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Biodegradability of wastewater determines microbial assembly mechanisms in full-scale wastewater treatment plants

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ABSTRACT

Wastewater treatment plants (WWTPs) are critical for maintaining sustainable development in modern societies, wherein microbial populations residing in activated sludge (AS) are responsible for the removal of pollutants from wastewater. The biodegradability [biological oxygen demand/chemical oxygen demand (B/C ratio)] of influent, as a measure of the degree of available energy and toxicity to microorganisms in AS, has been hypothesized to drive AS microbial community assembly. However, the validity of this hypothesis has not been tested in full-scale WWTPs. In this study, we assessed the pollutant removal loads, the microbial community diversity, the relative importance of deterministic and stochastic assembly processes, and bio-interactions within the communities by analyzing 195 AS samples comprising nearly 5 000 000 16S rRNA sequences. Our results indicate that the effects of B/C ratio on pollutant removal loads can be perfectly reflected through biological properties, implying that B/C ratio determined WWTPs performance through affecting microbial community. Very low and/or very high B/C ratios result in low microbial diversity, strong stochastic processes, and large, complex networks, leading to low pollutant removal load of treatment. A B/C ratio of around 0.5 was optimal for system stability and efficiency. Based on the results of this study, the authors propose using the B/C ratio as an indispensable index to assess system performance and to provide an indicator of an impending process upset before function deteriorates significantly. This study provides a specific measure that can be used to evaluate strategies for process optimization and operation of WWTPs.

1. Introduction

Biological wastewater treatment plants (WWTPs) play key roles in biodegrading organic materials, transforming toxic compounds into harmless products, and removing carbon and nutrients (Gentile et al., 2007; Wang et al., 2011). As such, they are an important application area of biotechnology (Wagner and Loy, 2002) and critical to maintaining sustainable development. So, a better understanding of how to maintain the stable function of WWTPs when environmental conditions are so dynamic due to constantly changing wastewater influent is crucial. It is widely accepted that activated sludge (AS) microbial communities are responsible for most of the nutrient removal (Wagner and Loy, 2002) and that the diversity, structure and composition of these communities determine WWTP performance (Briones and Raskin, 2003; Falk et al., 2009; Griffin and Wells, 2017; Miura et al., 2007; Wagner and Loy, 2002). However, the underlying mechanisms are still regarded as a black box. Understanding microbial community assembly mechanisms is a promising way to help uncover key parameters in regulating system performance in WWTPs.

Microbial assembly is believed to be simultaneously influenced by deterministic processes such as selection imposed by environmental filtering or/and biotic interactions, and stochastic processes such as dispersal limitation, birth-death events or drift (Caruso et al., 2011; Dumbrell et al., 2010; Langenheder and Szekely, 2011; Myers and Harms, 2011; Ofiteru et al., 2010; Stegen et al., 2012). To date, in both natural and engineered ecosystems, there has been considerable debate about the relative contributions of deterministic and stochastic processes on microbial community assembly (Griffin and Wells, 2017). For instance, some studies (Ju and Zhang, 2015; Valentin-Vargas et al., 2012) showed that

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deterministic factors had a bigger impact on community assembly in WWTPs, whereas others (Ofiteru et al., 2010) showed that stochastic processes such as random immigration were the dominant force. This inconsistency could be due to variations in the degree of influence of stochastic and deterministic processes on AS microbial assemblage from different environmental conditions at the WWTPs studies.

Microbial communities in WWTPs are affected by many factors (Chen et al., 2017; Pholchan et al., 2010; Wang et al., 2012; Xia et al., 2014), including influent characteristics, operational parameters, process configuration and environmental conditions. Influent characteristics has been shown to be the most important factor shaping the microbial community structure and composition (Chen et al., 2017; Ibarbalz et al. 2013, 2016). Furthermore, microbial communities from industrial and municipal WWTPs were different (Ibarbalz et al., 2013) because each had distinct influent components and organic loading, which resulted in differing bacterial growth and metabolism (Nielsen et al., 2010). Because not all of the organic matters can be broken down by microorganisms through biodegradation, biodegradability can be used to represent the level of available nutrients and toxicity of influent (Mangkoedihardjo, 2006) and it is much easier to measure than wastewater composition. A number of methods have been established to estimate wastewater biodegradability, among which the ratio of biological oxygen demand (BOD) to chemical oxygen demand (COD) (referred to as B/C ratio hereafter) is one of the most well-adopted surrogates (Amat et al., 2009).

Many investigations have demonstrated that the B/C ratio significantly affects WWTP system performance. A lower B/C ratio is suggestive of insufficient food and the presence of poorly biodegradable substances that may be toxic to microbes and inhibit microbial activity (Mangkoedihardjo, 2006; Mohan et al., 2005; Samudro and Mangkoedihardjo, 2010), resulting in a high COD in the effluent, inadequate denitrification (Bolzonella et al., 2001; De Lucas et al., 2005), deterioration of bio-phosphate removal (Mulkerrins et al., 2004; Ra et al., 2000; Tong and Chen, 2007), and even system breakdown (Mangkoedihardjo, 2006). It is likely that biodegradability (Ju et al., 2014) and food availability (Xia et al., 2016) could affect microbial interactions because various species/populations interact with each other through the flow of energy, matter, and information (Montoya et al., 2006). However, the effects of influent biodegradability on the microbial community remain unclear.

In this study, high-throughput 16S rRNA amplicon sequencing was used to obtain comprehensive microbial community information to address the following questions: (1) Does biodegradability of wastewater affect system performance of WWTPs? (2) Do microbial community properties vary with biodegradability? (3) What are the possible links between microbial community properties and system performance? Our work identified a previously undocumented dimension of the effects of wastewater biodegradability on microbial assembly and interactions, and built a conceptual model which offers insights into links between the best microbial community properties and best WWTP performance. Overall, these findings provide the basis for improving the efficiency and functional stability of WWTPs from a new dimension and represent an important step forward in achieving better reactor design, operation and control.

2. Materials and methods

2.1. Sampling

A total of 195 AS samples were collected from 58 full-scale WWTPs located in 15 cities of China using a uniform sampling protocol as shown below. Briefly, each WWTP was sampled only once and the whole survey was finished in the summer of 2014. AS samples were taken from inlet, middle and outlet of the aeration phase, respectively in each WWTP and most of these three samples were about 5 m apart. Details of the 58 WWTPs, including pollutant concentration in influent and effluent, activated sludge properties, and design and operational parameters, are listed in Supplementary Table S1.

All AS samples were briefly settled on site and then immediately transported to the laboratory on ice. A 2-ml aliquot of activated sludge sample was centrifuged at 15 000×g for 10 min and the pellets were stored at −80 °C until DNA was extracted. Another 100-ml aliquot of AS was centrifuged and supernatants were used to measure common chemical parameters including ammonia, nitrite, nitrate, total nitrogen (TN), total phosphorus (TP) and COD. Temperature, pH, dissolved oxygen (DO), and conductivity of activated sludge were measured in situ. The pollutant concentrations of influents and effluents (ammonia, nitrite, nitrate, TN, TP, BOD, and COD) of WWTPs were directly obtained from the WWTP staffs. Other information about the WWTPs, including geographic coordinates, age of plant, hydraulic retention time (HRT), sludge retention time (SRT), volume of aeration tanks, proportion of industrial wastewater, and the recycling ratio were either obtained from the WWTPs or from online sources.

2.2. DNA extraction, Illumina sequencing and data processing

Microbial genomic DNA was extracted using PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). DNA quality was assessed by an ND-2000 spectrophotometer (Nanodrop Inc., Wilmington, DE) and quantified using PicoGreen on a FLUO star Optima (BMG Labtech, Jena, Germany).

DNA samples were amplified with a set of primers targeting the variable region 4 (V4) of the 16S rRNA gene by applying an effective and robust two-step PCR as previously described (Wu et al., 2015). The forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were utilized for the first-step PCR. Reagents and program parameters for PCR amplification can be seen in Supplementary Table S2 and Table S3. After first-step amplification, the triplicate first-step PCR products were pooled together and purified using 75 μl Agencourt AMPure XP beads (Beckman, USA) to remove primer dimers and other contaminants. Purified PCR products were recovered in 50 μl water and 15 μl were used as template the second round PCR using the same reaction buffer as above but with barcode primers and the same amplification protocol except for 15 cycles.

After the second-step amplification, PCR products from triplicate reactions were pooled and quantified by PicoGreen. Subsequently, 200 ng of DNA from each sample were combined and loaded to 1% agarose gel. The amplicon band was removed and the target genes were extracted using a MinElute Gel Extraction Kit (QIAGEN Sciences, Germantown, MD, USA) to remove agarose. Finally, the mixture was loaded into a MiSeq reagent cartridge (Illumina, San Diego, CA, USA) and sequenced (2 × 250 bp paired-end kit) at The University of Oklahoma (Norman, OK, USA). Raw sequences are available from the NCBI Sequence Read Archive with accession number PRJNA509305.

Raw sequences were processed using a sequencing analysis pipeline that was built on the Galaxy platform (http://www.ou.edu/ieg/tools/data-analysis-pipeline) Briefly, raw sequencing data were separated based on sample barcodes. Quality trimming was done using Btrim (Kong, 2011). Forward and reverse reads were merged into full-length sequences by FLASH (Magoc and Salzberg, 2011). Sequences were removed if they were too short or contained ambiguous bases. The operational taxonomic units (OTUs) were classified using UPARSE at the 97% similarity level with singletons removed. To compare the 211 samples at the same sequencing depth, normalization of the sequence number was conducted by extracting 25 600 sequences randomly from each sample for all the following analyses. The taxonomic assignment was conducted by RDP classifier (Wang et al., 2007) with a minimal 50% confidence estimate for the downstream analysis.

2.3. Data analyses

Shannon index (H), evenness, and estimated richness in the pool (Chao2) were used to evaluate microbial taxonomic diversity. Null model was used to explore the relative influence of deterministic and stochastic processes on microbial assembly. Here we only considered the influence of selection, dispersal, and drift. Speciation was ignored because it has little influence within communities (Stegen et al., 2013). Analysis was performed in R following the method developed by Stegen et al. 2012, 2013, 2015. The related processes included homogeneous selection, variable selection, dispersal limitation, homogenizing dispersal, and undominated (Stegen et al., 2015). The item ‘Undominated’ was used to refer to the scenario in which neither dispersal nor selection was the primary cause. About calculation, there are two steps to estimate the relative influences of each ecological processes. First, the difference between observed between-community version of the β-mean-nearest taxon distance (βMNTD) and the mean of the null distribution was measured in units of standard deviation to estimate the influences of Selection. Second, the deviation between observed Bray-Curtis distance and a null distribution of Bray-Curtis values was standardized to estimate the influences of Drift and Limitation. More details of the calculation can be found in the Reference (Stegen et al., 2015).

$$\text{Pollutant removal load} = \frac{\textit{gross of pollutant removal}}{\textit{total biomass}} = \frac{\left[\textit{pollutant in influent}\ \left(g/m^3\right) - \textit{pollutant in effluent}\ \left(g/m^3\right)\right]\left[\textit{influent flow rate}\ \left(m^3/d\right)\right]}{\left[\textit{aeration tank volume}\ \left(m^3\right)\right]\left[\textit{reactor biomass}\ \left(g/m^3\right)\right]} \qquad \text{Equation 1}$$

Microbial association networks were constructed to reveal possible interactions between microbes based on the random matrix theory (RMT) algorithm by using the Molecular Ecological Network Analysis Pipeline (MENAP) (http://www.ou.edu/ieg/tools/data-analysis-pipeline). For each network, only OTUs detected in more than 66.7% of the samples were used for network construction to remove poorly represented OTUs and reduce network complexity. Then 100 corresponding random networks were generated, each having the same network size and average number of links for each network. The Z-test was used to test differences in indices between the constructed networks and random networks. To characterize modularity properties, each network was separated into modules by the fast-greedy modularity optimization. Each module in the network considered a subset of species from similar ecological niches (Zhou et al., 2011) and may perform similar functions (Luo et al. 2006a, 2006b; Newman, 2006). A modularity value was used to measure how well a network can be separated into modules (Deng et al., 2012) and is a fundamental characteristic of biological networks (Dethlefsen et al., 2008). Other crucial parameters usually used to describe the topological properties of network are as follows. Average clustering coefficient and average connectivity can be used to indicate the complexity of the network (Deng et al., 2012) and average geodesic distance/efficiency is a measure of the efficiency of information or mass transport within a network (Deng et al., 2012). Gephi (0.9.1) was used for visualization of network nodes and connectivity.

3. Results

3.1. Overview of influent biodegradability and its impacts on system performance

A total of 195 samples were collected from 58 WWTPs located in 15 cities. The influent B/C ratio and pollutant removal loads were calculated for each WWTP. We defined pollutant removal load as the estimated amount of pollutant removed per unit of biomass (Equation (1)).

Overall, a high pollutant removal load was observed when B/C ratios ranged from 0.4 to 0.6. The highest BOD and TP removal loads were observed when the B/C ratio was 0.55 and 0.49 (Fig. 1a). Similarly, COD, NH_4^+ and TN removal load also showed significant Parabolic distributions with B/C ratios (Fig. S1) and a high removal load of these pollutants was observed when the B/C ratio was between 0.4 and 0.6. The optimal B/C ratio for COD, NH_4^+ and TN removal was between 0.48 and 0.53. In comparison, the pollutant removal load was very low when the B/C ratio was below 0.3 or above 0.6 (Fig. 1a and Fig. S1). These results suggest that biodegradability of wastewater has strong effects on WWTP performance.

The WWTPs were divided into five groups based on their B/C ratios: below 0.3 (group A, 18 samples), between 0.3 and 0.4 (group B, 24 samples), between 0.4 and 0.5 (group C, 65 samples), between 0.5 and 0.6 (group D, 61 samples), and above 0.6 (group E, 27 samples). Fisher-LSD results indicated that Group A had the significantly lowest removal loads, while Group D had the highest (Table S4). More than two thirds of the samples (126 in total) were from WWTPs with B/C ratios between 0.4 and 0.6 (Fig. 1b), indicating efficient pollutant removal conditions.

3.2. Impacts of B/C ratio on microbial community diversity and composition

Given that the efficiency and stability of WWTPs largely depends on the AS microbial community, it is important to investigate the differences in biological properties at different B/C ratios. To avoid bias during comparisons among different B/C ratio groups, all groups were resampled to the same number of samples based on the smallest group size, that is, 18 samples were randomly extracted from each group for downstream biological analyses.

Studies have shown that system stability and function depend on not only species richness but also evenness or composition of the microbial community (Griffiths and Philippot, 2013; Wittebolle

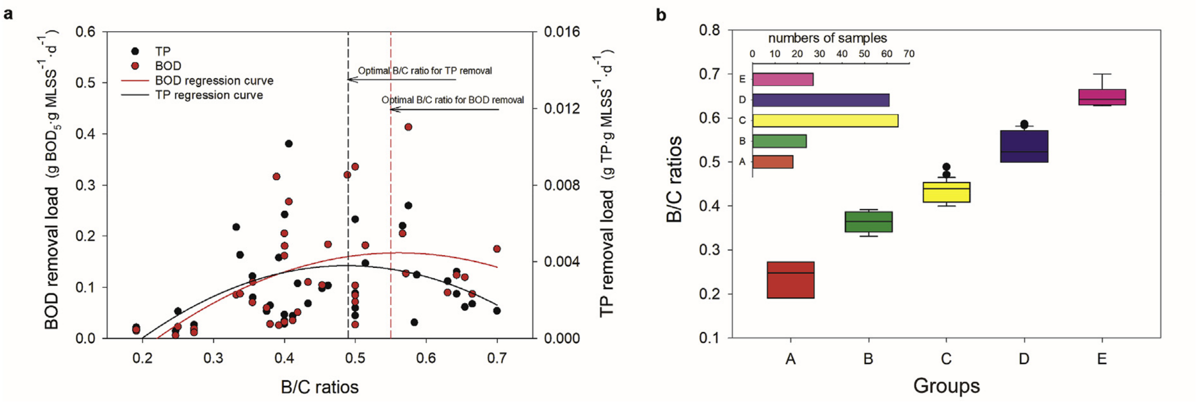

Fig. 1. (a) Relationship between pollutant removal loads and B/C ratio in WWTPs. (b) Distribution of influent biodegradability in WWTPs (upper left plot) and average B/C ratio (ratio of BOD to COD; main plot) in each group. Red circles represent the COD removal load and the black circles represent TP removal load. Pollutant removal load was calculated by the equation (pollutant concentration in influent - pollutant concentration in effluent) × actual flow rate/mixed liquor suspended solids (MLSS) × volume of aeration tank). Dashed lines indicate where pollutant removal loads reached maximum. The letters represent WWTPs grouped based on their B/C ratios, A, below 0.3; B, between 0.3 and 0.4; C, between 0.4 and 0.5; D, between 0.5 and 0.6; E, above 0.6. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

et al., 2009). Here, Chao 2, Pielou evenness (J), and Shannon index (H) were adopted. The average value of Chao2 for each group was generated from 1000 random resamplings. J and H indices were generated in a similar manner. ANOVA results indicated that all three indices were significantly different among the B/C ratio groups ($P < 0.001$). Interestingly, biodegradability of influent exhibited a curvilinear relationship with Shannon's diversity index (H) rather than a simple linear relationship (Fig. 2). Initially, H index increased gradually with increasing B/C ratio and a maximum H index was observed when the average B/C ratio was 0.53 (group D). However, H index dropped when the B/C ratio exceeded 0.6. The same trend could be seen for relationship of the B/C ratio to richness and evenness (J) (Fig. S2), which demonstrates that there is a relationship between the B/C ratio and biodiversity in AS.

Dissimilarity test showed that microbial community composition among groups had significant difference (Tables S5 and S6). Notably, *Actinobacteria* had the largest proportion of the population in group D compared with other groups (Fig. S3). As *Actinobacteria* is proposed to be important bacteria for phosphorus removal (Wagner et al., 1994), it may enhance biological phosphorus removal at B/C ratios around 0.5. And this observation is supported by the TP removal efficiencies observed in this study (Fig. 1a).

3.3. Impact of B/C ratio on the relative importance of deterministic and stochastic processes

Since the B/C ratio had significant effects on microbial diversity and composition, the forces involved in determining microbial community composition and structure were subsequently investigated by using the null model developed by Stegen et al., (2013). Given that group A contained 18 samples coming from 6 WWTPs (5 WWTPs but 6 treatment systems, treated as 6 WWTPs), 18 samples from 6 WWTPs were randomly selected each time for Group B-E to calculate the relative contributions of different assembly processes. In detail, results of Group B and E were generated from all possible permutations and combinations and results of Group C and D were derived from 1000 permutations because of too much possibility. Results of each permutation for each group can be found in Table S7. In agreement with our hypothesis, these two assembly processes occurred simultaneously and their relative influence changed under different operating conditions (Fig. 3). With increasing B/C ratio, deterministic processes tended to predominate, despite the decrease was observed when the B/C ratio was over 0.6 (Fig. 3).

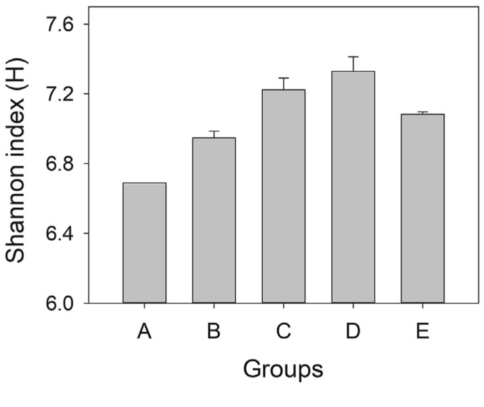

Fig. 2. Alpha diversity of each B/C ratio group in WWTPs. Each bar represents the mean Shannon index (H) calculated from 1000-time random resampling with 18 samples for each time and error bar represents standard deviation of 1000-time random resampling. The letters represent WWTPs grouped based on their B/C ratios, A, below 0.3; B, between 0.3 and 0.4; C, between 0.4 and 0.5; D, between 0.5 and 0.6; E, above 0.6.

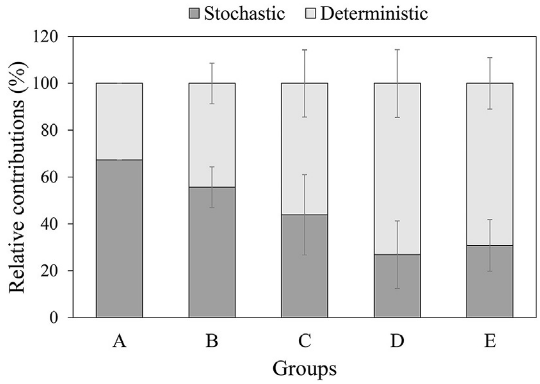

Fig. 3. The relative contribution of microbial community assembling processes in WWTPs. The letters represent WWTPs grouped based on their B/C ratios, A, below 0.3; B, between 0.3 and 0.4; C, between 0.4 and 0.5; D, between 0.5 and 0.6; E, above 0.6. "Deterministic" represents deterministic processes and "stochastic" represents stochastic process.

Remarkably, this trend of deterministic processes was highly consistent with that of alpha diversity. It is worth noting that stochastic processes could explain as much as 60% of assembly process when the B/C ratio was below 0.3. By comparing against Midas database (Microbial Database for Activated Sludge), which give abundance information of individual genera to allow an assessment of immigration on population structure (McIlroy et al., 2015), we found that immigrated taxa may become key members of the AS microbial community. For example, *P. simplicispira*, which is reported to be more predominant in influent rather than activated sludge (McIlroy et al., 2015), was the most abundant and frequent genus detected in group A. Its high abundance in Group A may arise from continuous influent into WWTPs (Ofiteru et al., 2010) and it ability to metabolize fatty acids, sugars, and proteins (McIlroy et al., 2015).

3.4. Impacts of B/C ratio on bacterial interactions

As one of the deterministic processes, biological interactions were proposed as major drivers during microbial assembly (Ju and Zhang, 2015). Identifying the interactions occurring among microorganisms and keystone species is essential for better understanding microbial community diversity and functions (Hallam and Mccutcheon, 2015; Ren et al., 2015). We thus examined microbial interactions by employing MENA network analysis. The same thresholds (St) was applied for each of the group (0.86) to allow for comparisons among the groups (Table 1).

Networks were constructed based on B/C ratios and all five of these networks exhibited scale-free characteristics, as indicated by power law R^2 s larger than 0.8. In addition, significant differences between these five networks and their corresponding random networks, with identical network sizes and average numbers of links, were observed (Table 1), suggesting that the network structures were non-random. Surprisingly, it seems that B/C ratios played a decisive role in microbial interactions because the networks were substantially different in terms of network composition among the five groups. For example, the average geodesic distance, average clustering coefficient, and modularity (as well as many other network parameters) of these pMENs were significantly different (Table 1).

Specifically, microbes with a B/C ratio of around 0.5 (group D) tended to form many, but small, modules (Fig. 4) with a large modularity, smaller clustering coefficient and a shorter average geodesic distance (Table 1). In contrast, microbial interactions in groups A and E tended to form several large, complex modules, indicating that the microbial communities in these groups contained fewer functional groups compared with the communities in group D. Also, there were less connections among modules in groups D than in those of other groups. However, instead of the tight links among modules observed in group A, connectivity among modules in group E was looser (Fig. 4). A majority of the links in all groups were positive, ranging from 69.12% to 80.94% (Fig. 5), indicating that most species tended to co-occur rather than co-exclude. The proportion of positive links between species reached a maximum when the B/C ratio was around 0.5. This may indicate that a B/C ratio of 0.5 could promote microbial co-occurrence, which benefits to microbial survival. Moreover, the trend of negative interactions supported our hypothesis that the decrease in diversity under conditions of high B/C ratio results from competition.

In order to identify putative keystone taxa critical to maintaining the community structure and function (Olesen et al., 2007), nodes were classified into four categories based on their within-module (Zi) and among-module connectivity (Pi) (Deng et al., 2016): peripherals, connectors, module hubs and network hubs (see definitions in Fig. 6 legend). A majority of nodes in each network were peripherals with most of their links inside their own modules (Fig. 6). Network hubs were not identified in all groups. Multiple nodes were classified as module hubs in groups A and E but only one module hub was detected in group D (Fig. 6). This implies that the communities in groups A and E “employ” active and important keystone species that transfer information or produce intermediate metabolites in order to maintain such large and complex module structures. As such, these modules and/or networks may fall apart if these putative keystone species disappear (Albert et al., 2000; Power et al., 1996). In contrast, module structures in group D did not have an excessive reliance on particular species, but rather groups of species had similar roles and importance. Interestingly, Pi values of all nodes in group D were zero, indicating that nodes from different modules in this group had no connectivity at all.

Connectors were only detected in networks from group E. Compared with networks from group A, these connectors may play a role in communication among modules.

4. Discussion

4.1. The optimal B/C ratio for efficient system performance

The pollutant removal loads in the WWTPs were at an optimum when the B/C ratio was near 0.5. Further, properties of the microbial community with this B/C ratio (Group D) implied that conditions favored system stability and efficiency (Table S8). This demonstrates the utility of the B/C ratio as a proxy to evaluate actual performance of the WWTPs.

In WWTPs, microbial community have been considered playing determinant roles in achieving process stability and efficiency (Briones and Raskin, 2003; Falk et al., 2009) and our study was the first to uncover the underlying mechanisms. To obtain a deeper understanding of such microbial community properties, their biological significance, and possible links with system functions, we integrated favorable microbial community characteristics with

Table 1
Topological properties of the empirical pMENs of microbial communities in each group and their associated random pMENs.

No.	B/C ratio	Empirical networks							Random networks		
		St	R square of power-law	Network size	Avg. connectivity	Avg. geodesic distance	Avg. clustering coefficient	Modularity	Avg. geodesic distance ±SD	Avg. clustering coefficient ±SD	Modularity ±SD
A	<0.3	0.86	0.845	345	9.42	4.229	0.402	0.566	2.902 ± 0.021	0.090 ± 0.007	0.262 ± 0.015
B	0.3 −0.4	0.86	0.898	309	2.628	10.886	0.229	0.861	5.341 ± 0.121	0.009 ± 0.004	0.679 ± 0.008
C	0.4 −0.5	0.86	0.849	226	2.088	6.826	0.121	0.862	4.986 ± 1.278	0.012 ± 0.006	0.887 ± 0.015
D	0.5 −0.6	0.86	0.990	139	1.396	2.599	0.059	0.910	3.472 ± 0.813	0.008 ± 0.003	0.921 ± 0.013
E	>0.6	0.86	0.848	471	4.730	6.154	0.298	0.753	3.897 ± 0.032	0.019 ± 0.004	0.447 ± 0.005

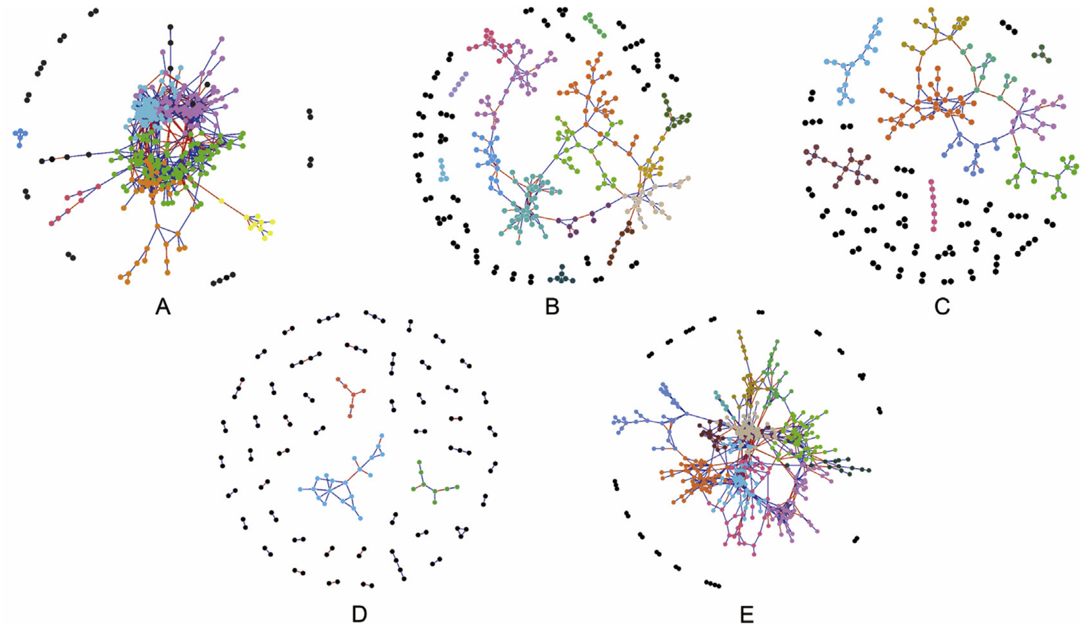

Fig. 4. Network in activated sludge at different B/C ratio based on pMENs, where nodes represent OTUs, and links between the nodes indicate significant correlation. The letters represent WWTPs grouped based on their B/C ratios, A, below 0.3; B, between 0.3 and 0.4; C, between 0.4 and 0.5; D, between 0.5 and 0.6; E, above 0.6. Modules are randomly colored at each ratio, and nodes in modules with less than 5 nodes are colored black. Positive links between nodes are colored blue and negative links are colored red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

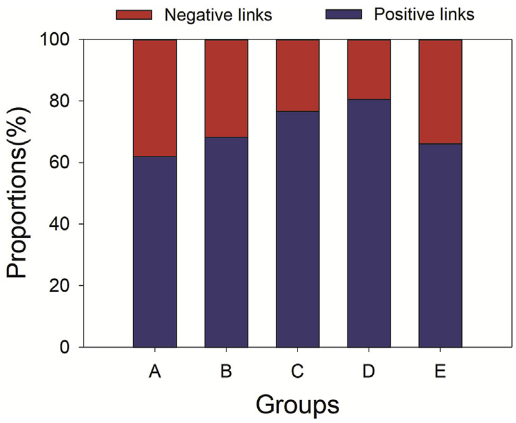

Fig. 5. Proportion of negative (red bars) and positive interactions (blue bars) in each group. The letters represent WWTPs grouped based on their B/C ratios, A, below 0.3; B, between 0.3 and 0.4; C, between 0.4 and 0.5; D, between 0.5 and 0.6; E, above 0.6. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

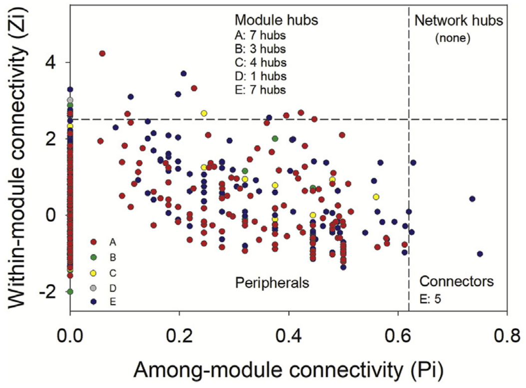

Fig. 6. Classification of nodes to identify putative keystone species within networks. Each symbol represents an OTU from the five networks with different B/C ratios and different colors represent different B/C ratio group. Module hubs are nodes with $Zi > 2.5$ but $Pi < 0.62$, whereas connectors are nodes with $Pi > 0.62$ but $Zi < 2.5$. Network hubs are nodes with $Pi > 0.62$ and $Zi > 2.5$, whereas peripherals are nodes with $Pi < 0.62$ and $Zi < 2.5$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

desirable WWTP performance. A conceptual model was then built to improve current knowledge on fundamental ecological principles in water engineered systems (Fig. 7).

4.2. Links between microbial community diversity and system performance

Overall, microbial communities with an optimal B/C ratio exhibited the highest biodiversity (including richness, evenness, and Shannon's diversity), the least influence from stochastic processes, the highest modularity value, highest average geodesic efficiency, and smallest clustering coefficient compared to those with lower or higher B/C ratios. The high diversity at the optimal B/C ratio is possibly because the microbial communities benefited from ideal nutrient availability and environmental conditions, which may support a larger number of bacteria and thus more taxa. In comparison, when the B/C ratio is lower than the optimum, microbial communities have less available nutrients and a higher concentration of toxic substances, resulting in an adverse effect on diversity. In contrast, when the B/C ratio was higher, there was likely increased competition and dominant taxa contributed to the decline of richness and evenness. This hypothesis is supported by the fact that the relative abundance of OTU 27 coming from *Paludibacter* increased three times from Group D (0.75%) to Group E (2.35%) (Fig. S4) and became the most abundant bacteria in Group E.

It has been shown that the evenness of a microbial community can promote system functions that allow the community to be resistant to environmental stress (Wittebolle et al., 2009). In

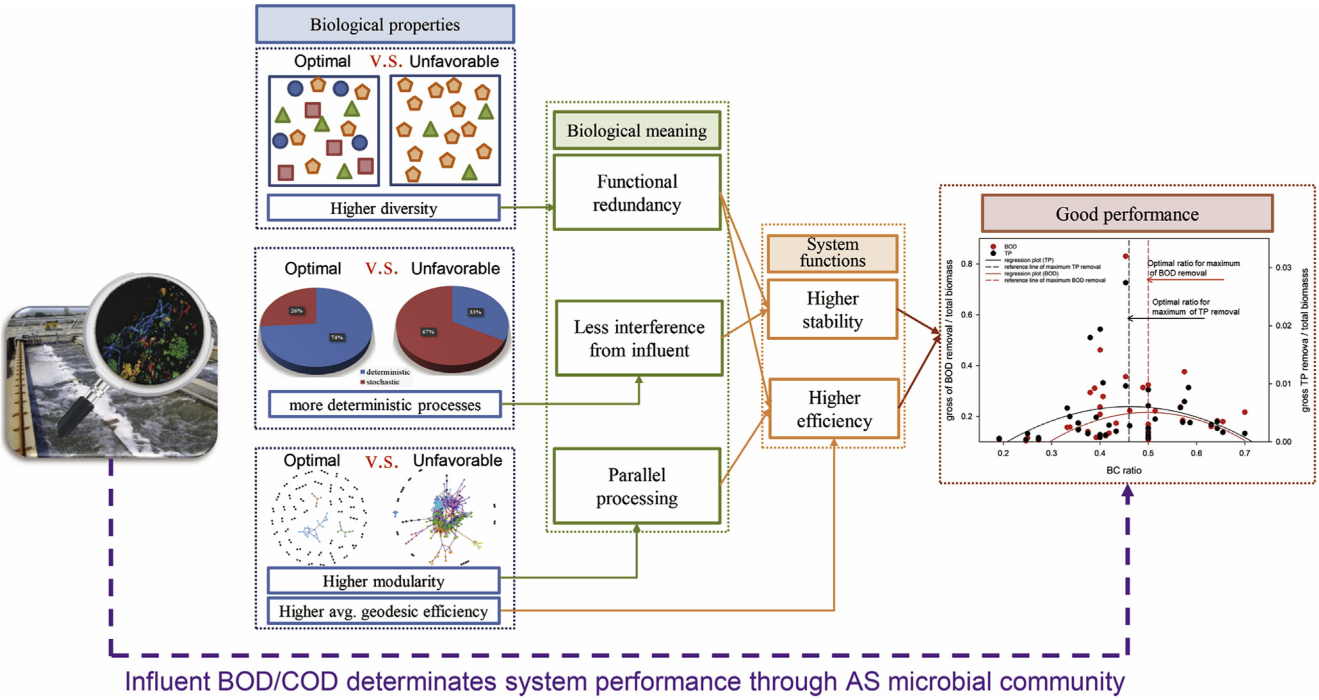

Fig. 7. Conceptual models of possible links between microbial community properties and system performance. “B/C ratio” means the ratio of BOD_5 to COD and “avg.” is abbreviation of average.

addition, highly diverse communities, containing many unique members within different trophic groups, are functionally redundant, which is also important in maintaining system function and stability in response to disturbance (Briones and Raskin, 2003; Fernandez et al., 2000; Valentin-Vargas et al., 2012). Therefore, microbial communities at the optimal B/C ratio, which have a larger number of species at an even distribution, have a better chance at maintaining a stable community structure and function in the face of changing environmental conditions (i.e. pH, substrate, and temperature changes, etc) (Loreau et al., 2001).

4.3. Links between microbial assembly and system performance

Null model results suggested that deterministic processes had more of an influence on microbial assembly when pollutant removal load was high, whereas stochastic processes dominated when influent biodegradability was lower than 0.5. These results agreed with recent study which demonstrated that reactors had better performance with dominant deterministic assembly (Zhang et al., 2019). It has been reported that influents are an important source of new microbes for the activated sludge system (McLellan et al., 2010) and continuous seeding with influent wastewater microorganisms can cause shifts in the bioreactor microbial communities (Seib et al., 2016). In addition, the vigorous and continuous mixing the AS likely disturbs the microbial community and increases the probability of microbial immigration from the influent. Consequently, community functional changes may occur as species with a given trait are replaced by other species with different traits, which may not be as beneficial to the WWTP system. As such, influent immigration is expected to play a major role in the stochastic process. Because of these potential deleterious effects, an extremely low B/C ratio should be considered a warning sign of an unstable system. In contrast, microbial communities with an optimal B/C ratio of 0.5 had the ability to resist interference from influent microorganisms and were able to maintained stability of treatment system.

4.4. Links between microbial interactions and system performance

Networks from communities with lower and higher biodegradability (a B/C ratio or less or more than 0.5) had more complex, but fewer modules, whereas those from communities with optimal biodegradability conditions (a B/C ratio around 0.5) had smaller, but more numerous modules. Based on the network parameters of each group, microbial communities with optimal B/C ratios likely had multiple functions and accomplished parallel processing, which allowed a more efficient removal of pollutants and system performance. There are several possible reasons why groups with lower and higher B/C ratios had more complex networks than those of groups with an optimal B/C ratio. Firstly, nutrient availability is probably an important driver (Henzi et al., 2009). When biodegradable sources are scarce, a wide range of microorganisms need to cooperate to consume the relatively undesirable available carbon sources in order to enhance food availability (Seo et al., 2009). For example, *Flavobacterium* sp., which is capable of metabolizing high-molecular-weight polycyclic aromatic hydrocarbons, was prevalent and abundant in group A (Kanaly and Harayama, 2000). In addition, microbes may need tighter connections to resist the harsh environmental conditions in these systems. Another possible reason is the greater influence of stochastic processes in community assembly. These communities face greater disturbance from microbial immigration and other stochastic effects and the accumulation of these types of disturbances affects small, highly integrated modules more than larger, less integrated modules (Ravasz et al., 2002). When food was plentiful, and the living environment was moderate (less toxic), food-limitation was no longer an important driver (Henzi et al., 2009) and associations among modules decreased dramatically. Under these conditions, microbes likely do not need to resist as many external disturbances, and thus are less likely to form complex modules and tight connectivity. Instead, there is less interaction and dependency between microbes, allowing the microbes to function more efficiently, since they did not need to rely on other community members to obtain required nutrients. The short average geodesic distance and high geodesic efficiency under this condition supports this point. Nevertheless, a high B/C ratio could cause excessive growth of some bacteria in AS and some species may become dominant through competition. These dominant species could consume a large amount of a given resource, like food, oxygen, or other necessary elements, resulting in more complex network modules because of negative interactions between species. Environmental filtering could also contribute to this scenario (Shi et al., 2016). An undesirable environment may promote the development of niches populated by adaptive and/or dominant taxa, which would concurrently yield decreased richness, greater interactions, greater co-variations and overall result in more complex networks. But the underlying mechanisms that how high B/C ratio affected microbial communities still need further research.

Interestingly, a decreased average clustering coefficient was accompanied by increased richness of each group ($r = -0.965$, $P = 0.008$) (Fig. S5), indicating that as the community became richer the networks became less clustered and members had fewer connections with their neighbors. But the underlying reasons need to be revealed in the future.

5. Conclusions

The importance of the microbial communities associated with AS in WWTPs in pollutant removal load and functional stability has been long recognized, but the links between microbiological properties and system performance are not well known. Evident impacts of influent biodegradability on system performance and on multiple properties of the AS microbial community were observed in our study. Both the α-diversity and proportion of deterministic processes involved in microbial community assembly showed a curvilinear relationship with the B/C ratio, and the indices related to these biological properties reached a maximum at a B/C ratio of 0.5. The functional redundancy of communities resulting from the higher α-diversity and less influence of microorganisms in the influent with more deterministic processes involved in microbial community assembly, favored functional stability of the system. The topological properties of the observed networks in the different B/C ratio groups were distinctly different. The network for the samples with optimal biodegradability conditions (B/C ratio of 0.5) had simple, but more numerous modules, more positive links between species, and fewer keystone species, all of which were beneficial to system efficiency and stability. In contrast, networks for samples with B/C ratios higher or lower than the optimum had more complex modules, fewer positive links and more keystone species, which result in less stable systems. As expected, the highest pollutant removal load was observed when the B/C ratio was around 0.5. Accordingly, B/C ratio is proposed to be used as an indispensable index to assess system performance and as an indicator of an impending process upset before function deteriorates significantly.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Albert, R., Jeong, H., Barabasi, A.L., 2000. Error and attack tolerance of complex networks. Nature 406 (6794), 378–382.

Amat, A.M., Arques, A., Garcia-Ripoll, A., Santos-Juanes, L., Vicente, R., Oller, I., Maldonado, M.I., Malato, S., 2009. A reliable monitoring of the biocompatibility of an effluent along an oxidative pre-treatment by sequential bioassays and chemical analyses. Water Res. 43 (3), 784–792.

Bolzonella, D., Innocenti, L., Pavan, P., Cecchi, F., 2001. Denitrification potential enhancement by addition of anaerobic fermentation products from the organic fraction of municipal solid waste. Water Sci. Technol. 44 (1), 187–194.

Briones, A., Raskin, L., 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Curr. Opin. Biotechnol. 14 (3), 270–276.

Caruso, T., Chan, Y.K., Lacap, D.C., Lau, M.C.Y., Mckay, C.P., Pointing, S.B., 2011. Stochastic and deterministic processes interact in the assembly of desert microbial communities on a global scale. ISME J. 5 (9), 1406–1413.

Chen, Y., Lan, S., Wang, L., Dong, S., Zhou, H., Tan, Z., Li, X., 2017. A review: driving factors and regulation strategies of microbial community structure and dynamics in wastewater treatment systems. Chemosphere 174, 173–182.

De Lucas, A., Rodriguez, L., Villasenor, J., Fernandez, F.J., 2005. Denitrification potential of industrial wastewaters. Water Res. 39 (15), 3715–3726.

Deng, Y., Jiang, Y.H., Yang, Y., He, Z., Luo, F., Zhou, J., 2012. Molecular ecological network analyses. BMC Bioinf. 13, 113.

Deng, Y., Zhang, P., Qin, Y., Tu, Q., Yang, Y., He, Z., Schadt, C.W., Zhou, J., 2016. Network succession reveals the importance of competition in response to emulsified vegetable oil amendment for uranium bioremediation. Environ. Microbiol. 18 (1), 205–218.

Dethlefsen, L., Huse, S., Sogin, M.L., Relman, D.A., 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol. 6 (11), 2383–2400.

Dumbrell, A.J., Nelson, M., Helgason, T., Dytham, C., Fitter, A.H., 2010. Relative roles of niche and neutral processes in structuring a soil microbial community. ISME J. 4 (3), 337–345.

Falk, M.W., Song, K.G., Matiasek, M.G., Wuertz, S., 2009. Microbial community dynamics in replicate membrane bioreactors - natural reproducible fluctuations. Water Res. 43 (3), 842–852.

Fernandez, A.S., Hashsham, S.A., Dollhopf, S.L., Raskin, L., Glagoleva, O., Dazzo, F.B., Hickey, R.F., Criddle, C.S., Tiedje, J.M., 2000. Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. Appl. Environ. Microbiol. 66 (9), 4058–4067.

Gentile, M.E., Jessup, C.M., Nyman, J.L., Criddle, C.S., 2007. Correlation of functional instability and community dynamics in denitrifying dispersed-growth reactors. Appl. Environ. Microbiol. 73 (3), 680–690.

Griffin, J.S., Wells, G.F., 2017. Regional synchrony in full-scale activated sludge bioreactors due to deterministic microbial community assembly. ISME J. 11 (2), 500–511.

Griffiths, B.S., Philippot, L., 2013. Insights into the resistance and resilience of the soil microbial community. FEMS Microbiol. Rev. 37 (2), 112–129.

Hallam, S.J., Mccutcheon, J.P., 2015. Microbes don't play solitaire: how cooperation trumps isolation in the microbial world. Environ. Microbiol. Rep. 7 (1), 26–28.

Henzi, S.P., Lusseau, D., Weingrill, T., van Schaik, C.P., Barrett, L., 2009. Cyclicity in the structure of female baboon social networks. Behav. Ecol. Sociobiol. 63 (7), 1015–1021.

Ibarbalz, F.M., Figuerola, E.L., Erijman, L., 2013. Industrial activated sludge exhibit unique bacterial community composition at high taxonomic ranks. Water Res. 47 (11), 3854–3864.

Ibarbalz, F.M., Orellana, E., Figuerola, E.L.M., Erijman, L., 2016. Shotgun metagenomic profiles have a high capacity to discriminate samples of activated sludge according to wastewater type. Appl. Environ. Microbiol. 82 (17), 5186–5196.

Ju, F., Xia, Y., Guo, F., Wang, Z.P., Zhang, T., 2014. Taxonomic relatedness shapes bacterial assembly in activated sludge of globally distributed wastewater treatment plants. Environ. Microbiol. 16 (8), 2421–2432.

Ju, F., Zhang, T., 2015. Bacterial assembly and temporal dynamics in activated sludge of a full-scale municipal wastewater treatment plant. ISME J. 9 (3), 683–695.

Kanaly, R.A., Harayama, S., 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J. Bacteriol. 182 (8), 2059–2067.

Kong, Y., 2011. Btrim: a fast, lightweight adapter and quality trimming program for next-generation sequencing technologies. Genomics 98 (2), 152–153.

Langenheder, S., Szekely, A.J., 2011. Species sorting and neutral processes are both important during the initial assembly of bacterial communities. ISME J. 5 (7), 1086–1094.

Loreau, M., Naeem, S., Inchausti, P., Bengtsson, J., Grime, J.P., Hector, A., Hooper, D.U., Huston, M.A., Raffaelli, D., Schmid, B., Tilman, D., Wardle, D.A., 2001. Ecology - biodiversity and ecosystem functioning: current knowledge and future challenges. Science 294 (5543), 804–808.

Luo, F., Zhong, J.X., Yang, Y.F., Scheuermann, R.H., Zhou, J.Z., 2006a. Application of random matrix theory to biological networks. Phys. Lett. A 357 (6), 420–423.

Luo, F., Zhong, J.X., Yang, Y.F., Zhou, J.Z., 2006b. Application of random matrix theory to microarray data for discovering functional gene modules. Phys. Rev. E 73 (3).

Magoc, T., Salzberg, S.L., 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27 (21), 2957–2963.

Mangkoedihardjo, S., 2006. Biodegradability improvement of industrial wastewater using hyacinth. J. Appl. Sci. 6, 1409–1414.

McIlroy, S.J., Saunders, A.M., Albertsen, M., Nierychlo, M., McIlroy, B., Hansen, A.A., Karst, S.M., Nielsen, J.L., Nielsen, P.H., 2015. MiDAS: the field guide to the microbes of activated sludge. Database (Oxford) 2015, bav062.

McLellan, S.L., Huse, S.M., Mueller-Spitz, S.R., Andreishcheva, E.N., Sogin, M.L., 2010. Diversity and population structure of sewage-derived microorganisms in wastewater treatment plant influent. Environ. Microbiol. 12 (2), 378–392.

Miura, Y., Hiraiwa, M.N., Ito, T., Itonaga, T., Watanabe, Y., Okabe, S., 2007. Bacterial community structures in MBRs treating municipal wastewater: relationship between community stability and reactor performance. Water Res. 41 (3), 627–637.

Mohan, S.V., Prasad, K.K., Rao, N.C., Bhaskar, Y.V., Babu, V.L., Rajagopal, D., Sarma, P., 2005. Biological Treatment of Low-Biodegradable Composite Chemical Wastewater Using Upflow Anaerobic Sludge Blanket (UASB) Reactor: Process Monitoring.

Montoya, J.M., Pimm, S.L., Sole, R.V., 2006. Ecological networks and their fragility. Nature 442 (7100), 259–264.

Mulkerrins, D., Dobson, A.D.W., Colleran, E., 2004. Parameters affecting biological phosphate removal from wastewaters. Environ. Int. 30 (2), 249–259.

Myers, J.A., Harms, K.E., 2011. Seed arrival and ecological filters interact to assemble high-diversity plant communities. Ecology 92 (3), 676–686.

Newman, M.E.J., 2006. Modularity and community structure in networks. Proc. Natl. Acad. Sci. U.S.A. 103 (23), 8577–8582.

Nielsen, P.H., Mielczarek, A.T., Kragelund, C., Nielsen, J.L., Saunders, A.M., Kong, Y., Hansen, A.A., Vollertsen, J., 2010. A conceptual ecosystem model of microbial communities in enhanced biological phosphorus removal plants. Water Res. 44 (17), 5070–5088.

Ofiteru, I.D., Lunn, M., Curtis, T.P., Wells, G.F., Criddle, C.S., Francis, C.A., Sloan, W.T., 2010. Combined niche and neutral effects in a microbial wastewater treatment community. Proc. Natl. Acad. Sci. U.S.A. 107 (35), 15345–15350.

Olesen, J.M., Bascompte, J., Dupont, Y.L., Jordano, P., 2007. The modularity of pollination networks. Proc. Natl. Acad. Sci. U. S. A. 104 (50), 19891–19896.

Pholchan, M.K., Baptista, J.D., Davenport, R.J., Curtis, T.P., 2010. Systematic study of the effect of operating variables on reactor performance and microbial diversity in laboratory-scale activated sludge reactors. Water Res. 44 (5), 1341–1352.

Power, M.E., Tilman, D., Estes, J.A., Menge, B.A., Bond, W.J., Mills, L.S., Daily, G., Castilla, J.C., Lubchenco, J., Paine, R.T., 1996. Challenges in the quest for keystones. Bioscience 46 (8), 609–620.

Ra, C.S., Lo, K.V., Shin, J.S., Oh, J.S., Hong, B.J., 2000. Biological nutrient removal with an internal organic carbon source in piggery wastewater treatment. Water Res. 34 (3), 965–973.

Ravasz, E., Somera, A.L., Mongru, D.A., Oltvai, Z.N., Barabasi, A.L., 2002. Hierarchical organization of modularity in metabolic networks. Science 297 (5586), 1551–1555.

Ren, D.W., Madsen, J.S., Sorensen, S.J., Burmolle, M., 2015. High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. ISME J. 9 (1), 81–89.

Samudro, G., Mangkoedihardjo, S., 2010. Review on bod, cod and bod/cod ratio: a triangle zone for toxic, biodegradable and stable levels. Int. J. Acad. Res. 2 (4).

Seib, M.D., Berg, K.J., Zitomer, D.H., 2016. Influent wastewater microbiota and temperature influence anaerobic membrane bioreactor microbial community. Bioresour. Technol. 216, 446–452.

Seo, J.S., Keum, Y.S., Li, Q.X., 2009. Bacterial degradation of aromatic compounds. Int. J. Environ. Res. Public Health 6 (1), 278–309.

Shi, S.J., Nuccio, E.E., Shi, Z.J., He, Z.L., Zhou, J.Z., Firestone, M.K., 2016. The interconnected rhizosphere: high network complexity dominates rhizosphere assemblages. Ecol. Lett. 19 (8), 926–936.

Stegen, J.C., Lin, X., Fredrickson, J.K., Konopka, A.E., 2015. Estimating and mapping ecological processes influencing microbial community assembly. Front. Microbiol. 6, 370.

Stegen, J.C., Lin, X., Konopka, A.E., Fredrickson, J.K., 2012. Stochastic and deterministic assembly processes in subsurface microbial communities. ISME J. 6 (9), 1653–1664.

Stegen, J.C., Lin, X.J., Fredrickson, J.K., Chen, X.Y., Kennedy, D.W., Murray, C.J., Rockhold, M.L., Konopka, A., 2013. Quantifying community assembly processes and identifying features that impose them. ISME J. 7 (11), 2069–2079.

Tong, J.A., Chen, Y.G., 2007. Enhanced biological phosphorus removal driven by short-chain fatty acids produced from waste activated sludge alkaline fermentation. Environ. Sci. Technol. 41 (20), 7126–7130.

Valentin-Vargas, A., Toro-Labrador, G., Massol-Deya, A.A., 2012. Bacterial community dynamics in full-scale activated sludge bioreactors: operational and ecological factors driving community assembly and performance. PLoS One 7 (8), e42524.

Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D., Schleifer, K.H., 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus Acinetobacter and its application for in situ monitoring in activated sludge. Appl. Environ. Microbiol. 60 (3), 792–800.

Wagner, M., Loy, A., 2002. Bacterial community composition and function in sewage treatment systems. Curr. Opin. Biotechnol. 13 (3), 218–227.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73 (16), 5261–5267.

Wang, X., Hu, M., Xia, Y., Wen, X., Ding, K., 2012. Pyrosequencing analysis of bacterial diversity in 14 wastewater treatment systems in China. Appl. Environ. Microbiol. 78 (19), 7042–7047.

Wang, X.H., Wen, X.H., Yan, H.J., Ding, K., Zhao, F., Hu, M., 2011. Bacterial community dynamics in a functionally stable pilot-scale wastewater treatment plant. Bioresour. Technol. 102 (3), 2352–2357.

Wittebolle, L., Marzorati, M., Clement, L., Balloi, A., Daffonchio, D., Heylen, K., De Vos, P., Verstraete, W., Boon, N., 2009. Initial community evenness favours functionality under selective stress. Nature 458 (7238), 623–626.

Wu, L., Wen, C., Qin, Y., Yin, H., Tu, Q., Van Nostrand, J.D., Yuan, T., Yuan, M., Deng, Y., Zhou, J., 2015. Phasing amplicon sequencing on Illumina Miseq for robust environmental microbial community analysis. BMC Microbiol. 15, 125.

Xia, Y., Hu, M., Wen, X., Wang, X., Yang, Y., Zhou, J., 2016. Diversity and interactions of microbial functional genes under differing environmental conditions: insights from a membrane bioreactor and an oxidation ditch. Sci. Rep. 6, 18509.

Xia, Y., Wang, X., Wen, X., Ding, K., Zhou, J., Yang, Y., Zhang, Y., 2014. Overall functional gene diversity of microbial communities in three full-scale activated sludge bioreactors. Appl. Microbiol. Biotechnol. 98 (16), 7233–7242.

Zhang, Z., Deng, Y., Feng, K., Cai, W., Li, S., Yin, H., Xu, M., Ning, D., Qu, Y., 2019. Deterministic assembly and diversity gradient altered the biofilm community performances of bioreactors. Environ. Sci. Technol. 53 (3), 1315–1324.

Zhou, J., Deng, Y., Luo, F., He, Z., Yang, Y., 2011. Phylogenetic molecular ecological network of soil microbial communities in response to elevated CO2. mBio 2 (4).