Oligotrophic Nitrification and Denitrification Bacterial Communities in a Constructed Sewage Treatment Ecosystem and Nitrogen Removal of *Delftia tsuruhatensis* NF4

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Abstract

Oligotrophic nitrifiers and denitrifiers play important roles in the removal of nitrogen from wastewater. Here, we studied the dominant bacterial populations of the sewage treatment ecosystem (STE) water from different processes and those of culture on oligotrophic heterotrophic nitrification (OHN) medium and oligotrophic aerobic denitrification (OAD) medium, using co-analysis of Illumina HiSeq DNA sequencing and traditional culture methods. The results showed that the STE water had no dominant population of oligotrophic nitrifiers or oligotrophic denitrifiers. However, after culturing on OHN medium and OAD medium, the core genera *Pseudomonas*, *Aeromonas*, and *Acinetobacter* that have the nitrogen removal capacity in oligotrophic environments, dominated in the bacterial community. The principal component analysis (PCA) showed that the bacterial community in the constructed rapid infiltration (CRI) effluent water of STE had high similarity with those of cultures on OHN medium and OAD medium, which prompt the special purification role of nitrogen in the CRI system. The sodium alginate immobilized OAD bacteria strain *Delftia tsuruhatensis* NF4 was isolated from the CRI system, with total nitrogen (TN) removal efficiency of 43.3% in sterilized STE influent water, and 60.1% in OAD medium on day three. The immobilization significantly influenced the TN and nitrate removal efficiency in OAD medium ($p < 0.05$), but not in sterilized STE influent water ($p > 0.05$). This study would lay the foundation for resource discovery of oligotrophic heterotrophic nitrifiers and aerobic denitrifiers in STE and further functional application of them on the bioremediation of wastewater.

K e y   w o r d s: oligotrophy, heterotrophic nitrifier, aerobic denitrifier, Illumina, *Delftia tsuruhatensis*

Introduction

The nutritional status, like the amounts of carbon (C) and nitrogen (N) in the environments, will regulate the nitrifiers and denitrifiers community in the system (Meyer-Reil et al. 2000). Numerous oligotrophic microorganisms have been isolated from oligotrophic environments, such as water reservoirs, deserts, and oceans (Anderson et al. 2003; Huang et al. 2015a; Montiel-González et al. 2017). Oligotrophic nitrifiers and denitrifiers have important theoretical and engineering application values for micro-polluted water treatment (Su et al. 2015). Notably, some oligotrophic denitrifiers were isolated from copiotrophic environments. The oligotrophic denitrifiers from the topsoil of agricultural field sand played a significant role in denitrification (Hashimoto et al. 2006). Lee et al. (2011) had successfully used oligotrophic nitrifiers in the bioremediation of over-fertilized land. The highly relative abundance of these bacteria has been reported in a study of wastewater treatment facilities (MacRae et al. 1991). In certain cases, the oligotrophs were a predominant population, pointing to efficient purification processes in oil refinery wastewaters (Petrovic et al. 1986; Kolarević et al. 2011). However, there is a lack of study on oligotrophic nitrifiers or denitrifiers microbiome in the sewage treatment ecosystem (STE).

The STE is composed of three purification crafts, including grille filtration, constructed rapid infiltration (CRI), and an artificial wetland in turn. The CRI

Abbreviations

OHN – oligotrophic heterotrophic nitrification
OAD – oligotrophic aerobic denitrification
CRI – a constructed rapid infiltration
STE – sewage treatment ecosystem

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is mainly constructed of river sand and coarse deposits. The dry and flood alternation of CRI provide physical, chemical, and biological reactions to remove pollutant when wastewater passes through the osmosis layers (Hunt et al. 2003; Andres et al. 2013). The nitrogen (N) in sewage is efficiently decreased in CRI and further purified in the artificial wetland (He et al. 2005; Chen et al. 2009). During the nitrification and denitrification process, ammonia is biologically oxidized to nitrate, which is then reduced to N gas using organic matter as the electron donor. Over the last decades, large efforts have been made to study biological processes developed for N removal in sewage treatment (Kuba et al. 1997; Li et al. 2017). Although currently selected aerobic denitrification bacteria have good nitrogen removal ability, most strains can only grow well at a high ratio of C to N. Hence, it is important to study the oligotrophic nitrifiers and denitrifiers microbiome in the STE.

The nutritional composition of the culture medium is important for effectively maintaining the microorganisms in nature. It has been recently reported that medium components would functionally influence the growth of microbiome communities (Li et al. 2018). Studies have found that the genus could strongly be enriched within the intestinal bacterial communities by different nutritional environments (Flint et al. 2017). The oligotrophic heterotrophic nitrifiers and oligotrophic aerobic denitrifiers are mainly known from their culturable microorganisms. We know that traditional pure-culture methods can identify only a few micro-

organisms (Ward et al. 1990). Recently, the culture-independent molecular biological methods, such as Illumina HiSeq DNA sequencing has been used besides conventional isolation techniques for the analysis of microbial community structures (Watson et al. 2014). So far, there has been no report on the bacterial community in STE using both the Illumina HiSeq DNA sequencing and traditional culture methods.

The major objectives of this study were to (1) compare the dominant community of OHN and OAD bacteria in STE water, with those of water samples after laboratory incubation on OHN medium and OAD medium; (2) evaluate the denitrification ability of an OAD strain *Delftia tsuruhatensis* NF4 in oligotrophic medium *in vitro* and STE influent water.

Experiental

Materials and Methods

Study site and sample preparation. The water samples were collected from the Feng Huang He Ergou Wastewater Treatment Ecosystem (Chengdu, China). There were three sampling sites in this ecosystem (Fig. 1), including site A: the sewage treatment ecosystem influent water (IC) (30°44′14″N, 104°4′24″E), site B: the CRI effluent water (CRIC) (30°44′15″N, 104°4′21″E), and site C: the sewage treatment ecosystem effluent water (EC) (30°44′15″N, 104°4′17″E). At each sam-
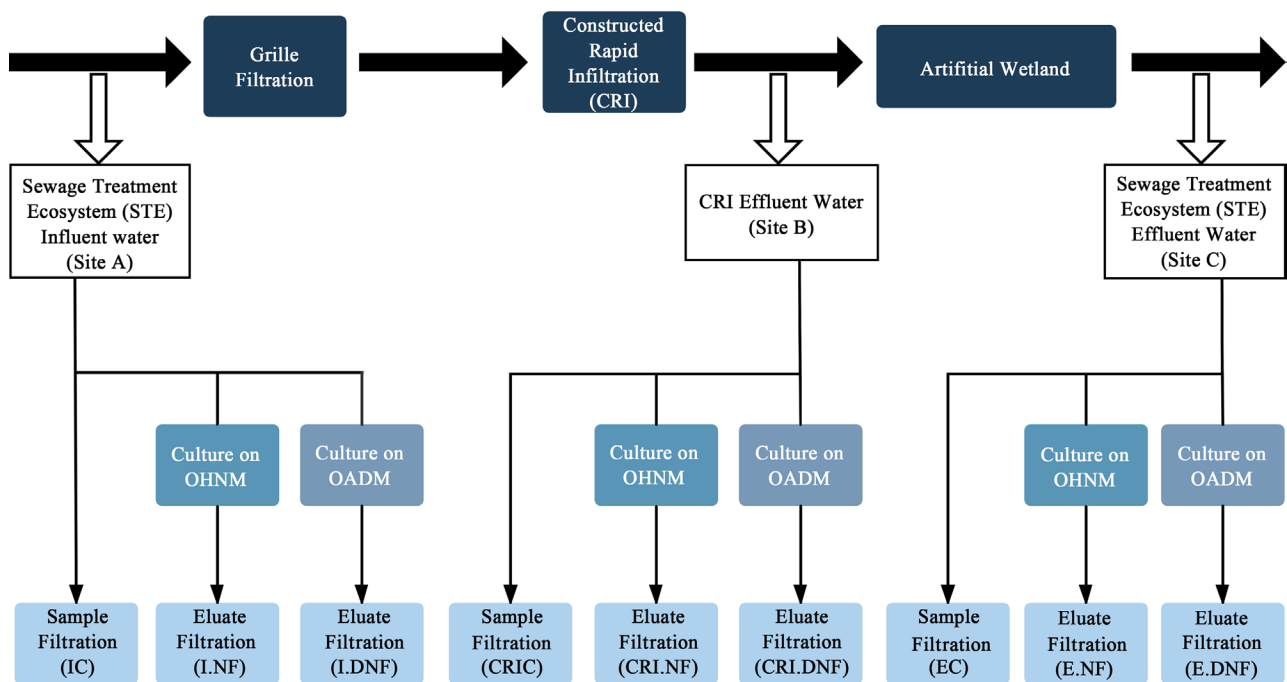

Fig. 1. Water sample sites in constructed Sewage Treatment Ecosystem (STE) of Feng Huang He Ergou River. The source of nine samples, including IC, I.NF, I.DNF, CRIC, CRI.NF, CRI.DNF, EC, E.NF, and E.DNF. The bold thick arrows indicate the water flow direction in STE, and the bold thick hollow arrows indicate the sampling site.

Table I
Sample names and processing methods in the study.

Sample source	Processing methods	Sample names
STE Influent Water	Filtration of STE influent water samples with filter membrane	IC
	Filtration of eluate from OHN medium	I.NF
	Filtration of eluate from OAD medium	I.DNF
CRI Effluent Water	Filtration of CRI effluent water samples with filter membrane	CRIC
	Filtration of eluate from OHN medium	CRI.NF
	Filtration of eluate from OAD medium	CRI.DNF
STE Effluent Water	Filtration of STE effluent water samples with filter membrane	EC
	Filtration of eluate from OHN medium	E.NF
	Filtration of eluate from OAD medium	E.DNF

OHN – oligotrophic heterotrophic nitrification; OAD – oligotrophic aerobic denitrification;
CRI – constructed rapid infiltration; STE – sewage treatment ecosystem

pling site, 1 liter of water was collected and stored in a sterile polyethylene bottle (Kangwei, Beijing, China), put in a cooler (8°C), and transferred to the laboratory within 24 h. A 0.5 liter of water was used for physical and chemical parameters determination. To compare the bacterial community of original water with that of cultured on OHN medium and OAD medium, 2 ml of the water samples were cultured on OHN medium (CH_3COONa 0.1 g/l, NH_4Cl 0.014 g/l, K_2HPO_4 0.04 g/l, $MgCl_2 \cdot 6H_2O$ 0.02 g/l, $CaCl_2$ 0.02 g/l) and OAD medium (CH_3COONa 0.1 g/l, $NaNO_3$ 0.02 g/l, K_2HPO_4 0.02 g/l, $MgCl_2 \cdot 6H_2O$ 0.01 g/l, $CaCl_2$ 0.01 g/l), separately (Huang et al. 2015b), using the same inoculum as in the water samples for filtration. After culturing at 37°C for 48 h, the lawn culture on the medium was washed with sterile water three times and collected as the washing solution. These washing solutions and the 2 ml water samples were filtered individually using filter membranes (pore size of 0.22 μm) and then stored at –20°C. The sampling preparations are shown in Table I.

Measurement of physical and chemical parameters of water. The water quality parameters were analyzed in the laboratory according to the standard procedures and methods recommended issued by the Ministry of Environmental Protection Agency, China (China Environmental Protection Bureau 1989) and the Ministry of Land and Resources of China (2015). A portable dissolved oxygen meter (JPB-607A, INESA, Shanghai, China) was used to measure dissolved oxygen concentration (DO), and a pH meter (PHS-320, Fangzhou, Chengdu, China) was used to measure pH values. The conductivity was measured with a conductivity meter (MIK-TDS210, Meacon, Hangzhou, China). The turbidity was measured at 680 nm with a spectrophotometer (UV5100, METASH, Shanghai, China). The acidic potassium permanganate oxidation method was used to measure the potassium permanganate index. Total nitrogen (TN) and total phosphorus

(TP) were also measured with the potassium persulfate oxidation method by ultraviolet spectrophotometry (UV5100, METASH, Shanghai, China) after heating (121°C, 30 min) of the water samples. The nitrate was measured with the same spectrophotometer at a UV wavelength of 220 nm and 275 nm, while the ammonia was measured with Nessler's reagent spectrophotometry at a wavelength of 540 nm and 420 nm. All analyses were carried out in triplicate.

Illumina HiSeq Sequencing. Bacterial genomic DNA of water samples and cultured samples were extracted by the CTAB method, and specific primers with Barcode, were designed for the 16S rRNA gene V3-V4 region using 341 F: 5'-ACTCCTACGGGAG-GCAGCA-3', 806 R: 5'-GGACTACJVGGGTWTCT-TAT-3'. The DNA library was constructed using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific, MI, USA). The constructed library was quantified with Qubit Fluorometric Quantification (Thermo Fisher Scientific, MI, USA). The Illumina HiSeq sequencing was performed with Thermo Fisher's Ion S5 XL (Thermo Fisher Scientific, MI, USA). The initial sequences data obtained were quality-filtered, using Cutadapt v1.9.1, http://cutadapt.readthedocs.io/en/stable/) to filter low quality reads. The clean reads were clustered into Operational Taxonomic Units (OTUs) with an identity of 97%. The MOTHUR 3.6 (Schloss et al. 2009) and SSUrRNA database (Wuyts et al. 2002) were used to perform annotation (set threshold value to 0.8–1). OTUs' abundance analysis was performed according to annotated OTUs.

Isolation and identification of an oligotrophic aerobic denitrifying strain. A 2 ml CRI effluent water sample was smeared on OAD solid medium and incubated at 37°C for 24 h. The strain NF4 with high N removal ability in denitrification medium with $NaNO_3$ as the sole N source was identified using the 16S rRNA gene sequencing.

Table II
Physical and chemical parameters of the STE influent, CRI effluent, and STE effluent water samples.

Sample Name	DO (mg/l)	Turbidity (NTU)	Conductivity (S/m)	pH	COD_{Mn} (mg/l)	TN (mg/l)	NO_3^--N (mg/l)	NH_4^+-N (mg/l)	TP (mg/l)
IC	1.47 ± 0.0	16.00 ± 3.3	0.98 ± 0.0	7.83 ± 0.0	17.46 ± 1.6	19.4 ± 0.0	0.98 ± 0.0	16.6 ± 0.5	0.92 ± 0.0
CRIC	3.92 ± 0.1	0.27 ± 0.2	0.79 ± 0.0	6.96 ± 0.3	6.46 ± 0.1	2.36 ± 0.1	0.72 ± 0.0	0.64 ± 0.0	0.30 ± 0.0
EC	5.37 ± 0.1	0.27 ± 0.2	0.77 ± 0.0	7.59 ± 0.0	4.98 ± 0.2	0.31 ± 0.0	0.10 ± 0.0	0.10 ± 0.0	0.22 ± 0.3
ANOVA	***	**	***	***	***	***	***	***	***

DO – dissolved oxygen; COD_{Mn} – permanganate index; TN – total nitrogen; TP – total phosphorus. Data showed as means ± standard deviations ($n = 3$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ represent statistical significance using One-way ANOVA

Nitrogen removal experiments. The method by Wang et al. (2012) on sodium alginate immobilization was used to immobilize *Delftia tsuruhatensis* NF4. The free cells and immobilized cells of *D. tsuruhatensis* NF4 had the same cell concentration, with OD_{600} of 0.185. The IC water was pasteurized at 100°C for 15 sec. The immobilized and free *D. tsuruhatensis* NF4 cells were cultivated in OAD medium and sterilized STE influent water separately, with inoculum 5% (V/V), shaking under a temperature of 30°C, the speed at 140 r/min, separately for three days. The NO_3^--N and TN concentrations were measured daily. The sterilized pellets were used as control. The assays were performed in triplicate ($n = 3$).

Statistical analysis. To compare the means of water quality parameters, and the nitrate and TN removal efficiency, the statistical analysis was performed using one-way ANOVA followed by a Tukey HSD post-hoc test using Rstudio (version 3.5, Rstudio Inc, San Francisco, USA). The significance level was set at $\alpha = 0.05$. Alpha diversity of Illumina HiSeq sequencing analysis, including Chao1, ACE, rarefaction plots, and PCA plots were performed using QIIME and displayed with Rstudio.

Results and Discussion

Water quality parameters. According to the Surface Water Environmental Quality Standard (GB3838-2002), the NH_4^+-N, TN, and TP concentration of STE influent water exceeded class V water quality requirements. After three steps of treatment procedure in the sewage treatment ecosystem, the concentrations of NH_4^+-N (from 16.6 ± 0.5 mg/l to 0.10 ± 0.0 mg/l), TP (from 0.92 ± 0.0 mg/l to 0.22 ± 0.3 mg/l) and COD_{Mn} (from 17.46 ± 1.6 mg/l to 4.98 ± 0.2 mg/l) declined dramatically ($p < 0.001$), indicating a significant improvement of water quality (Table II). Total N concentration significantly decreased from 19.4 ± 0.0 mg/l to 0.31 ± 0.0 mg/l after the STE ($p < 0.001$). The turbidity (from 16 ± 3.3 NTU to 0.3 ± 0.2 NTU) and conductivity (from 0.98 ± 0.0 S/m to 0.77 ± 0.0 S/m) decreased during the sewage treatment ($p < 0.001$, $p < 0.01$, separately). DO were increased significantly from 1.47 ± 0.0 μmol/l to 5.37 ± 0.1 μmol/l

($p < 0.001$), representing the improvement of water quality. Further detection of water quality in different seasons and precipitation conditions will be more helpful for the study of STE availability and stability.

Alpha diversity analysis. The rarefaction analysis indicated the relationship between the sequencing numbers and the representative OTU numbers. As shown in Fig. 2a, the rarefaction curves tend to reach a plateau around 30,000, which means that more sequencing numbers would not generate more OTUs, and the sequencing depth was sufficient (Lazarevica et al. 2009). The sequencing of STE water and STE culture samples yielded a total of 509,464 sequences recovered from all nine samples. The richness estimators (Chao1 and ACE) were calculated to analyze community richness (Table III). It was shown that after culturing of CRIC and IC samples on OHN medium and

Table III
Effective reads, OTUs, and richness estimators (Chao1 and ACE) of nine samples.

Sample Names	Effective reads	OTUs	Chao1	ACE
IC	46433	171	161.5	163.5
I.NF	47463	142	161.0	158.9
I.DNF	80100	137	139.3	141.9
CRIC	54424	131	132.6	137.9
CRI.NF	59932	118	117.8	126.0
CRI.DNF	64800	103	123.9	135.2
EC	48924	88	85.2	89.7
E.NF	50389	132	128.3	130.5
E.DNF	56999	125	121.0	122.4

IC – Filtration of sewage treatment ecosystem (STE) influent water samples with filter membrane; I.NF – Filtration of STE influent water samples eluate from oligotrophic heterotrophic nitrification (OHN) medium; I.DNF – Filtration of STE influent water samples eluate from oligotrophic aerobic denitrification (OAD) medium; CRIC – Filtration of constructed rapid infiltration (CRI) effluent water samples with filter membrane; CRI.NF – Filtration of CRI effluent water samples eluate from OHN medium; CRI.DNF – Filtration of CRI effluent water samples eluate from OAD medium; EC – Filtration of STE effluent water samples with filter membrane; E.NF – Filtration of STE effluent water samples eluate from OHN medium; E.DNF – Filtration of STE effluent water samples eluate from OAD medium
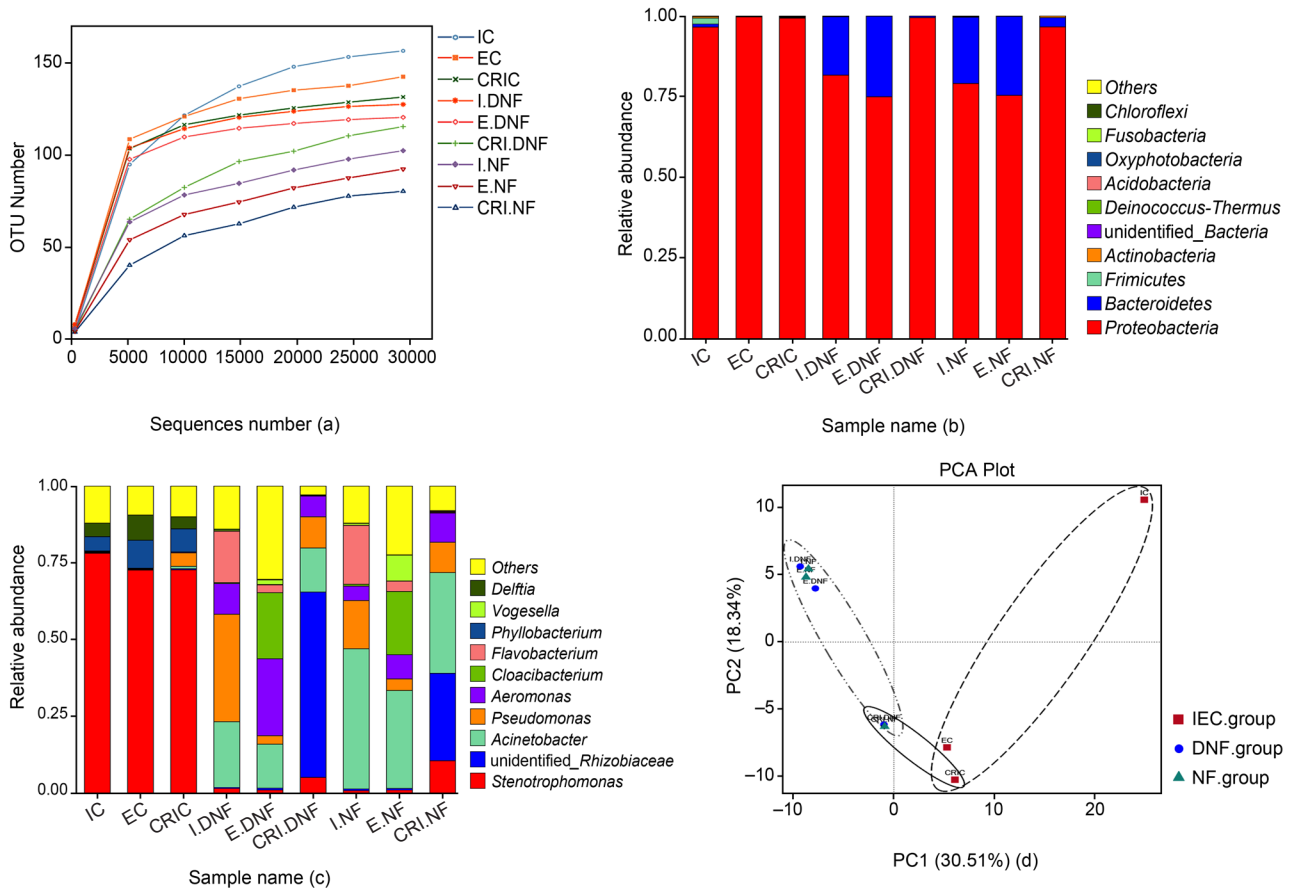
Fig. 2. 2a) The rarefaction curve representing bacterial diversity of IC, I.NF, I.DNF, CRIC, CRI.NF, CRI.DNF, EC, E.NF, and E.DNF;
2b) The phylum community abundance of nine samples, including IC, I.NF, I.DNF, CRIC, CRI.NF, CRI.DNF, EC, E.NF, and E.DNF;
2c) The genera community abundance of nine samples, including IC, I.NF, I.DNF, CRIC, CRI.NF, CRI.DNF, EC, E.NF, and E.DNF;
2d) Principal component analysis (PCA) of the bacterial community using an unweighted clustering method. The dashed line oval indicates the cluster of IC, EC, and CRIC. The dotted dashed line oval indicates the cluster of NF and DNF groups. The solid line oval indicates the cluster of CRI.NF, CRI.DNF and CRIC, which represent a closer cluster of community structures.

IC – Filtration of sewage treatment ecosystem (STE) influent water samples with filter membrane; I.NF – Filtration of STE influent water samples eluate from oligotrophic heterotrophic nitrification (OHN) medium; I.DNF – Filtration of STE influent water samples eluate from oligotrophic aerobic denitrification (OAD) medium; CRIC – Filtration of constructed rapid infiltration (CRI) effluent water samples with filter membrane; CRI.NF – Filtration of CRI effluent water samples eluate from OHN medium; CRI.DNF – Filtration of CRI effluent water samples eluate from OAD medium; EC – Filtration of STE effluent water samples with filter membrane; E.NF – Filtration of STE effluent water samples eluate from OHN medium; E.DNF – Filtration of STE effluent water samples eluate from OAD medium

OAD medium separately, the richness of bacterial communities decreased. This might be due to oligotrophic medium screening. Buton et al. (1993) had found that the environment of poor nutrition, like low organic carbon content, would limit the bacterial populations. Inversely, the bacterial community richness of cultures on OHN medium and OAD medium was higher than those of STE effluent water samples. After treating with grille filtration, CRI and artificial wetland, the potassium permanganate index of EC decreased significantly (Table II, $p < 0.001$), which may have led to lower bacterial diversity. It implied that after growth on medium, low content of some bacteria from EC was reproduced on the medium to reach the measurable level. These findings are consistent with the observed OTUs, Chao1, and ACE richness estimators. Cho and Giovannoni (2004) found that none of the 44 strains of *Proteobacte-*

ria from ocean samples could form colonies when they were inoculated on nutritional agar medium, but seven strains of them could grow in the oligotrophic medium. Therefore, the culture of bacteria on the oligotrophic medium might lead to a higher or lower diversity of the bacterial community, depending on the different sample sources.

Taxa distribution at the phylum level. *Proteobacteria* was the most abundant phylum in all samples. *Proteobacteria* are the prominent phylum in pharmaceutical, petroleum refinery, wastewater treatment plants, steel industrial wastewater, and sewage (Ma et al. 2015; Zhang et al. 2019). The CRI.NF and CRI.DNF communities, with *Proteobacteria* occupying 96.6% and 99.5% individually, were similar to CRI bacterial communities (with *Proteobacteria* occupying 99.3%). On the contrary, the relative abundance of *Proteobacteria* in I.NF

and I.DNF were 79.1% and 81.8% separately, which was lower than in IC (96.6%). The same trend was found in the E.NF and E.DNF, with *Proteobacteria* occupying 75.4% and 74.9% respectively, compared to 99.7% in EC (Fig. 2b). In E.NF and E.DNF, *Bacteroidetes* increased to the relative abundance of 18.1–25% after culture on OHN medium and OAD medium, compared with only 0.3–0.4% before. *Bacteroidetes* from aquatic sediment has been studied for its nitrogen removal ability (Xie et al. 2013). *Actinobacteria*, *Deinococcus-Thermus*, *Acidobacteria*, *Oxyphotobacteria*, *Fusobacteria*, *Chloroflexi*, unidentified_*Bacteria*, and other phylum total represent only 0.01–0.74% of the whole bacterial community in all samples. From the above results, CRI.NF, CRI.DNF and CRIC showed higher similarity community structure at the phylum level, compared to those samples from EC and IC. To our knowledge, the main studies of OHN and OAD bacteria are mainly focused on phylum *Proteobacteria, Actinobacteria,* and *Firmicutes* (Huang et al. 2013; Srivastava et al. 2016; Zhou et al. 2016; Zhao et al. 2017; Wang et al. 2019).

Core genera in all the samples. At the genus level, *Stenotrophomona*s and *Phyllobacterium* dominated, being 80.1–82.7% (average of 81.5%) of total populations of IC, CRIC, and EC water samples (Fig. 2c). *Stenotrophomona*s is responsible for heterotrophic nitrification and aerobic denitrification (Young et al. 2014) and its role in semi-anaerobic denitrification was found from an industrial wastewater study (Yu et al. 2009). *Phyllobacterium* has been mainly studied for its nitrogen-fixing function (Gonzalez-Bashan et al. 2000), while limited studies on its role in nitrification and denitrification were reported. *Delftia* was another one of the top three populations in IC and EC, accounting for 4.4% and 8.2%, separately. *Delftia* is a promising OAD bacterium, which has been isolated from the reservoir sediment (Zhang and Zhou 2016). *Pseudomonas* accounted for 4.4% of the assigned sequences in CRIC. *Pseudomonas* had characteristics of aerobic denitrification that utilizes ammonium and nitrate simultaneously under the oligotrophic niche (Zhu et al. 2012). *Acinetobacter*, *Aeromonas*, *Pseudomonas*, and unidentified_*Rhizobiaceae* had an average abundance of 0.5–5.7% in IC, CRIC, and EC samples, and the population of these bacteria dramatically increased after subsequent culture on medium. Based on the above results, the water samples from STE had relative dominance of heterotrophic nitrifiers and aerobic denitrifiers, while the oligotrophic aerobic denitrifers were not dominant. The top three relatively abundant bacterial populations were summarized at phylum, class, order, family and genus level in Table IV.

After incubation on OHN medium, *Acinetobacter* and *Pseudomonas* population increased significantly and became the dominant genera in I.NF and CRI.NF, with average occupation of 52.1% (42.7–61.4%) (Fig. 2c; Table IV). *Acinetobacter* participates in N removal and heterotrophic nitrification at low nutrient conditions (Su et al. 2015). Another one of the top three populations in I.NF was *Flavobacterium* (Table IV). The heterotrophic nitrification and aerobic denitrification of *Flavobacterium* were found in saline sewage in the constructed wetland (Fu et al. 2018) and in an oligotrophic freshwater lake (Nam et al. 2017). The unidentified_*Rhizobiaceae* was another one of the top three genera in CRI.NF and E.NF, which occupied 28.5% and 22.5% individually (Table IV). Members of *Rhizobiaceae* were studied for their nitrification and aerobic denitrification performance (Hamdi and Tewfik 1969; Okada et al. 2005; Li et al. 2018). Some *Rhizobiaceae* were isolated in oligotrophic environments (Tomczyk-Żak and Zielenkiewicz 2015). The unidentified_*Rhizobiaceae* might play important roles in sewage treatment processes and do require comprehensive evaluation. *Aeromonas,* accounting for 7.9% occupation, was another one of the top three populations in E.NF (Table IV). *Aeromonas* has been studied for ammonia removal from the oligotrophic aquatic system through heterotrophic nitrification and aerobic denitrification (Velez et al. 2018). Therefore, using the OHN medium, the bacterial communities tend to concentrate on OHN and OAD bacteria, including *Pseudomonas, Acinetobacter* and *Aeromonas,* etc., not only OHN bacterial flora.

After culturing on OAD medium, *Pseudomonas*, *Acinetobacter* and *Flavobacterium* were the top three dominant in I.DNF, occupying 73.2% of the total population, which were also dominant in I.NF (with 80.6% occupation) (Fig. 2c; Table IV). *Acinetobacter, Pseudomonas*, and unidentified_*Rhizobiaceae* were the top three populations in CRI.DNF, with the occupation of 84.9% was also dominant in CRI.NF (71.2% occupation) (Fig. 2c; Table IV). *Acinetobacter* and *Aeromonas* were the members of the top three populations in E.DNF, which was similar to E.NF, with 39.4% and 39.8% individually. Another one of the top three genera in E.DNF was *Cloacibacterium*. *Cloacibacterium* had denitrification ability under aerobic conditions (Huang et al. 2014) and was reported for its contribution to nitrification by few studies (He et al. 2019). From these results, using OAD medium, the communities tend to focus on both OHN and OAD bacterial communities, not just OAD bacterial bacteria. Meanwhile, the high similarity between communities on OHN and OAD media implied that bifunctional flora was ubiquitous after screening with these two mediums. The results were consistent with PCA (Fig. 2d).

Oligotrophs are not restricted to certain microbial groups or specialized genera. The *Sphingopyxis alaskensis*, *Caulobacter* spp., *Rhodococcus erythropolis*, *Staphylococcus citreus*, *Bacillus megaterium*, *Proteus vulgaris*,

Table IV

The relative abundance of the top three populations at different levels of the taxonomy.

Tax-onomy	IC			CRIC			EC		
	Top1 (%)	Top2 (%)	Top3 (%)	Top1 (%)	Top2 (%)	Top3 (%)	Top1 (%)	Top2 (%)	Top3 (%)
Phylum	*Proteobacteria* (96.6)	*Firmicutes* (17.4)	*Bacteroidetes* (0.96)	*Proteobacteria* (99.3)	*Firmicutes* (0.17)	*Bacteroidetes* (0.16)	*Proteobacteria* (99.7)	*Bacteroidetes* (0.14)	*Firmicutes* (0.09)
Class	*Gammaproteobacteria* (83.8)	*Alphaproteobacteria* (12.8)	*Clostridia* (0.97)	*Gammaproteobacteria* (83.7)	*Alphaproteobacteria* (15.6)	*Bacteroidia* (0.16)	*Gammaproteobacteria* (81.7)	*Alphaproteobacteria* (18.0)	*Bacteroidia* (0.13)
Order	*Xanthomonadales* (78.0)	*Sphingomonadales* (6.1)	*Rhizobiales* (5.2)	*Xanthomonadales* (72.7)	*Rhizobiales* (7.8)	*Pseudomonadales* (5.8)	*Xanthomonadales* (72.7)	*Rhizobiales* (7.8)	*Sphingomonadales* (5.8)
Family	*Xanthomonadaceae* (78.0)	*Rhizobiaceae* (4.9)	*Others* (11.4)	*Xanthomonadaceae* (72.6)	*Others* (8.5)	*Rhizobiaceae* (7.8)	*Xanthomonadaceae* (72.7)	*Rhizobiaceae* (7.8)	Others (8.5)
Genus	*Stenotrophomonas* (78.0)	*Sphingomomas* (12.1)	*Phyllobacterium* (4.7)	*Stenotrophomonas* (72.7)	*Phyllobacterium* (10.0)	*Sphingomomas* (7.5)	*Stenotrophomonas* (72.6)	*Sphingomomas* (9.5)	*Delftia* (9.1)

IC – Filtration of sewage treatment ecosystem (STE) influent water samples with filter membrane; CRIC – Filtration of constructed rapid infiltration (CRI) effluent water samples with filter membrane; EC – Filtration of STE effluent water samples with filter membrane

Table IV (Continue)

Tax-onomy	I.NF			CRI.NF			E.NF		
	Top1 (%)	Top2 (%)	Top3 (%)	Top1 (%)	Top2 (%)	Top3 (%)	Top1 (%)	Top2 (%)	Top3 (%)
Phylum	*Proteobacteria* (79.0)	*Bacteroidetes* (20.6)	*Firmicutes* (0.09)	*Proteobacteria* (96.6)	*Bacteroidetes* (2.9)	*Actinobacteria* (0.12)	*Proteobacteria* (75.4)	*Bacteroidetes* (24.5)	*Firmicutes* (0.06)
Class	*Gammaproteobacteria* (77.0)	*Bacteroidia* (20.6)	*Alphaproteobacteria* (2.0)	*Gammaproteobacteria* (66.2)	*Alphaproteobacteria* (30.3)	*Bacteroidia* (2.9)	*Gammaproteobacteria* (73.8)	*Bacteroidia* (24.5)	*Alphaproteobacteria* (1.6)
Order	*Pseudomonadales* (61.4)	unidentified_*Gammaproteobacteria* (6.7)	*Aeromonadales* (5.7)	*Pseudomonadales* (42.7)	*Rhizobiales* (28.6)	*Aeromonadales* (11.5)	*Pseudomonadales* (35.6)	*Flavobacteriales* (24.0)	unidentified_*Gammaproteobacteria* (18.3)
Family	*Moraxellaceae* (45.7)	*Flavobacteriaceae* (19.1)	*Pseudomonadaceae* (15.8)	*Moraxellaceae* (32.8)	*Rhizobiaceae* (28.7)	*Xanthomonadaceae* (10.5)	*Moraxellaceae* (31.9)	unidentified_*Flavobacteriales* (20.6)	*Burkholderiaceae* (9.9)
Genus	*Acinetobacter* (46.7)	*Flavobacterium* (20.5)	*Pseudomonas* (15.8)	*Acinetobacter* (32.1)	unidentified_*Rhizobiaceae* (28.5)	*Pseudomonas* (10.5)	*Acinetobacter* (31.9)	unidentified_*Rhizobiaceae* (22.5)	*Aeromonas* (20.6)

I.NF – Filtration of STE influent water samples eluate from oligotrophic heterotrophic nitrification (OHN) medium; CRI.NF – Filtration of CRI effluent water samples eluate from OHN medium; E.NF – Filtration of STE effluent water samples eluate from OHN medium

Table IV (Continue)

Tax-onomy	I.DNF			CRI.DNF			E.DNF		
	Top1 (%)	Top2 (%)	Top3 (%)	Top1 (%)	Top2 (%)	Top3 (%)	Top1 (%)	Top2 (%)	Top3 (%)
Phylum	*Proteobacteria* (81.7)	*Bacteroidetes* (18.1)	*Firmicutes* (0.05)	*Proteobacteria* (99.5)	*Bacteroidetes* (0.4)	*Actinobacteria* (0.07)	*Proteobacteria* (74.9)	*Bacteroidetes* (25.0)	*Firmicutes* (0.05)
Class	*Gammaproteobacteria* (80.4)	*Bacteroidia* (18.1)	*Alphaproteobacteria* (1.2)	*Alphaproteobacteria* (60.9)	*Gammaproteobacteria* (38.6)	*Bacteroidia* (0.39)	*Gammaproteobacteria* (72.9)	*Bacteroidia* (25.0)	*Alphaproteobacteria* (1.9)
Order	*Pseudomonadales* (56.5)	*Flavobacteriales* (17.0)	*Aeromonadales* (11.6)	*Rhizobiales* (60.5)	*Pseudomonadales* (24.5)	*Aeromonadales* (7.7)	*Aeromonadales* (29.6)	*Flavobacteriales* (24.1)	*Pseudomonadales* (17.1)
Family	*Pseudomonadaceae* (35.1)	*Moraxellaceae* (21.4)	*Flavobacteriaceae* (16.7)	*Rhizobiaceae* (60.5)	*Moraxellaceae* (14.3)	*Pseudomonadaceae* (10.2)	*Aeromonadaceae* (25.1)	unidentified_*Flavobacteriales* (21.5)	*Moraxellaceae* (14.3)
Genus	*Pseudomonas* (34.8)	*Acinetobacter* (20.3)	*Flavobacterium* (16.7)	unidentified_*Rhizobiaceae* (61.4)	*Acinetobacter* (12.7)	*Pseudomonas* (6.1)	*Aeromonas* (22.5)	*Cloacibacterium* (20.9)	*Acinetobacter* (16.8)

I.DNF – Filtration of STE influent water samples eluate from oligotrophic aerobic denitrification (OAD) medium; CRI.DNF – Filtration of CRI effluent water samples eluate from OAD medium; E.DNF – Filtration of STE effluent water samples eluate from OAD medium

Lactobacillus lactis, Pseudomonas aeruginosa, Aeromonas aerophile, Acinetobacter sp., etc. have been shown to grow in carbon deficient medium (Jain et al. 1995; Giovannoni and Stingl 2007; Huang et al. 2013; Matsuoka et al. 2018; Wilhelm et al. 2018). In our study, *Pseudomonas, Aeromonas, Acinetobacter* occupied with a dominance of OHN and OAD bacterial communities with 66.1% in I.NF, 56.4% in I.DNF, 42.1% in E.DNF, and 43.5% in E.NF. These three genera have been studied for their roles in OHN and OAD processes (Zhu et al. 2012; Su et al. 2015; Velez et al. 2018). For CRI.NF, these three genera occupied 52.2% and unidentified *Rhizobiaceae* also occupied an abundance of 28.5% (Table IV). In CRI.DNF samples, these three genera comprised 31.3%, and unidentified *Rhizobiaceae* occupied the dominant of 60.4%. It showed that culturing the STE water on OHN and OAD media resulted in the OHN and OAD bacterial communities becoming more concentrated.

Principal Component Analysis (PCA). PCA revealed that the bacterial community structures varied significantly after the culture of EC and IC samples on OHN medium and OAD medium. Principle component 1 (PC1) and Principle component 2 (PC2) explained 39.51% and 18.34% of the total variance, respectively. There was less similarity between IC, EC, and CRIC (IEC group) (Fig. 2d dashed line oval), which indicated the discrepant bacterial communities among STE influent, CRI effluent and STE effluent water samples. It also showed closer clusters of NF and DNF groups (Fig. 2d dotted dashed line oval) and this was consistent with the summarized populations (Table IV), indicating the similarity of bacterial community between I.DNF and I.NF, CRI.DNF and CRI.NF, E.DNF and E.NF. Interestingly, the bacterial community of the CRI.NF, CRI.DNF, and CRIC showed the

most similarity of the bacterial community, compared to the other two clusters above, which was well characterized with their closer distribution (Fig. 2d solid line oval). In CRI, ammonia was absorbed and undergoes nitrification and further denitrification (Wang et al. 2006; Baram et al. 2012). We speculated that CRI's special structure might provide a natural enrichment for nitrifiers and denitrifiers, which provide environments for the concentration of OAD and OHN bacterial communities in nature.

The nitrogen removal efficiency. The nitrate removal efficiency by immobilized and free *D. tsuruhatensis* NF4 in sterilized STE influent water were 94.2% (from 1.31 ± 0.2 mg/l to 0.08 ± 0.03 mg/l) and 65.9% (from 1.13 ± 0.6 mg/l to 0.39 ± 0.2 mg/l) respectively on day 3 (Fig. 3), while those in OAD medium were 99.4% (from 3.21 ± 0.3 mg/l to 0.02 ± 0.02 mg/l) and 58.4% (from 3.09 ± 0.02 mg/l to 1.28 ± 0.3 mg/l), respectively (Fig. 4). The TN removal efficiency by immobilized and free *D. tsuruhatensis* NF4 was lower than those of nitrate in two types of water (Fig. 3 and Fig. 4). The TN removal efficiency by immobilized cells in sterilized STE influent water was 43.3% (from 19.98 ± 3.3 mg/l to 11.33 ± 2.0 mg/l), compared with those of 26.0% (from 21.07 ± 1.8 mg/l to 15.61 ± 2.6 mg/l) of free cells. In OAD medium, the TN removal efficiency by immobilized cells was 60.1% (from 3.31 ± 0.5 to 1.32 ± 0.5 mg/l), compared with those of 23.0% (3.96 ± 0.6 mg/l to 3.05 ± 0.2 mg/l) of free cells. The nitrate and TN concentrations of the controls were not decreased. With one-way ANOVA, the immobilization had significantly influenced the nitrate and TN removal in OAD medium ($p < 0.05$), while no significant in sterilized STE influent water ($p > 0.05$). The results showed that the immobilized *D. tsuruhatensis* NF4 had higher denitrification
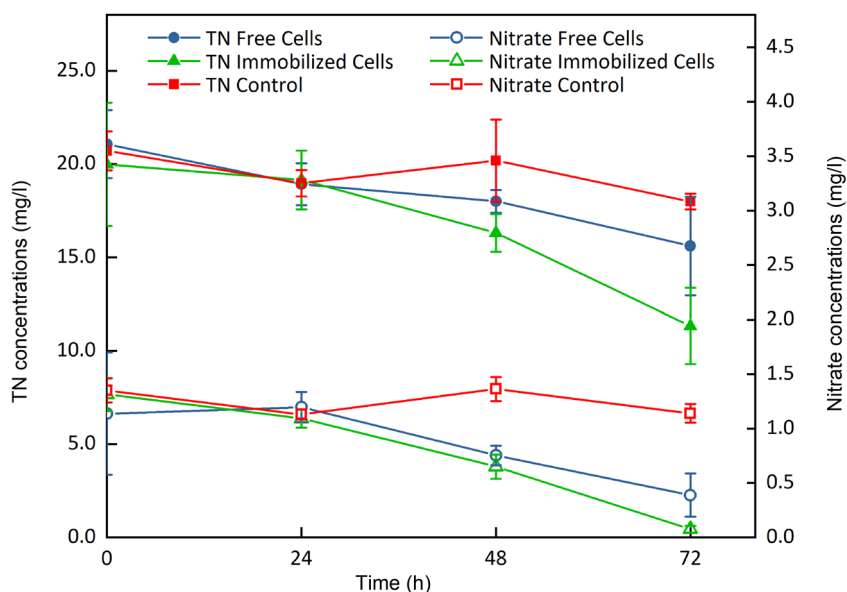

Fig. 3. Nitrate and TN removal efficiency with *Delftia tsuruhatensis* NF4 in sterilized STE influent water.
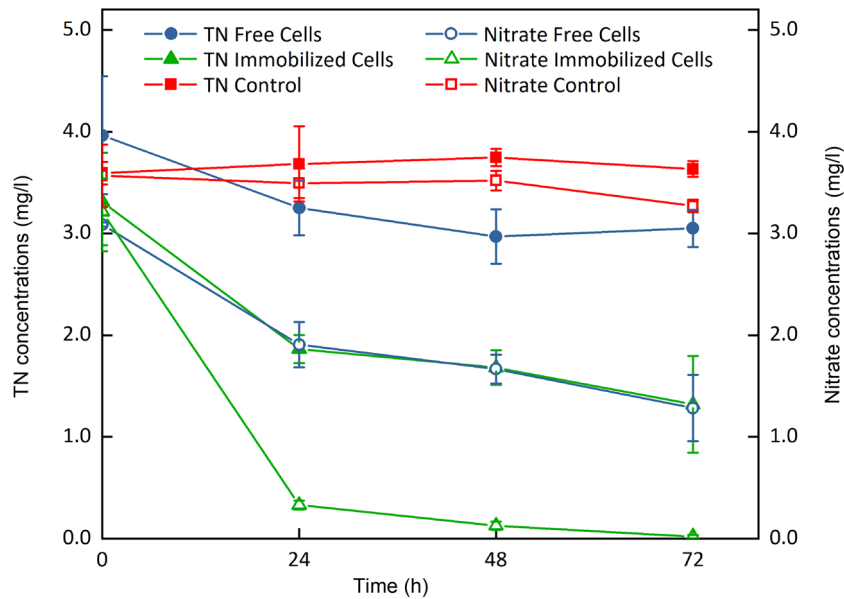
Fig. 4. Nitrate and TN removal efficiency with *Delftia tsuruhatensis* NF4 in the oligotrophic aerobic denitrification medium.

efficiency than free cells in the two different water environments (Fig. 3 and Fig. 4). The contribution of denitrification to TN and nitrate removal from water has been investigated by other scientists (Gao et al. 2010; Wang et al. 2019). Sun et al. (2015) studied bioceramic immobilized aerobic denitrifier *Pseudomonas stutzeri* T13, and found that the nitrate removal rate of immobilized strain was higher than the free state under oligotrophic conditions. It has also been reported that NO_2^--N removal can be enhanced by immobilized *Bacillus megaterium* (Gao et al. 2018). Oligotrophic aerobic denitrifiers have been used to study the N removal rate in both sterilized and unsterilized reservoir water (Huang et al. 2015a), while the N removal of oligotrophic aerobic denitrifiers in both wastewater and oligotrophic medium had not been studied before. Further studies of different oligotrophic aerobic denitrifiers will help to prompt wastewater treatment *in situ*.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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