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The Stochastic Assembly of *Nitrobacter winogradskyi*-Selected Microbiomes with Heterotrophs from Sewage Sludge or Grassland Soil

Hendrikus J. Laanbroek,[a,b] Noriko A. Cassman,[a] Rosalinde M. Keijzer,[a] Eiko E. Kuramae[a,b]

[a]Microbial Ecology Department, Netherlands Institute of Ecology (NIOO), Wageningen, The Netherlands
[b]Ecology and Biodiversity, Institute of Environmental Biology, Utrecht University, Utrecht, The Netherlands

ABSTRACT Chemolitho-autotrophic microorganisms like the nitrite-oxidizing *Nitrobacter winogradskyi* create an environment for heterotrophic microorganisms that profit from the production of organic compounds. It was hypothesized that the assembly of a community of heterotrophic microorganisms around *N. winogradskyi* depends on the ecosystem from which the heterotrophs are picked. To test this hypothesis, pure cultures of *N. winogradskyi* were grown in continuously nitrite-fed bioreactors in a mineral medium free of added organic carbon that had been inoculated with diluted sewage sludge or with a suspension from a grassland soil. Samples for chemical and 16S rRNA gene amplicon analyses were taken after each volume change in the bioreactor. At the end of the enrichment runs, samples for shotgun metagenomics were also collected. Already after two volume changes, the transformations in community structure became less dynamic. The enrichment of heterotrophs from both sewage and soil was highly stochastic and yielded different dominant genera in most of the enrichment runs that were independent of the origin of the inoculum. Hence, the hypothesis had to be refuted. Notwithstanding the large variation in taxonomic community structure among the enrichments, the functional compositions of the communities were statistically not different between soil- and sludge-based enrichments.

IMPORTANCE In the process of aerobic nitrification, nitrite-oxidizing bacteria together with ammonia-oxidizing microorganisms convert mineral nitrogen from its most reduced appearance, i.e., ammonium, into its most oxidized form, i.e., nitrate. Because the form of mineral nitrogen has large environmental implications, nitrite-oxidizing bacteria such as *Nitrobacter winogradskyi* play a central role in the global biogeochemical nitrogen cycle. In addition to this central role, the autotrophic nitrite-oxidizing bacteria also play a fundamental role in the global carbon cycle. They form the basis of heterotrophic food webs, in which the assimilated carbon is recycled. Little is known about the heterotrophic microorganisms that participate in these food webs, let alone their assembly in different ecosystems. This study showed that the assembly of microbial food webs by *N. winogradskyi* was a highly stochastic process and independent of the origin of the heterotrophic microorganisms, but the functional characteristics of the different food webs were similar.

KEYWORDS *Nitrobacter winogradskyi*, nitrite oxidation, heterotrophic microorganisms, microbiome, soil, sewage sludge, sewage

It is common knowledge that photolitho-autotrophic algae as primary producers are at the root of complex food webs, which further consist of heterotrophic consumers. As pointed out by Cole (1) in his classic review on interactions between algae and heterotrophic bacteria in aquatic ecosystems, several mechanisms may be involved in the transfer of organic matter from the algae to the heterotrophic microorganisms: (i) microbes may parasitize on algal cells, (ii) microbes might profit from the lysis of death algal cells, and (iii)

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Address correspondence to Hendrikus J. Laanbroek, R.Laanbroek@nioo.knaw.nl.

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microbes may consume carbon released during algal cell growth. This process in which organic carbon is transferred from algae to heterotrophic microorganisms is also known as the microbial loop (2). Microbial food webs are not only supported by photolitho-autotrophic algae but may also be sustained by chemolitho-autotrophic microorganisms that grow at the expense of reduced inorganic compounds. The carrying capacity of a chemolitho-autotrophic community can be large. For example, members of the chemolitho-autotrophic, nitrite-oxidizing *Nitrospinae* phylum fix 15 to 45% of the inorganic carbon in the dark ocean (3). In addition, Kindaichi et al. (4) showed that a chemolitho-autotrophic nitrifying community, which comprised both ammonia- and nitrite-oxidizing bacteria, supported an equally large heterotrophic community in a carbon-limited biofilm fed with only ammonium. Although less is known about the food web rooted in chemolitho-autotrophs, the mechanisms of carbon transfer are likely the same as observed in food webs that are supported by photolitho-autotrophs, i.e., parasitism, and consumption of organic compounds released from lysed or active cells. In a follow-up of the study of Kindaichi et al. (4) mentioned above, the same authors showed by application of microautoradiography combined with fluorescence *in situ* hybridization that the heterotrophic community used both dead biomass and metabolites of the chemolitho-autotrophic nitrifying community (5). Functions that facilitate the processing of carbon compounds from chemolitho-autotrophic microorganisms should be present in every ecosystem where these chemolitho-autotrophs are active as these microorganisms shape their chemical environment by producing organic compounds from carbon dioxide. However, due to deterministic factors, such as species traits, interspecies interactions, and environmental conditions, as well as stochastic factors, such as colonization, extinction, and speciation, each ecosystem with its intrinsic environmental characteristics will have its specific community of microbial species (6). Hence, it is hypothesized that the assembly of a new community of heterotrophic, escorting, or satellite microorganisms around a chemolitho-autotrophic bacterium depends on the ecosystem from which the heterotrophs are picked.

To test this hypothesis, we inoculated pure cultures of *Nitrobacter winogradskyi* that are actively growing in strictly nitrite-fed bioreactors with diluted sludge from a sewage treatment plant for domestic wastewater or with a suspension from a calcareous grassland soil. 16S rRNA gene amplicon sequence analysis was applied to follow the assembly of communities of heterotrophic, escorting microorganisms around *N. winogradskyi* during the enrichment runs. Shotgun metagenomics was employed to establish the sets of functional genes that emerge in the different enrichment cultures. Because actively growing and CO_2-assimilating *N. winogradskyi* is the only source of carbon in the different enrichment runs, the pool of organic compounds produced should be independent of the ecosystem from which the escorting communities were derived and so should be the sets of emerging functional genes. Continuously fed bioreactors are an excellent choice for studying interspecies interactions as nonparticipating species that are present in the inoculum will leave the bioreactor in the absence of a suitable carbon and energy source. Only heterotrophic microorganisms that grow at the expense of organic carbon produced by *N. winogradskyi* will survive the dilution in the bioreactor with a freshly applied mineral medium. At the same time, active *N. winogradskyi* cells will maintain the concentration of nitrite in the bioreactors below the toxicity level of most bacteria (7). The carbon compounds originally present in the inoculum will be diluted with the same washout rate. In our experiments, the enrichments were finished after 10 volume changes. At this time, 0.0005% of the microbial cells that were originally present in the slurries will still be present, while the rest of the heterotrophic microbial community will consist of microbes growing at the expense of carbon compounds derived from the chemolitho-autotrophs. The same percentage applies to the carbon compounds that were originally present in the soil and the sludge.

RESULTS

Bioreactor performance. In the first series of enrichments with runs Sewage 1, Sewage 2, and Soil 1, it took several volume changes before the ammonium concentrations in the enrichment culture reached their minimum values being on average 0.04

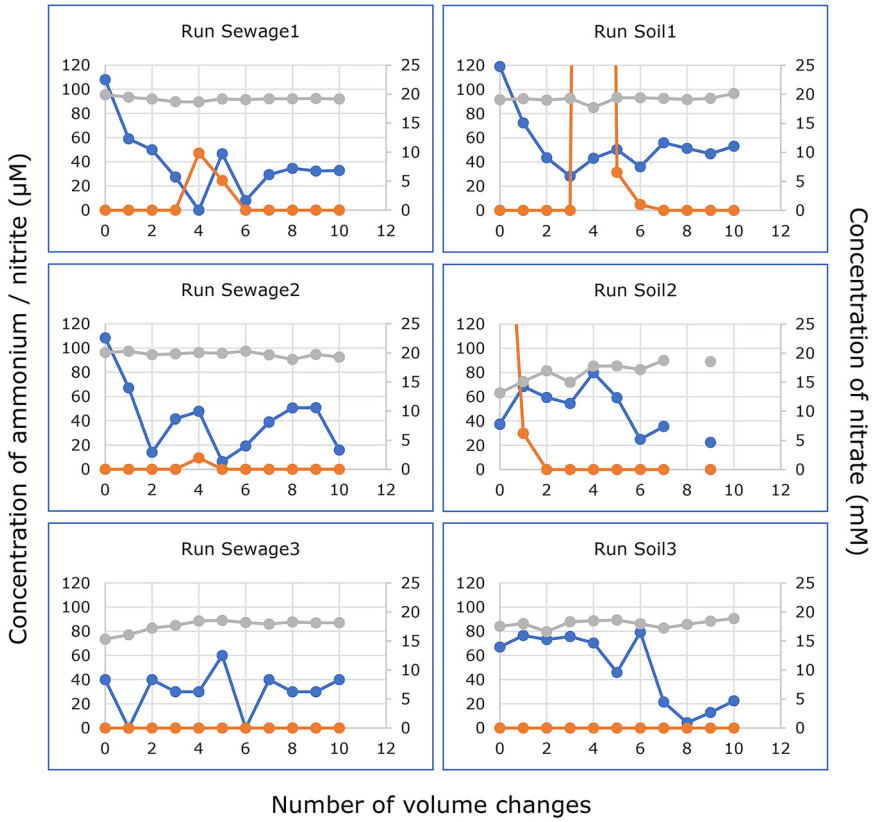

FIG 1 Concentrations of ammonium (blue), nitrite (orange), and nitrate (gray) were measured in samples collected after each volume change during the enrichment runs in nitrite-fed bioreactors with *Nitrobacter winogradskyi* that had been inoculated diluted sewage sludge (runs Sewage 1, Sewage 2, and Sewage 3) or with a grassland soil suspension (runs Soil 1, Soil 2, and Soil 3). Some points are missing in the figure due to exceeding the scale of the *y*-axes, i.e., Run Soil 1, $t = 4$ (with nitrite 1.9 mM) and Run Soil 2, $t = 0$ (with nitrite 0.33 mM), or due to missing data, i.e., Run Soil 2, $t = 8$ and $t = 10$.

(± 0.01 standard deviation [SD]) mM from the 6th volume change onward (Fig. 1). The nitrite concentrations in the enrichments remained low from the start on but accumulated occasionally for a short period. Interrupting the supply of nitrite from the reservoir to the culture for a few hours was sufficient to stimulate nitrite oxidation again, even in the case of run Soil 1 where nitrite once accumulated overnight until 1.92 mM.

In the second series of enrichments with runs Sewage 3, Soil 2, and Soil 3, the behavior of the mineral nitrogen concentrations were as expected with an almost complete conversion of nitrite and ammonium to nitrate without notable accumulation of nitrite (Fig. 1). The ammonium concentrations left in the cultures was on average 0.04 (± 0.01 SD) mM from the 6th volume change on.

The balances of mineral nitrogen recovered in the bioreactors during the enrichment runs were generally between 80 and 100% (Fig. S1 in Supplemental File 1). The mineral nitrogen balances of runs Sewage 3 and Soil 2 were low at the start of the runs but reached an equilibrium with higher recovery percentages after 4 volume changes, although the recovery of nitrogen during run Soil 2 remained low compared to the other enrichment runs. Remarkably, the percentages of recovered nitrogen in the first series of enrichments with runs Sewage 1, Sewage 2, and Soil 1 were generally higher than those observed in the second series of enrichment runs (Fig. S1 in Supplemental File 1).

Limited biofilm formation was observed in the bioreactors at the end of the enrichment runs, which was independent of the nature of the inoculum, i.e., sewage or soil.

16S rRNA gene amplicons: *N. winogradskyi* versus escorting microorganisms. The MiSeq analyses yielded 489,927 amplicons of the partial 16S rRNA gene (range 1,055

to 27,438) from 53 different samples collected at the start of each enrichment run or taken after each volume change during the runs. Whereas most DNA samples yielded 16S rRNA gene amplicons, no amplicons were obtained from run Sewage 1 except from the sample collected after 10 volume changes. For this reason, run Sewage 1 has been omitted from further calculations with the 16S rRNA gene amplicons. Based on 97% mutual similarity, 383 operational taxonomic units (OTUs) could be distinguished. The OTU, which was affiliated with *N. winogradskyi*, comprised on average 53.9% of the total community of OTUs. The remaining OTUs belonged to taxa that had been introduced with the inoculum and will be further referred to as *N. winogradskyi*-escorting microorganisms or shortly as escorting microorganisms. Of the OTUs belonging to the escorting microorganisms, 294 were affiliated with Bacteria, 2 with Archaea (both members of the family Halobacteriaceae), whereas 87 OTUs could not be assigned to Archaea or Bacteria.

With more than 80% of the total reads affiliated with escorting microorganisms, reads belonging to the bacterial classes Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria were most abundant with 27.5, 23.2, and 30.0%, respectively, of the total. Reads associated with the bacterial classes Actinobacteria, Flavobacteriia, Sphingobacteriia, Cytophagia, and Deinococci comprised also more than 1% of the total reads. Three OTUs comprising 1.5% of the total community of escorting microorganisms, were affiliated with the ammonia-oxidizing *Nitrosomonas* genus (class Betaproteobacteria).

The percentage of escorting microorganisms increased during the enrichment runs. The median percentage of amplicons affiliated with escorting bacteria at the start of the enrichments amounted to 8.1% but quickly increased to median percentages of 37.8 to 56.3% after two volume changes (Fig. S2 in Supplemental File 1). However, due to the large variation in the percentages of amplicons affiliated with escorting microorganisms, only the median values measured after 2, 4, 5, 6, and 8 volume changes were significantly different from those observed at the start ($P < 0.05$, Dunn's *post hoc* test). The nature of the inoculum, i.e., diluted sewage sludge or grassland soil suspension, had no significant effect on the median percentages of escorting bacteria (Kruskal-Wallis test: H = 13.49, $P = 0.1971$).

Diversity within assemblies of escorting microbes based on 16S rRNA gene amplicons. The composition of the original communities of escorting microbial communities at the start of the enrichment runs can be retrieved from the 16S rRNA gene amplicons generated at time zero. In total, 66 and 47 different OTUs were detected at the start of the various runs inoculated with sewage and soil, respectively. No significant effect of the origin of the starting material, i.e., sewage or soil, was observed on alpha diversity indices in the starting communities (Table S1 in Supplemental File 1). With respect to beta diversity, no significant differences in escorting community composition were observed between enrichment runs that started with diluted sewage sludge or with a grassland soil suspension (one-way analysis of similarities [ANOSIM] test: $R = -0.4167$, $P = 1.0000$). While 8 of the 25 OTUs that were most dissimilar between sewage- and soil-based enrichment runs, were found in both the sewage- and the soil-based communities, 5 were only found in sewage-based enrichment runs and 12 in soil-based runs (Table S2 in Supplemental File 1). With 11 unassigned OTUs, the list of the 25 most dissimilar OTUs sewage- and soil-based enrichment runs contained a relatively large number of nonclassified OTUs (Table S3 in Supplemental File 1). Of these 11 unassigned OTUs, 6 appeared only in the soil-based enrichment runs (i.e., OTUs 075, 135, 279, 410, 730, and 804), 4 only in the sewage-based runs (i.e., OTUs 123, 189, 535 and 793), while OTU402 was found in both sewage- and soil-based runs.

The development of escorting communities was rather nonlinear, although large differences in the direction and the size of community changes were observed between the individual enrichment runs as can be seen in a biplot of the results of a principal coordinates analysis (PCoA) of the composition of the escorting communities during the enrichment runs (Fig. 2). The variance explained by both axes in this biplot is 34.8% (20.4 and 14.4%, respectively) of the total variance observed between the escorting communities. The communities detected after 2 or more volume changes

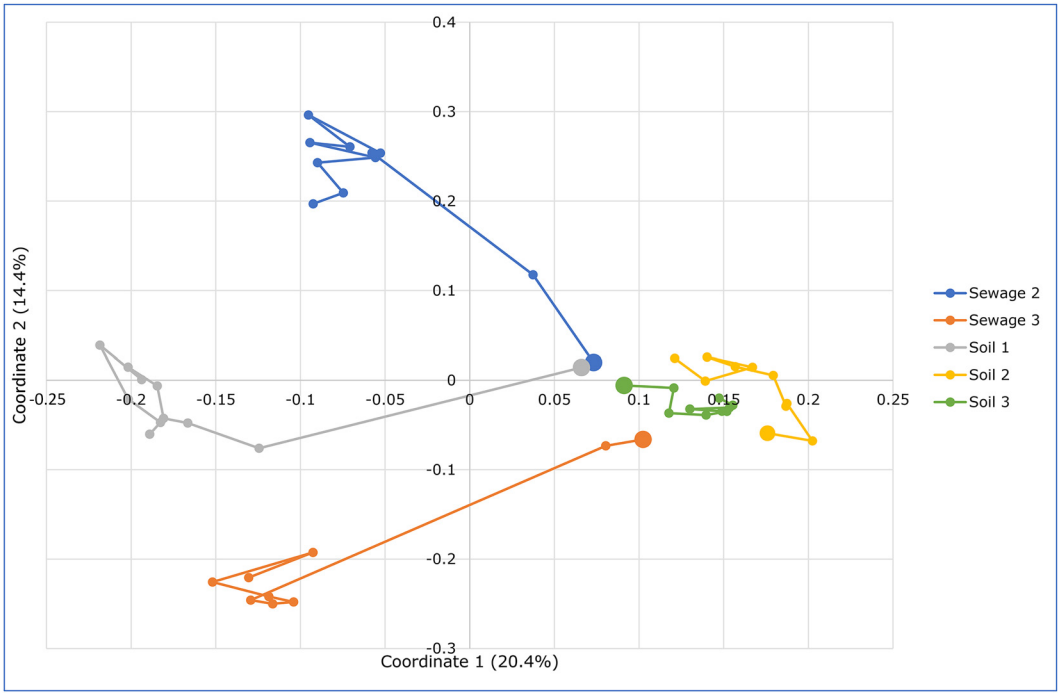

FIG 2 Principal component analysis (PCoA) based on Bray-Curtis distance dissimilarity of 16S rRNA amplicons of escorting communities sampled during the enrichment runs that started with diluted sewage sludge (runs Sewage 2 and Sewage 3) or with a grassland soil suspension (runs Soil 1, Soil 2, and soil 3). Dots represent communities at successive volume changes connected by lines. Starting points are indicated by enlarged symbols. N.B. Enrichment runs sewage 1 is missing due to the lack of sequencing data.

were significantly different from the initial escorting communities observed at the start (Dunn's *post hoc* test, $P < 0.05$), but they were not significantly different from each other. After two volume changes, all enrichments reached a less dynamic phase, in which the changes in community structure were relatively small. In this phase, the runs were significantly different from each other (one-way ANOIM test: $R = 0.9957$, $P = 0.0001$). The origin of the inoculums, i.e., sludge or soil, had a significant effect on the community composition of the escorting microorganisms in the less dynamic stage of the runs (one-way ANOSIM test: $R = 0.2485$, $P = 0.0003$). However, any combination of 2 and 3 random runs gave a significant effect on the community composition of the escorting microorganisms after two volume changes in the reactors. Hence, likely due to the largely stochastic nature of the enrichment runs, we were not able to show a definite effect of the sample origin of their outcomes. Noteworthy in Fig. 2, is the separation of the enrichment runs in two groups being affected by the *x*-axis. The group that was affected by the *x*-axis all originate from runs in the first series of enrichments, while the second group comes from the second series. The composition of the communities of escorting microorganisms enriched in the final phase of the enrichment runs was significantly affected by these "PCoA groups" (one-way ANOSIM test: $R = 0.4672$, $P = 0.001$). But again, each combination of 2 and 3 random runs gave a significant difference in the community composition of the escorting microorganisms between the two groups. In addition, no significant difference in community composition was found between the two series at the start of the enrichment runs (one-way ANOSIM test: $R = 0.6667$, $P = 0.2110$).

In the period starting after 2 volume changes in the bioreactors, 250 and 310 different OTUs were detected in total in the enrichment runs that were inoculated with sewage and soil, respectively. The 25 OTUs that were most dissimilar between sewage- and soil-based enrichment runs, contributed 71.7% to the total dissimilarity between both types of runs (Table 1). The most dissimilar OTU between sewage and soil was OTU444, which mainly

TABLE 1 Mean relative numbers of 16S rRNA gene amplicons observed in the top-25 of escorting taxa that are most dissimilar between sewage- and soil-based enrichment runs as determined by similarity percentages (SIMPER)-based analyses with samples collected in the less dynamic phase of the runs (i.e., after volume changes 2 to 10)

Taxon	Average dissimilarity[a]	Contribution (%)	Cumulative %	Mean sewage	Mean soil	Affiliation (genus)
OTU444	12.65	14.71	14.71	0.24	0.01	*Zoogloea*
OTU384	7.25	8.44	23.14	0.05	0.16	*Acinetobacter*
OTU118	5.98	6.95	30.10	0.11	0.02	*Pseudomonas*
OTU039	4.16	4.84	34.93	0.08	0.05	Unassigned[c]
OTU650	2.89	3.37	38.30	0.03	0.06	Unassigned[d]
OTU710	2.77	3.23	41.53	0.04	<0.01	*Aeromonas*
OTU233	2.76	3.20	44.73	<0.01	0.07	*Rhodobacter*
OTU315	2.44	2.83	47.56	<0.01	0.04	*Sphingopyxis*
OTU317	2.22	2.59	50.15	<0.01	0.05	*Rhodococcus*
OTU705	2.02	2.35	52.50	0.05	0.02	*Nitrosomonas*
OTU200	1.94	2.26	54.75	<0.01	0.04	*Stenotrophomonas*
OTU791	1.39	1.62	56.37	<0.01	0.03	*Ochrobactrum*
OTU460	1.28	1.49	57.86	0.02	<0.01	*Pseudomonas*
OTU782	1.27	1.48	59.34	0.02	<0.01	*Flavobacterium*
OTU620	1.19	1.39	60.72	<0.01	0.02	Unassigned[e]
OTU171	1.19	1.38	62.10	0.02	<0.01	*Brevundimonas*
OTU615	1.07	1.25	63.35	0.02	0.01	*Flavobacterium*
OTU519	1.02	1.19	64.54	<0.01	0.03	*Phenylobacterium*
OTU293	1.02	1.18	65.72	<0.01	0.02	Unassigned[f]
OTU674	0.90	1.05	66.76	<0.01	0.02	*Lacibacter*
OTU614	0.90	1.04	67.81	ND[b]	0.02	*Deinococcus*
OTU217	0.88	1.03	68.83	0.01	0.01	Uncultured[g]
OTU204	0.87	1.01	69.84	0.01	0.02	Unassigned[h]
OTU784	0.82	0.95	70.80	0.01	0.01	*Pseudomonas*
OTU083	0.81	0.94	71.73	0.01	0.01	*Sphingopyxis*

[a]Overall average dissimilarity: 86.01.
[b]ND, not detected.
[c]Family Rhizobiaceae.
[d]Family Comamonadaceae.
[e]Family Xanthomonadaceae.
[f]Family Comamonadaceae.
[g]Family env.OPS 17.
[h]Family Intrasporangiaceae.

appeared in sewage-based runs and was affiliated with the genus *Zoogloea* of the class Betaproteobacteria (Table S4 in Supplemental File 1). This main dissimilar OTU was followed by OTU384, which emerged predominantly in the soil-based runs and is affiliated with the genus *Acinetobacter* of the class Gammaproteobacteria (Table S4 in Supplemental File 1). In comparison with the list of 25 most dissimilar OTUs between sewage and soil-based runs from the start of the enrichment runs (Table S3 in Supplemental File 1), the list of 25 most dissimilar OTUs from the period after two volume changes did not contain unassigned microbial taxa anymore (Table S4 in Supplemental File 1). Of the original list of 25 most dissimilar OTUs from the start, only 6 were recovered in the list of 25 most dissimilar OTUs after 2 volume changes in the bioreactors, i.e., OTUs 039, 200, 204, 317, 614, and 705. These OTUs were affiliated with an unassigned genus of the family Rhizobiaceae, the genus *Stenotrophomonas*, an unassigned genus of the family Intrasporangiaceae, the genus *Rhodococcus*, the genus *Deinococcus*, and the genus *Nitrosomonas*, respectively (Table S4 in Supplemental File 1).

Finally, looking at the samples collected after 10 volume changes, it appeared that the alpha diversity indices of the different enrichment runs were not significantly affected by the origin of the starting material (Table S5 in Supplemental File 1).

Functional homologs. Shotgun sequencing on the total DNA collected from each bioreactor after 10 volume changes, i.e., at the end of the enrichment runs also enabled the exploration of differences in functional homology between the enrichment runs. Overall, the sequencing yielded read lengths of 227 to 336 Mbp distributed into 931,468 to 1,368,353 reads after quality control (Table S6 in Supplemental File 1). The distribution of the different KEGG metabolism genes was similar between soil- and sewage-based

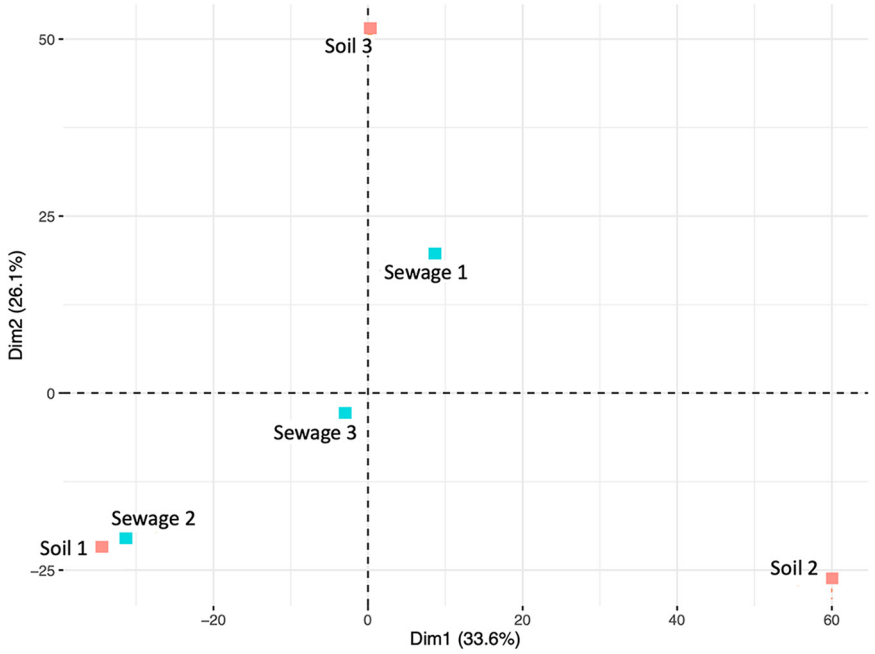

FIG 3 Principal component analysis (PCA) based on Bray-Curtis distance dissimilarity of shotgun metagenomes of samples collected at the end of their enrichments in strictly nitrite-fed reactors with *Nitrobacter winogradskyi* cultures that had been inoculated with a grassland soil suspension or diluted sewage sludge. The dissimilarity had been calculated at the level of functional orthologs (KOs).

samples (Fig. S3 in Supplemental File 1). The analyses of classical univariate statistical comparisons using the Mann-Whitney/Kruskal-Wallis test showed that 16 functional orthologous functions (KOs) seemed to be statistically different ($P < 0.05$) between sewage- and soil-based samples. However, when adjusting the *P* value to FDR (i.e., false discovery rate) of 0.05, no statistical differences were observed between the two types of origin. The similar patterns between soil- and sewage-based samples based on metagenome profiles are shown in the principal component analysis (Fig. 3) and heatmap analysis (Fig. 4). In the metagenome data set, a total of 35 KOs were only present in the *N. winogradskyi* reference genome (Table S7 in Supplemental File 1), while a total of 5,644 KOs were found that were specific for the escorting community (Fig. S4 in Supplemental File 1).

Taxonomy based on metagenomic sequencing. The shotgun metagenomes used for comparing functional homologs between enrichment runs lend themselves also to a taxonomic analysis between the runs. In total, 5,479 different taxa were detected, of which 384 were removed before the taxonomic analyses started because they were classified as animals, diatoms, fungi, plants, and protists or because they amounted to less than 10 copies in 1 or 2 enrichment runs. The remaining 5,095 prokaryotic taxa belonged mainly to the bacteria (95.8%), while a smaller part belonged to the Archaea (4.2%). Of the alpha diversity indices based on shotgun libraries of the individual enrichment runs, only the number of individuals was significantly affected by the origin of the starting material with lower numbers in the sewage-based runs (Table S8 in Supplemental File 1). In contrast, all alpha diversity indices obtained after 10 volume changes were significantly affected by the library of choice, i.e., 16S rRNA gene amplicons or shotgun metagenomics (Table S9 in Supplemental File 1). Except for the evenness index, all indices were significantly larger for the shotgun libraries.

The top-25 list of taxa that are most dissimilar between the enrichment runs that started with sewage or soil represented 45.7% of the overall average dissimilarity between these series (Table 2). Remarkably, two genera stand out in the top-25 list, i.e., *Acidovorax* and *Sphingopyxis* belonging to the bacterial phyla Betaproteobacteria and Alphaproteobacteria, respectively (Table S10 in Supplemental File 1). Except for *Sphingopyxis* sp. QXT-31, *Sphingopyxis* species

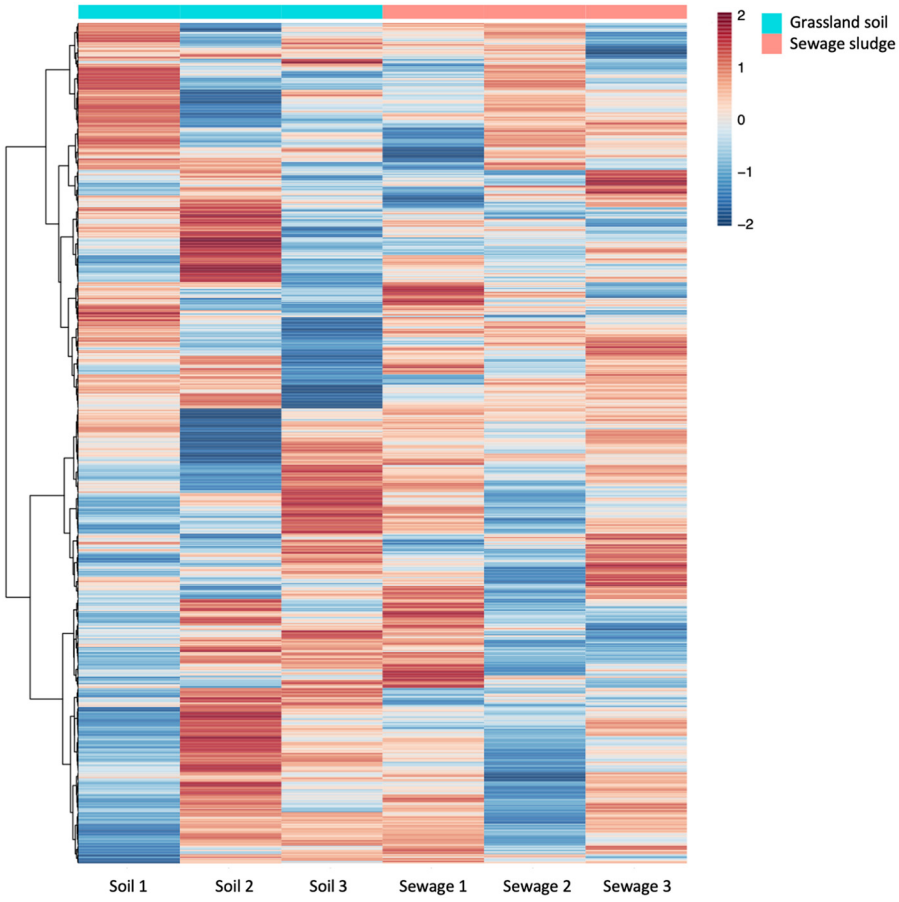

FIG 4 Hierarchical clustering of orthologous functions (KOs) based on Euclidean distance measure and Ward clustering algorithm visualized in the heatmap of metagenomes of samples collected at the end of their enrichments in strictly nitrite-fed reactors with *Nitrobacter winogradskyi* cultures that had been inoculated with a grassland soil suspension or diluted sewage sludge.

were most prominent in sewage-based runs. In contrast, *Acidovorax* species were most noticeable in soil-based runs.

On average, 99.99% of the sequences that presumably belonged to nitrite-oxidizing microorganisms were affiliated with *N. winogradskyi*. *Nitrobacter hamburgensis* was the second most numerous nitrite-oxidizing microorganism with 0.01% of the total. The other chemolitho-autotrophic microorganisms that were enriched were the ammonia-oxidizing microorganisms. Their numbers were more variable between the runs, although sequences affiliated with *Nitrosospira multiformis* were most numerous in all but two enrichment runs (Fig. S5 in Supplemental File 1). In enrichment run Soil 1, *N. multiformis* was replaced by *Nitrosomonas europaea* as the most abundant ammonia-oxidizing microorganism. The ratio between ammonia-oxidizing Thaumarchaea and Betaproteobacteria was on average 0.29 (range from 0.08 to 0.58).

DISCUSSION

The outcome of the enrichment runs disproved the hypothesis that the assembly of a heterotrophic microbiome of *N. winogradskyi* should be dependent on the origin of the heterotrophic microorganisms. The highly stochastic nature of the individual enrichments makes it impossible to demonstrate a significant effect of the origin of the starting material, which was either diluted sewage sludge or a grassland soil suspension, on the outcome of the enrichments. However, despite the large taxonomic variation observed between the outcome of the enrichment runs, we demonstrated that the distribution of KEGG functional homologs or genes was largely similar for all

TABLE 2 Mean relative numbers of 16S rRNA gene amplicons observed in the top-25 of escorting taxa that are most dissimilar between sewage- and soil-based enrichment runs as determined by SIMPER-based analyses in samples collected at the end of the runs (i.e., after 10 volume changes)

Taxon	Average dissimilarity[a]	Contribution (%)	Cumulative %	Mean sewage	Mean soil
Shinella sp. HZN7	4.43	7.03	7.03	0.01	0.09
Sphingopyxis sp. QXT-31	3.65	5.79	12.82	0.02	0.07
Sphingopyxis macrogoltabida	1.47	2.34	15.15	0.03	0.02
Sphingopyxis sp. EG6	1.36	2.16	17.31	0.03	0.01
Acidovorax sp. KKS102	1.35	2.14	19.45	<0.01	0.03
Stenotrophomonas maltophilia	1.26	1.99	21.44	0.03	0.01
Sphingopyxis fribergensis	1.25	1.98	23.42	0.03	0.01
Pseudomonas veronii	1.23	1.95	25.37	<0.01	0.02
Ensifer adhaerens	1.18	1.87	27.23	0.02	0.01
Sphingopyxis lindanitolerans	1.08	1.72	28.95	0.03	0.01
Acidovorax sp. RAC01	0.94	1.49	30.44	<0.01	0.02
Simplicispira suum	0.93	1.48	31.92	<0.01	0.02
Sphingopyxis sp. PAMC25046	0.93	1.48	33.41	0.02	0.01
Bradyrhizobiaceae bacterium SG-6C	0.92	1.46	34.86	0.02	<0.01
Acidovorax carolinensis	0.90	1.43	36.29	<0.01	0.02
Achromobacter xylosoxidans	0.90	1.42	37.72	0.02	<0.01
Xanthomonas euvesicatoria	0.88	1.39	39.10	0.02	<0.01
Acidovorax sp. T1	0.86	1.37	40.47	<0.01	0.02
Acidovorax sp. 1608163	0.69	1.10	41.57	<0.01	0.01
Sinorhizobium sp. RAC02	0.59	0.94	42.51	<0.01	0.01
Methyloversatilis sp. RAC08	0.52	0.82	43.33	<0.01	0.01
Pseudomonas sp. LH1G9	0.51	0.80	44.13	<0.01	0.01
Variovorax paradoxus	0.49	0.78	44.91	0.01	0.01
Sphingopyxis sp. MG	0.49	0.77	45.68	0.01	<0.01

[a]Overall average dissimilarity: 63.06.

runs and, hence, independent of the composition of the escorting community. Although not triggered by the choice of starting material, dissimilar microbial communities do possess similar functional genes.

Another notable outcome of the enrichment runs is the observation that in some runs, i.e., in runs Sewage 1, Sewage 2, and Soil 1, the composition of the communities of escorting microorganisms undergoes a rapid change, whereas in the other runs, i.e., in runs Soil 2 and Soil 3, the community structures remain more stable from the start on.

The role of substrate kinetics during community assembly. The establishment of escorting communities of microbes in an environment created by actively growing *N. winogradskyi* cells that were hitherto free of heterotrophic microorganisms depends on deterministic and stochastic factors (6). Only those microbes likely survive washout in the bioreactors that grow at the expense of organic carbon produced from CO_2 by *N. winogradskyi*. Hence, deterministic factors, such as species traits and interspecies interactions will have played their role in the establishment of new communities from diluted sewage sludge and grassland soil suspension. However, stochastic factors such as species composition of the inoculum also played their role. The original sewage and soil samples were 5×10^{-8} times diluted at the start of the enrichment runs, which may have caused differences in species composition at the start. It is less likely that the conservation of samples of diluted sewage sludge and soil suspensions in glycerol at −80°C would have caused the differences. Whenever this way of conservation would have been selected for certain species, it would have been the same for each enrichment run. Hence, stochastic factors such as historical contingency or priority effects also played their role in the assembly of new communities.

At the time of inoculation, the heterotrophic microorganisms from sewage and soil encountered an environment largely created by the nitrite-oxidizing *N. winogradskyi*. This environment, which likely contained an excess of organic compounds produced by this nitrite oxidizer, enables the selection of heterotrophic microorganisms based on growth rates. In this first phase of the enrichments, the selection of fast-growing heterotrophic microorganisms seems to be a stochastic process and relatively large changes in

community composition can occur during the first two volume changes in the bioreactors as can be observed from the PCoA analysis. After this initial period changes in the compositions of the escorting communities became less dynamic. Bittleston et al. (8), who studied the assembly of communities in microcosms containing bacterial communities collected from wild pitchers of the carnivorous pitcher plant *Sarracenia purpurea*, observed that the diversity of assembled communities was determined by the diversity at early preassembly stages. Only after one transfer in the microcosms, the non-metabolically active species were pruned and the final diversity of the community was determined. Hence, like in our experiment, the long-lasting effects of early conditions and biota led to strong differences in final community composition and ecosystem function emphasizing the importance of historical contingency.

At the time of more stable escorting communities, the supply of organic compounds produced by the nitrite-oxidizing *N. winogradskyi* became likely growth-limiting for the heterotrophic microorganisms and at the same time dependent on the rate of release of organic compounds from the nitrite-oxidizing *N. winogradskyi*, that grew at a growth rate similar to the dilution rate in the bioreactor. Such an imposed growth rate is likely much lower than the (sub)maximum growth rates of the heterotrophic microorganisms reached in the first phase of the enrichments. Under such conditions of constant low growth rates, i.e., 0.0014 h^{-1}, as realized in the bioreactor by pumping continuously fresh mineral medium, the affinity of heterotrophic microorganisms for organic compounds becomes an important selection factor (9). However, according to the concept of specific affinity as formulated by Healey (10), a low affinity for a compound is compensated by a high maximum substrate uptake rate. Because of this, heterotrophic species with high relative biomass remain competitive with respect to scavenging small amounts of organic compounds released by the nitrite oxidizer and the community composition will consequently hardly change.

Neutral or deterministic assembly processes? Autotrophic microorganisms such as the nitrite-oxidizing bacterium *N. winogradskyi*, create their environment by the release of organic compounds. Therefore, the selection of escorting heterotrophic microorganisms is determined by the environment created by *N. winogradskyi*. In this way, the enrichment process could be described as deterministic. However, the final composition of the escorting community was highly variable and therefore likely determined by stochastic processes as well. Because changes in the composition of the individual enrichment runs became already less dynamic after two volume changes, stochastic processes mainly played their role at the start of the runs. Associated with this, it cannot be excluded that the diluted and frozen sewage and soil samples were already mutually different before they were used for inoculation of the actively growing *N. winogradskyi* cultures. Although the outcome of the enrichment runs was highly variable by stochastic processes, only a limited number of genera came to the fore in the different runs. Hence, deterministic factors such as species traits must also have played a role in the assembly of escorting communities. Studying microbial communities in a wastewater treatment plant, Ofiteru et al. (11) observed population dynamics that were consistent with neutral community assembly, meaning that chance and random immigration play an important and predictable role in shaping the communities in an open biological system such as a wastewater treatment plant. In studying population dynamics in an anaerobic digester fed with a sterile model substrate, i.e., cellulose, to minimize the inflow of microorganisms, Vanwonterghem et al. (12) observed community dynamics that were strongly linked with reactor performance and with synchronized populations over long periods. These authors concluded that deterministic factors such as operational conditions, substrate availability, and interspecies interactions are important in the assembly of communities in their relatively closed anaerobic digester.

Notwithstanding, the stochastic enrichment of heterotrophic communities in our strictly nitrite-fed bioreactors, the composition of the enriched functions as represented by the enriched functional genes was always similar and independent of the origin and the composition of the enriched heterotrophic community. Hence, the different taxonomic guilds that

had been enriched had the same functions regardless of their origin. Navarrete et al. (13) studying the soil ecosystem under fire disturbance also found microbial redundancy carried out by different guilds of bacteria. Subculturing of natural microbial communities from various soils and plant leaf surfaces on a single limiting resource for approximately 60 generations led to highly diverse multispecies communities with comparable community-level functions (14). Studying the composition and function of microbial communities in replicated miniature aquatic systems contained within the foliage of wild bromeliads, Louca et al. (15) found that all communities exhibited a remarkably similar functional structure, which contrasted with a highly variable taxonomic composition within individual functional groups. Based on this and other studies in additional ecosystems, Louca et al. (15) suggested that the metabolic functional potential of microbial communities is closely related to environmental constraints such as the availability of electron acceptors for respiration, while the taxonomic variation within individual functional groups is only poorly explained by environmental conditions, and these authors concluded that similar environments should promote similar microbial community functions while allowing for taxonomic variation within individual functional groups. The same seems to hold for the heterotrophic escorting microorganisms in the microbiome of the nitrite-oxidizing, organic carbon-producing *N. winogradskyi*.

Ammonia-oxidizing microorganisms. A striking difference between the 16S rRNA sequences and the shotgun metagenome libraries concerns the taxonomy of dominant ammonia-oxidizing bacteria. Whereas only ammonia-oxidizing taxa affiliated with the genus *Nitrosomonas* were encountered in the 16S rRNA gene amplicon libraries, the dominant taxa of ammonia-oxidizing bacteria in the shotgun metagenomes library were more diverse with the dominance of *N. multiformis* in most of the enrichment runs. Because ammonia-oxidizing Thaumarchaea is generally smaller than the ammonia-oxidizing Betaproteobacteria, French et al. (16) suggested that ammonia-oxidizing archaea should exceed the ammonia-oxidizing bacteria in abundance by at least 10 to 100 times to contribute equally to the oxidation of ammonia in an environment. Because the ratio between ammonia-oxidizing Thaumarchaea and Betaproteobacteria was always lower than 1 in our study, Thaumarchaea was likely not responsible for the oxidation of ammonia in the bioreactors. The average ammonium concentration of 0.04 mM observed in the bioreactors from the 6th volume change onwards is at the same level as observed before in continuously ammonium-fed chemostats with ammonium-oxidizing bacteria growing at the same dilution rate as employed in the present experiments (17).

Conclusions. We demonstrated that, despite the largely stochastic assembly of communities of escorting microorganisms in continuously nitrite-fed bioreactors, the functional compositions of the communities were statistically not different between soil- and sludge-based enrichments, suggesting a large redundancy in functional characteristics among the enriched heterotrophic microorganisms.

MATERIALS AND METHODS

Origin of the microorganisms. The basis of the enrichment experiments consisted of a pure culture of *Nitrobacter winogradskyi* strain ATCC 25391, which is growing in a mineral medium with sodium nitrite as an energy source. The suspensions of heterotrophic microorganisms originated from a calcareous grassland soil (upper 5 cm) near the village of Brummen in the floodplain of the River IJssel or from sewage sludge of a municipal wastewater treatment plant near the village of Bennekom, respectively. Both locations are situated in the center of the Netherlands. The Brummen soil had been used before by our group in nitrification-related studies (18–20). Immediately after sampling in July 2011, the soil and sludge samples were stepwise 10,000 times diluted in Milli-Q water and frozen in glycerol at a ratio of 1:1 (vol/vol) at −80°C in Eppendorf cups of 1.5 mL. Freezing and conservation of glycerol at −80°C may have killed some species, but the composition of the community of surviving species will be the same at every inoculation of the bioreactors over longer periods. For each enrichment run, which may start several months after each other, one cup was defrosted and added to the bioreactors as described below.

Enrichment experiments. The enrichment experiments were performed in 3-L bioreactors (ADI 1010 Bioreactor, Applikon Biotechnology, UK) without any organic carbon source added. The mineral medium consisted of 20 mM sodium nitrite, 0.1 mM ammonium chloride, 10 mM sodium chloride, 1 mM potassium chloride, 0.2 mM magnesium sulfate heptahydrate, and 1 mM calcium chloride dihydrate, and 1 mL/L of a trace element solution (21), all in demineralized water. Ammonium was always added to prevent selection in favor of nitrite- and nitrate-assimilating heterotrophic microorganisms. After

autoclaving of the bulk of mineral medium, sterile 0.4 mM mineral potassium dihydrogen phosphate was added. The complete mineral medium was continuously pumped at a dilution rate of 0.014 h^{-1} into the bioreactor, which had a working volume of 1.5 L. The pH was maintained at pH 7.5 with a combination of 1 M hydrochloric acid and 1 M sodium hydrogen carbonate, while the temperature was set at 25°C. The nitrite concentration in the culture was checked daily by a colorimetric method and when the nitrite concentration exceeded 0.05 mM, the supply of nitrite from the reservoir to the culture was interrupted for a few hours. During this time, nitrite oxidation improved and the level of nitrite in the bioreactor declined again to nondetectable concentrations.

The enrichment experiments started by inoculating a 1.5 L nitrite-fed, actively growing *N. winogradskyi* culture with a 1.5 mL defrosted diluted sludge or soil suspension. Hence, the original sludge and soil samples were 5×10^{-8} times diluted at the start of the experiments. The enrichments were performed in 2 series of 3 runs each. The first series with the runs Sewage 1, Sewage 2, and Soil 1 were completed from October to November 2013, and the second series with the runs Sewage 3, Soil 2, and Soil 3 from February to April 2015. Hence, each series consisted of runs with diluted sewage and with soil suspensions to exclude a specific effect of time on the outcome of the enrichment runs with sewage and soil samples, respectively. In the end, the enrichments series with diluted sewage sludge and those with a grassland soil suspension consisted both of triplicate runs. Immediately after inoculation and again after each volume change, 15 mL samples were collected from the bioreactors, immediately filtered through 0.22 μm nitrocellulose membrane filters (Merck Millipore Ltd., Tullagreen, Carrigtwohil, Cork, Ireland) and stored at 4°C in sterile 15 mL polypropylene tubes (Greiner Bio-One, Alphen aan de Rijn, the Netherlands). The filters themselves were stored at −80°C for DNA extraction. After 10 volume changes, a larger sample was taken for DNA shotgun analysis.

Chemical analyses. The concentrations of ammonium, nitrite, and nitrate in the liquid samples collected after each volume change in the bioreactors were determined with a Seal QuAAtro auto-analyzer (Beun-de Ronde, Alphen, The Netherlands).

DNA extraction. Total genomic DNA was extracted from the filtered material by homogenizing one broken filter in 1 mL cetyltrimethylammonium bromide (CTAB) buffer in MP Lysing Matrix tubes (MP Biomedicals, Santa Ana, CA, USA), subjected twice to disruption by bead-beating at a 5.0 m/s rotation for 60 s, and incubated at 37°C for 30 min in the presence of 5 μL proteinase K (20 mg/mL), while vortexing every 15 min. The samples were supplemented with 150 μL of 20% sodium dodecyl sulfate (SDS) solution and incubated at 65°C for 1 h in a thermoblock and vortexed every 15 to 20 min. After centrifugation at 10,000 $\times$ *g* for 10 min., approximately 500 μL of the supernatant was collected and directly added to the lysis buffer of the Maxwell16 DNA Tissue extraction kit (Promega, Fitchburg, Wisconsin USA). Automatic purification of genomic DNA was performed using the Tissue DNA extraction program. DNA quality and quantity were good and yielded high-quality sequences (both amplicons and shotgun metagenomes). The ratio of absorbance at 260 (A260)/A280 in the DNA samples was between 1.7 to 1.9, and the quantity ranged from 15 to 25 ng/μL.

Library preparation and analysis of 16S rRNA gene amplicon sequences. The primers 515F and 806R (22) containing multiplex tags were used to amplify the 16S rRNA partial gene fragment. PCR was carried out using 0.2 μL of 0.056 U fast StartExpTaq Polymerase (Roche Applied Sciences, Indianapolis, IN, USA), 2.5 μL dNTP (2 mM each), 0.25 μL of each primer, and 1.0 μL of DNA template. Thermocycling conditions included denaturing at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 53°C for the 30s, extension at 72°C for 60 s followed by a final extension at 72°C for 10 min. As the negative-control, water was used instead of DNA, and positive-control DNA of *Escherichia coli* was used. The PCR products were purified using a QIAquick PCR purification kit (Qiagen Technologies), checked for quality in agarose gel electrophoresis in TBE buffer, and quantified using Fragment analyzer TM - Automated CE system (Advanced Analytical Technologies, Inc.). Equimolar PCR products were pooled and sequenced using the Illumina HiSeq2000 platform (2 $\times$ 150 bp) (BGI Inc. China). PANDASeq (23) was used to merge paired-end reads with a minimum overlap of 10 bp and at least a Phred score of 25. Sequences were converted to FASTA format and concatenated into a single file for downstream analyses. MicrobiomeAnalyst (Dhariwal et al. (24)) was used to determine differences in relative abundances between treatments. Statistical hypothesis tests were performed using the Kruskal-Wallis test followed by linear discriminant analysis to evaluate the relevance or effect size of differential abundant features. The 16S rRNA data sets were rarefied to the same number of sequences per sample and used to construct Bray-Curtis dissimilarity matrixes generated using the "phyloseq" package in R. The matrixes were ordinated by principal coordinate analysis (PCoA) and the adonis function was used to calculate the permutational multivariate analysis of variance and verify the strength and statistical significance of groups among soil and swage.

Preparation and analysis of shotgun libraries. The shotgun sequencing of the total DNA was carried out in samples at 10 volume changes to determine the functional potential of the escorting satellite bacteria growing with *N. winogradskyi*. From each of the nine enrichment runs (n = 5 grassland soil, n = 4 sewage sludge), the total DNA samples were prepared as TruSeq Nano libraries. These shotgun libraries were sequenced as paired-end with 350 bp inserts on the Illumina HiSeq 2000 sequencer (Macrogen, South Korea). The shotgun metagenomes were analyzed on a Linux server with 64 nodes and 250 GB RAM. Read processing and analysis were carried out in a snakemake pipeline with Snakemake version 3.7.1 (25), combined with bash and Perl scripts (available upon request). The metagenome reads were trimmed of sequencing adapters and trimmed on either end of the base quality was below 20. Further, reads were kept if the average quality was above 25, and if the read was longer than 50 bp following trimming. Reads were separated based on 49-kmer similarity (*Nitrobacter winogradskyi* reads) or not (satellite metagenome reads) to the *N. winogradskyi* genome downloaded from NCBI. The trimming, filtering, and separation steps were carried out using bbduk version 35.82 (available at

sourceforge.net/projects/bbmap). The separated *N. winogradskyi* reads were classified as 100% *N. winogradskyi* using Kraken version 0.10.5-beta (26). Taxonomic compositions of the satellite metagenomes were characterized with diamond blastx version 0.8.20 against the NCBI-nr database (2016-10-04 release) with the Last Common Ancestor algorithm from MEGAN v6.5.8 using the mapping from August 2016. To explore the functional potential of the satellite metagenomes, the satellite reads were compared to the 2014-03-17 KEGG database using the uproc-dna command with UPROC version 1.2.0. From the UPROC results, the KEGG orthologous groups present in each sample were visualized using iPath version 2. The lists of KOs were uploaded to the EGGNOG server (27) on August 3, 2019 using the EGGNOG-mapper v2 (available at http://eggnog-mapper.embl.de).

Statistical analyses. Significant effects of the origin of the inoculum (i.e., soil or sludge) and time on the composition of the enriched escorting satellite community were tested by one-way Analysis of Similarities (ANOSIM) of the PAST version 4.02 statistical software package (28). The same PAST software package was used to establish significant differences between median values by the Kruskal-Wallis and Dunn's *post hoc* tests (the data were not normally distributed), to visualize the ordination of the enriched escorting assemblies by principal coordination analysis (PCoA) and to determine to establish explanatory independent variables in relation to community assembly. The shotgun data analyses were carried out using the tools in MicrobiomeAnalyst (24) platform to determine differences in functional profiles between treatments. The data were normalized by cumulative sum scaling (CSS) and the clustering of the samples was visualized by principal component analysis (PCA). The hierarchical clustering and heatmap visualization were determined by Euclidean Distance using the Ward clustering algorithm. The samples were compared by nonparametric Mann-Whitney/Kruskal-Wallis method with *P* values adjusted using the FDR method. The visualization of the specific KEGGs of satellites and *N. winogradskyi* was carried out in iPath (29).

Data availability. Raw sequences of the 16S rRNA gene amplicons and shotgun metagenomes were submitted to ENA under the project accession number PRJEB36484 (ERP119682).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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We declare no conflict of interests.

REFERENCES

1. Cole JJ. 1982. Interactions between bacteria and algae in aquatic ecosystems. Annu Rev Ecol Syst 13:291–314. https://doi.org/10.1146/annurev.es.13.110182.001451.
2. Azam F, Fenchel T, Field JG, Gray JS, Meyer-Reil LA, Thingstad F. 1983. The ecological role of water-column microbes in the sea. Mar Ecol Prog Ser 10:257–263. https://doi.org/10.3354/meps010257.
3. Pachiadaki MG, Sintes E, Bergauer K, Brown JM, Record NR, Swan BK, Mathyer ME, Hallam SJ, Lopez-Garcia P, Takaki Y, Nunoura T, Woyke T, Herndl GJ, Stepanauskas R. 2017. Major role of nitrite-oxidizing bacteria in dark ocean carbon fixation. Science 358:1046–1051. https://doi.org/10.1126/science.aan8260.
4. Kindaichi T, Ito T, Okabe S. 2004. Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization. Appl Environ Microbiol 70:1641–1650. https://doi.org/10.1128/AEM.70.3.1641-1650.2004.
5. Okabe S, Kindaichi T, Ito T. 2005. Fate of C-14-labeled microbial products derived from nitrifying bacteria in autotrophic nitrifying biofilms. Appl Environ Microbiol 71:3987–3994. https://doi.org/10.1128/AEM.71.7.3987-3994.2005.
6. Zhou J, Ning D. 2017. Stochastic community assembly: does it matter in microbial ecology? Microbiol Mol Biol Rev 81. https://doi.org/10.1128/MMBR.00002-17.
7. Philips S, Laanbroek HJ, Verstraete W. 2002. Origin, causus and effects of increased nitrite concentrations in aquatic environments. Rev Environ Sci Biotechnol 1:115–141. https://doi.org/10.1023/A:1020892826575.
8. Bittleston LS, Gralka M, Leventhal GE, Mizrahi I, Cordero OX. 2020. Context-dependent dynamics lead to the assembly of functionally distinct microbial communities. Nat Commun 11:1–10. https://doi.org/10.1038/s41467-020-15169-0.
9. Pirt SJ. 1975. Principles of microbe and cell cultivation. Blackwell Scientific Publications.
10. Healey FP. 1980. Slope of the Monod equation as an indicator of advantage in nutrient competition. Microb Ecol 5:281–286. https://doi.org/10.1007/BF02020335.
11. Ofiteru ID, Lunn M, Curtis TP, Wells GF, Criddle CS, Francis CA, Sloan WT. 2010. Combined niche and neutral effects in a microbial wastewater treatment community. Proc Natl Acad Sci U S A 107:15345–15350. https://doi.org/10.1073/pnas.1000604107.
12. Vanwonterghem I, Jensen PD, Dennis PG, Hugenholtz P, Rabaey K, Tyson GW. 2014. Deterministic processes guide long-term synchronised population dynamics in replicate anaerobic digesters. ISME J 8:2015–2028. https://doi.org/10.1038/ismej.2014.50.
13. Navarrete AA, Tsai SM, Mendes LW, Faust K, de Hollander M, Cassman NA, Raes J, van Veen JA, Kuramae EE. 2015. Soil microbiome responses to the short-term effects of Amazonian deforestation. Mol Ecol 24:2433–2448. https://doi.org/10.1111/mec.13172.
14. Goldford JE, Lu N, Bajić D, Estrela S, Tikhonov M, Sanchez-Gorostiaga A, Segrè D, Mehta P, Sanchez A. 2018. Emergent simplicity in microbial community assembly. Science 361:469–474. https://doi.org/10.1126/science.aat1168.
15. Louca S, Jacques SM, Pires AP, Leal JS, Srivastava DS, Parfrey LW, Farjalla VF, Doebeli M. 2017. High taxonomic variability despite stable functional structure across microbial communities. Nat Ecol Evol 1:1–12. https://doi.org/10.1038/s41559-016-0015.
16. French E, Kozlowski JA, Bollmann A. 2021. Competition between ammonia-oxidizing archaea and bacteria from freshwater environments. Appl Environ Microbiol 87:e01038-21. https://doi.org/10.1128/AEM.01038-21.

17. Bollmann A, Bar-Gilissen MJ, Laanbroek HJ. 2002. Growth at low ammonium concentrations and starvation response as potential factors involved in niche differentiation among ammonia-oxidizing bacteria. Appl Environ Microbiol 68:4751–4757. https://doi.org/10.1128/AEM.68.10.4751-4757.2002.
18. Verhagen FJ, Duyts H, Laanbroek HJ. 1992. Competition for ammonium between nitrifying and heterotrophic bacteria in continuously percolated soil columns. Appl Environ Microbiol 58:3303–3311. https://doi.org/10.1128/aem.58.10.3303-3311.1992.
19. Verhagen FJ, Duyts H, Laanbroek HJ. 1993. Effects of grazing by flagellates on competition for ammonium between nitrifying and heterotrophic bacteria in soil columns. Appl Environ Microbiol 59:2099–2106. https://doi.org/10.1128/aem.59.7.2099-2106.1993.
20. Verhagen FJ, Hageman PE, Woldendorp JW, Laanbroek HJ. 1994. Competition for ammonium between nitrifying bacteria and plant roots in soil in pots; effects of grazing by flagellates and fertilization. Soil Biol Biochem 26:89–96. https://doi.org/10.1016/0038-0717(94)90199-6.
21. Verhagen FJM, Laanbroek HJ. 1991. Competition for ammonium between nitrifying and heterotrophic bacteria in dual energy-limited chemostats. Appl Environ Microbiol 57:3255–3263. https://doi.org/10.1128/aem.57.11.3255-3263.1991.
22. Bergmann GT, Bates ST, Eilers KG, Lauber CL, Caporaso JG, Walters WA, Knight R, Fierer N. 2011. The under-recognized dominance of Verrucomicrobia in soil bacterial communities. Soil Biol Biochem 43:1450–1455. https://doi.org/10.1016/j.soilbio.2011.03.012.
23. Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. 2012. PANDAseq: paired-end assembler for illumina sequences. BMC Bioinformatics 13:31–37. https://doi.org/10.1186/1471-2105-13-31.
24. Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J. 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res 45:W180–W188. https://doi.org/10.1093/nar/gkx295.
25. Köster J, Rahmann S. 2012. Snakemake—a scalable bioinformatics workflow engine. Bioinformatics 28:2520–2522. https://doi.org/10.1093/bioinformatics/bts480.
26. Wood DE, Salzberg SL. 2014. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol 15:R46–12. https://doi.org/10.1186/gb-2014-15-3-r46.
27. Huerta-Cepas J, Forslund K, Coelho LP, Szklarczyk D, Jensen LJ, Von Mering C, Bork P. 2017. Fast genome-wide functional annotation through orthology assignment by eggNOG-mapper. Mol Biol Evol 34:2115–2122. https://doi.org/10.1093/molbev/msx148.
28. Hammer Ø, Harper DA, Ryan PD. 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4:9.
29. Darzi Y, Letunic I, Bork P, Yamada T. 2018. iPath3. 0: interactive pathways explorer v3. Nucleic Acids Res 46:W510–W513. https://doi.org/10.1093/nar/gky299.