0.8, two-sided $P < 0.05$) but are not associ-

Table 1. Associations between the abundances of mRNA sequence reads assigned to specific SEED subsystems level 2 functional categories and the effluent NH_4-N and BOD_5 concentrations of the WWTPs (absolute Spearman rank correlation coefficients > 0.90, Benjamini–Hochberg-adjusted two-sided $P < 0.05$).

Level 1 functional category	Level 2 functional category	Environmental metric	Spearman rank correlation coefficient	P[a]
Amino acids and derivatives	Glutamine, glutamate, aspartate, asparagine; ammonia assimilation	Effluent NH_4-N	−0.95	0.020
Membrane transport	–	Effluent NH_4-N	−0.93	0.020
Amino acids and derivatives	Arginine; urea cycle, polyamines	Effluent NH_4-N	−0.92	0.020
Motility and chemotaxis	Flagellar motility in Prokaryota	Effluent NH_4-N	−0.92	0.020
Clustering-based subsystems	Clustering-based subsystems	Effluent NH_4-N	−0.91	0.020
Regulation and cell signalling	–	Effluent NH_4-N	−0.91	0.020
Clustering-based subsystems	Chemotaxis, response regulators	Effluent NH_4-N	−0.90	0.021
Iron acquisition and metabolism	–	Effluent NH_4-N	−0.90	0.021
Nucleosides and nucleotides	Purines	Effluent NH_4-N	−0.90	0.024
Nitrogen metabolism	–	Effluent BOD_5	−0.99	0.0028
Stress response	Detoxification	Effluent BOD_5	−0.98	0.0082

a. Benjamini-Hochberg-adjusted two-sided P.

ated with any of the other operational or environmental metrics of the WWTPs (Table S5).

We next reasoned that if our hypothesis is correct, then specific non-shared functional annotations involved with nitrogen and carbon consumption should have mRNA sequence read abundances that are also associated with ambient nitrogen and carbon availability. To test this, we again used the non-shared functional annotations that were grouped into level 2 functional categories, which reduced the number of non-detection events across the different WWTP communities and enabled more robust statistical tests. We then tested for associations between the abundances of non-shared mRNA sequence reads assigned to each level 2 functional category and the effluent NH_4 and BOD_5 concentrations. We provide a complete list of the results from the statistical tests in Table S6. We found that nine level 2 functional categories have abundances of non-shared mRNA sequence reads that are significantly associated with the effluent NH_4-N concentrations (absolute Spearman rank correlation coefficients > 0.90, Benjamini–Hochberg-adjusted two-sided $P < 0.05$) (Table 1). We also found that two level 2 functional categories have abundances of non-shared mRNA sequence reads that are significantly associated with the effluent BOD_5 concentrations (absolute Spearman rank correlation coefficients > 0.90, Benjamini-Hochberg-adjusted two-sided $P < 0.05$) (Table 1). All of these associations are negative, thus demonstrating that WWTP communities with more non-shared mRNA sequence reads have lower effluent NH_4-N and BOD_5 concentrations. While none of these level 2 functional categories are associated with both the effluent NH_4-N and BOD_5 concentrations when using the stringent significance criteria applied in Table 1, they are associated with both the effluent NH_4-N and BOD_5 concentrations when using less stringent significance criteria (Table S6). This was expected given that the effluent NH_4-N and BOD_5 concentrations are significantly associated with each other (Table S5).

We then examined the identities of these functional annotations and level 2 functional categories, and asked whether they are likely to be involved with nitrogen and carbon consumption (Table 1). We found that this was indeed the case. Non-shared mRNA sequence reads assigned to the functional categories 'glutamine, glutamate, aspartate, asparagine; ammonia assimilation' and 'arginine; urea cycle, polyamines' have the largest and third largest absolute Spearman rank correlation coefficients with the effluent NH_4-N concentrations respectively (Table 1). These include mRNA sequence reads annotated to the assimilation and metabolism of NH_4 and alternative nitrogen sources. Non-shared mRNA sequence reads assigned to the functional category 'membrane transport; –' had the second largest absolute Spearman rank correlation coefficient with the effluent NH_4-N concentrations (Table 1). Twenty-one per cent of these mRNA sequence reads are annotated to ammonia monooxygenase, which oxidizes NH_4 and contributes to BOD_5. Finally, non-shared mRNA sequence reads assigned to the functional category 'nitrogen metabolism; –' had the largest absolute Spearman rank correlation coefficient with the effluent BOD_5 concentrations (Table 1). These include mRNA sequence reads annotated to the assimilation and metabolism of a wide variety of nitrogen sources. Together, these results indicate that WWTP communities that synthesize more non-shared mRNA sequence reads involved with assimilating and metabolizing nitrogen sources have lower effluent NH_4-N and BOD_5 concentrations.

Discussion

We sought to test a basic and general prediction in ecology: communities that contain more taxa are more

likely to have more functional traits, and a positive association is therefore expected between functional and taxonomic richness (Chapin *et al*., 1997; Naeem and Wright, 2003; Cadotte *et al*., 2011). Although a general ecological prediction, empirical tests of this prediction are lacking for WWTP communities and have generated inconsistent outcomes for other types of microbial communities. We demonstrated that functional and taxonomic richness measurements based on mRNA and 16S rRNA amplicon sequence reads are indeed positively associated with each other among independent WWTP communities (Figs 1 and S1). Thus, functional redundancy and inaccuracies in functional richness measurements were not sufficiently pervasive to mask the expected positive association when employing the techniques used in this study. Our results are, therefore, consistent with the hypothesis that taxonomic richness is indeed an important ecological determinant of functional richness.

Our approach for measuring functional and taxonomic richness has notable strengths and limitations. An important strength is that the functional and taxonomic richness measurements are based on mRNA and 16S rRNA amplicon sequence reads, respectively, rather than DNA sequence reads. We, therefore, excluded non-expressed functional traits and non-active taxa that might otherwise lead to ecologically and biologically misleading measures of functional and taxonomic richness. An important limitation of our approach, however, is that our functional richness measurements were based on only those mRNA sequence reads that could be assigned to SEED subsystems functional annotations, and they therefore excluded mRNA sequence reads that could not be assigned to SEED subsystems functional annotations but are nevertheless of potential functional importance. Although we cannot fully address this limitation, it is unclear how this limitation could systematically create a false positive outcome (i.e. how this limitation could lead to a positive association when it does not exist). This limitation is, therefore, unlikely to compromise our main conclusion that WWTP communities that contain more taxa are indeed more likely to have more functional traits.

We further postulated the hypothesis that correlated variation in functional and taxonomic richness is likely related to variation in ambient nitrogen and carbon availability. We demonstrated that this hypothesis is consistent with the functional and taxonomic attributes of the WWTP communities. Namely, we found that the functional annotations and OTUs that are responsible for the observed correlated variation in the functional and taxonomic richness measurements are not random assemblages, but instead have additional attributes that are also associated with ambient nitrogen and carbon availability. This hypothesis is also consistent with basic ecological principles. Competition for available nutrients affects functional and taxonomic richness (Egli, 1995; Kovárová-Kovar and Egli, 1998). For example, when preferred nitrogen and carbon sources are scarce, selection for the use of less favourable nitrogen and carbon sources should be enhanced, thus promoting functional and taxonomic richness. Conversely, functional and taxonomic richness affect nitrogen and carbon consumption via selection and complementarity effects (Bell *et al*., 2005; Cardinale, 2011; Hernandez-Raquet *et al*., 2013). The main consequence is that communities with higher functional and taxonomic richness are more likely to consume nitrogen and carbon sources at faster rates and to greater extents (Bell *et al*., 2005; Cardinale, 2011; Hernandez-Raquet *et al*., 2013), thus resulting in reduced nitrogen and carbon availability. There is likely a positive feedback between these two effects, where competition for available nitrogen and carbon sources promotes increased functional and taxonomic richness, which in turn leads to the more complete consumption and reduced availability of nitrogen and carbon sources. Although our experimental design cannot disentangle the relative contributions of these two effects, the scenario described above is consistent with our empirical observations, where WWTP communities with higher functional and taxonomic richness have lower effluent NH_4-N and BOD_5 concentrations (Fig. 2), and thus lower ambient nitrogen and carbon availability.

An additional and important outcome of our study is that the functional and taxonomic richness measurements are not associated with any single metric of the WWTPs that could be operationally controlled (Fig. 2). This includes the hydraulic and solids retention times, both of which have been hypothesized to affect functional and taxonomic richness by controlling the range of successful growth phenotypes (Saikaly and Oerther, 2004; Clara *et al*., 2005; Saikaly *et al*., 2005; Krakat *et al*., 2010). This also includes the influent NH_4-N and BOD_5 concentrations, which are associated with the compositions (Fig. 3) but not the richness (Fig. 2) of the functional annotations and OTUs. Thus, although functional and taxonomic richness are thought to be important determinants of the overall functional performance of WWTP communities (Fernandez *et al*., 2000; Hashsham *et al*., 2000; Cook *et al*., 2006; Gentile *et al*., 2007; Wittebolle *et al*., 2008; Werner *et al*., 2011; Yang *et al*., 2011; Hernandez-Raquet *et al*., 2013), it is not clear how they could be controlled to achieve desired performance objectives. It is plausible that combinations of operational metrics rather than any single operational metric could achieve control. Further investigations of additional WWTP communities could help identify these more complex relationships.

In conclusion, we addressed two important gaps in our knowledge about the ecology and functioning of WWTP communities. First, we provided empirical evidence for a

basic and general ecological prediction: communities that contain more taxa are more likely to have more functional traits. Second, we provided empirical support for the hypothesis that variation in functional and taxonomic richness is likely related to variation in ambient nitrogen and carbon availability. This hypothesis provides a plausible explanation for why positive associations between functional and taxonomic richness were observed among some microbial communities but not among others. Sufficient variation in ambient nutrient availability among microbial communities from a particular habitat type might be required to prevent functional redundancy and inaccuracies in functional richness measurements from masking the expected positive association. We believe that addressing these two knowledge gaps improves our basic understanding about the ecology and functioning of WWTP communities.

Experimental procedures

WWTP communities and sample collection

We previously obtained 10 independent WWTP communities from 10 different WWTPs located across Switzerland as part of a biotransformation experiment that we reported elsewhere (Helbling *et al*., 2012a). We selected the WWTPs in order to obtain a group of WWTPs with substantial variation in their operational and environmental metrics, with specific focus on variation in total suspended solids, solids retention time and influent source (Helbling *et al*., 2012a). We reasoned that greater variation in those metrics should correspond to greater variation in specific metabolic activities. In this study, we reasoned that greater variation in those metrics should also correspond to greater variation in functional and taxonomic richness, which is essential for achieving our main objectives. We summarize the relevant operational and environmental metrics for each WWTP in Table S2, and the influent source and process type for each WWTP in Table S7.

We sampled all 10 WWTPs using an identical and standardized protocol (Helbling *et al*., 2012a). We collected 1 l of activated sludge directly from the biological aeration basin of each WWTP, transported the activated sludge to Eawag in a loosely capped 2 l amber glass bottle, and added a magnetic stir bar to the 2 l bottle immediately upon arrival at Eawag. We then removed 1.5 ml samples 2 h later, collected cells from the samples by centrifugation, and archived the cells at −80°C with RNA*later* (Qiagen, Hilden, Germany). The total time from collecting the activated sludge at the WWTP to removing the 1.5 ml cell samples was approximately 5 h. While our sampling protocol manipulates the WWTP communities to some extent, it also enables a more direct comparison of the results from this study to those from our previous study (Helbling *et al*., 2012a).

RNA methods

We isolated total RNA from the archived cell samples using a conventional acid-phenol method (Johnson *et al*., 2005; Helbling *et al*., 2012b). We removed residual DNA by DNAse digestion using the TURBO DNA-*free* Kit (Applied Biosystems, Foster City, CA, USA). We verified DNA digestion by polymerase chain reaction (PCR) amplification of 16S rRNA genes using the bacterial-specific B27F forward (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R reverse (5′-TACCTTGTTACGACTT-3′) primers. We repeated DNA digestion until 16S rRNA gene amplicons were no longer visible by gel electrophoresis. We assessed the purities of the digested total RNA samples using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MS, USA). All of the digested total RNA samples had A_{260}/A_{280} and A_{260}/A_{230} ratios greater than 1.85. We assessed the integrities of the digested total RNA samples by gel electrophoresis. All of the digested total RNA samples had intact 16S and 23S rRNA bands.

We enriched mRNA from the digested total RNA samples using the MICROB*Express* Bacterial mRNA Enrichment Kit (Invitrogen, Carlsbad, CA, USA). We used a single application because multiple applications were reported to not provide substantial improvements (He *et al*., 2010). We verified mRNA enrichment by gel electrophoresis of 16S and 23S rRNA bands, and by measuring the reductions in the masses of total RNA using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). For all of the enriched mRNA samples, the intensities of the 16S and 23S rRNA bands were visibly reduced, and the masses of total RNA were reduced by 45–63%. We prepared one sequencing library for each enriched mRNA sample using the TruSeq RNA Sample Preparation Kit (version 2) (Illumina, San Diego, CA, USA) and a different sample-specific multiplex adaptor. We then pooled all 10 of the libraries together and loaded the pool onto a single lane of a HiSeq Flow Cell (version 3) (Illumina). We sequenced the libraries using a HiSeq2000 sequencer (Illumina) operated by the Quantitative Genomics Facility at ETH Zürich (Basel, Switzerland). We used single-read 150-cycle sequencing with the TruSeq SR Cluster Kit (version v3-cBot-HS) and the TruSeq SBS Kit (version v3-HS) (Illumina). We performed primary data analysis with RTA software (version 1.13.48) (Illumina). We deposited all of the enriched mRNA sequence reads into the MG-RAST database under project ID 6015.

We reverse-transcribed bacterial 16S rRNAs from the same digested total RNA samples using the Superscript III First Strand Synthesis Supermix (Invitrogen). Each 20 µl reverse transcription reaction contained 1 µl of purified total RNA and 100 nM of the bacterial-specific B534R reverse primer (5′-ATTACCGCGGCTGCTGGC-3′). We amplified the reverse transcription products using the Expand High Fidelity^{PLUS} PCR System (Roche Applied Science, Penzberg, Germany). Each 50 µl PCR reaction contained 2 µl of reverse transcription products and 200 nM each of the bacterial-specific B27F forward and B534R reverse primers. In addition to the target-specific sequences, these PCR amplification primers contained the shotgun adapter sequences required for pyrosequencing with the 454-GS FLX platform and sample-specific multiplex identifier sequences (Table S8). We performed PCR amplification using the following thermal cycling conditions: 5 min at 95°C; 15 cycles of 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C; 10 min at 72°C. This was the minimum number of PCR cycles needed to produce visible amplicon

bands of the expected size after gel electrophoresis. After PCR amplification, we pooled equivalent masses of 16S rRNA amplicons from each of the 10 microbial communities together and prepared a single emPCR library using the forward primer (shotgun adapter sequence A) (Table S8) and the GS emPCR Kit II (Roche 454 Life Sciences, Branford, CT, USA). We then loaded the resulting emPCR products onto 1/16th region of a 454 GS FLX Standard PicoTiterPlate (Roche 454 Life Sciences) and sequenced the products from the forward primer using a 454 GS-FLX sequencer (Roche 454 Life Sciences) operated by GATC Biotech (Konstanz, Germany). We deposited all of the bacterial 16S rRNA amplicon sequence reads into the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under BioProject ID number PRJNA232662.

Analysis of mRNA and 16S rRNA amplicon sequence reads

We analysed mRNA sequence reads using the MG-RAST metagenomics analysis server (version 3.2) with default parameters for quality and length filtering (Meyer *et al*., 2008). We functionally annotated the mRNA sequence reads as described by Fierer and colleagues (2012) for DNA sequence reads. Briefly, we compared singleton mRNA sequence reads with the SEED database (Overbeek *et al*., 2005) using BLASTX and an e-value cut-off of 1×10^{-2} (accessed on 18 May 2013). We then assigned the functionally annotated mRNA sequence reads to SEED subsystems functional annotations, level 3 functional categories or level 2 functional categories (Overbeek *et al*., 2005) as described by Fierer and colleagues (2012). We investigated level 3 and level 2 functional categories because they provide more conservative measures of differences between different WWTP communities. We provide a complete summary of the results from the quality filtering, length filtering and functional annotation processing steps in Table S9.

We analysed the 16S rRNA amplicon sequence reads using the MOTHUR software (Schloss *et al*., 2009). We selected flowgram files containing ≤ 1 mismatch to the multiplex identifier sequence, ≤ 2 mismatches to the target-specific primer sequences, 0 ambiguous nucleotides, and flows between 450 and 720. We corrected and translated the selected flowgram files into DNA sequence reads using PyroNoise (Quince *et al*., 2009), denoised the sequence reads using AmpliconNoise (Quince *et al*., 2011) and removed chimera sequence reads using UCHIME with self-references (Edgar *et al*., 2011). We aligned the resulting sequence reads to the SILVA Gold reference set (Pruesse *et al*., 2007), trimmed the sequence reads to obtain comparable regions, and binned the sequence reads into OTUs using the furthest neighbour algorithm and a sequence similarity level of $\geq 97\%$.

Functional and taxonomic richness measurements

Prior to calculating functional and taxonomic richness, we corrected for differences in sequencing depth across the 10 WWTP communities by rarefying the functionally annotated mRNA and 16S rRNA amplicon sequence reads to 300 000 and 2500 respectively. We measured functional richness as described by Fierer and colleagues (2012) for DNA sequence reads. Briefly, we measured observed functional richness as the observed numbers of unique SEED subsystems functional annotations or level 3 functional categories. We measured observed taxonomic richness as the observed numbers of unique OTUs. We measured extrapolated functional and taxonomic richness using the ACE richness index (Chao, 1987). We compared the observed and ACE extrapolated richness measurements in order to assess the effect of incomplete sequencing on the rank ordering of the functional and taxonomic richness measurements. All of the reported functional and taxonomic richness measurements are the average values from 1000 independently rarefied sequence read datasets. We performed rarefaction and calculated richness in the *R* environment (R Development Core Team, 2013) using functions in the vegan (Oksanen *et al*., 2013) package.

Statistical and multivariate analyses

We performed all statistical and multivariate analyses in the *R* environment using functions in the stats (R Development Core Team, 2013) and vegan (Oksanen *et al*., 2013) packages. We used non-parametric statistical methods because the values of some of the operational and environmental metrics significantly deviate from a normal distribution (Shapiro–Wilk normality tests, $P < 0.05$). We used two-sided Spearman rank correlation tests to test for pairwise associations among the functional richness measurements (Table S1), the taxonomic richness measurements (Table S1), the operational and environmental metrics of the WWTPs (Table S2), and the abundances of functionally annotated mRNA sequence reads (Table S3). We used the Benjamini–Hochberg method to account for multiple comparisons (Benjamini and Hochberg, 1995).

We used NMDS (Minchin, 1987) to compare the differences in the compositions of functional annotations and OTUs. We performed NMDS using Bray–Curtis distances and log-transformed abundances of mRNA and 16S rRNA amplicon sequence reads. We used the envfit function with 999 permutations to test the significance of the associations between the NMDS ordinations and the operational and environmental metrics of the WWTPs (Table S2) (Oksanen *et al*., 2013). When values of specific operational and environmental metrics were missing, we performed NMDS and permutation tests with only those WWTPs that had non-missing values.

We used CCA and partial CCA to quantify the amount of variation in the compositions of the functional annotations and OTUs that could be explained by the effluent NH_4-N and BOD_5 concentrations. We performed CCA and partial CCA using log-transformed abundances of mRNA and 16S rRNA amplicon sequence reads. We used the anova function from the vegan package to test the significance of the constraints (Oksanen *et al*., 2013). We performed CCA and partial CCA with only those WWTPs that had measurements for both the effluent NH_4-N and BOD_5 concentrations (Table S2). We additionally performed CCA and partial CCA using shared and non-shared designations based on all 10 WWTP communities or based on only those WWTP communities for which we had both effluent NH_4-N and BOD_5 measurements. The CCA

and partial CCA results are qualitatively similar for both cases, and we therefore only report the results when using the shared and non-shared designations based on all 10 WWTP communities.

Acknowledgements

We thank Martin Ackermann, Samuel Bickel and George Wells for useful discussions and for critically reading preliminary versions of this manuscript. We thank Christian Beisel, Ina Nissen and Manuel Kohler of the Quantitative Genomics Facility at ETH Zürich for Illumina sequencing. Finally, we thank two anonymous reviewers for substantially improving the quality of this manuscript. This work was supported by grants from Eawag (Category: Seed) and the Korean-Swiss Science and Technology Cooperation.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Positive associations between the ACE extrapolated functional and taxonomic richness measurements of 10 independent WWTP communities (Spearman rank correlation coefficients ≥ 0.67, two-sided $P < 0.05$). The functional richness measurements are the ACE extrapolated numbers of (A) unique SEED subsystems functional annotations or (B) unique SEED subsystems level 3 functional categories. The taxonomic richness measurements are the ACE extrapolated numbers of unique OTUs.

Table S1. Functional and taxonomic richness measurements for each wastewater treatment plant community.

Table S2. Operational and environmental metrics of the wastewater treatment plants.

Table S3. mRNA sequence read abundances and shared/non-shared designation for each SEED subsystems functional annotation.

Table S4. Bacterial 16S rRNA amplicon sequence read abundances and shared/non-shared designation for each OTU.

Table S5. Pairwise association tests between the operational and environmental metrics of the wastewater treatment plants.

Table S6. Associations between the abundances of mRNA sequence reads assigned to specific SEED subsystems level 2 functional categories and the effluent NH4-N and BOD5 concentrations of the WWTPs.

Table S7. Influent source and process types for each wastewater treatment plant.

Table S8. Primers used for PCR amplification and sequencing of reverse-transcribed bacterial 16S rRNAs.

Table S9. Summary of metatranscriptomic analysis for each wastewater treatment plant community.