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1 **The stochastic assembly of *Nitrobacter winogradskyi*-selected microbiomes with**

2 **heterotrophs from sewage sludge or grassland soil**

3

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

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

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

48

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50 It is common knowledge that photolitho-autotrophic algae as primary producers are at the root of complex food
51 webs, which further consists of heterotrophic consumers. As already pointed out by Cole (1) in his classic review
52 on interactions between algae and heterotrophic bacteria in aquatic ecosystems, several mechanisms may be
53 involved in the transfer of organic matter from the algae to the heterotrophic microorganisms: 1) microbes may
54 parasitize on algal cells, 2) microbes might profit from the lysis of death algal cells, and 3) microbes may
55 consume carbon released during algal cell growth. This process in which organic carbon is transferred from
56 algae to heterotrophic microorganisms is also known as the microbial loop (2). Microbial food webs are not only
57 supported by photolitho-autotrophic algae, but may also be sustained by chemolitho-autotrophic microorganisms
58 that grow at the expense of reduced inorganic compounds. The carrying capacity of a chemolitho-autotrophic
59 community can be large. For example, members of the chemolitho-autotrophic, nitrite-oxidizing *Nitrospinae*
60 phylum fix 15 – 45% of the inorganic carbon in the dark ocean (3). In addition, Kindaichi and colleagues
61 showed that a chemolitho-autotrophic nitrifying community, which comprised both ammonia- and nitrite-
62 oxidizing bacteria, supported an equally large heterotrophic community in a carbon-limited biofilm fed with only
63 ammonium (4). Although less is known about the food web rooted on chemolitho-autotrophs, the mechanisms of
64 carbon transfer are likely the same as observed in food webs that are supported by photolitho-autotrophs, *i.e.,*
65 parasitism, and consumption of organic compounds released from lysed or active cells. In a follow-up of the
66 study of Kindaichi and colleagues mentioned above, the same authors showed by application of
67 microautoradiography combined with fluorescence *in situ* hybridization that the heterotrophic community used
68 both dead biomass and metabolites of the chemolitho-autotrophic nitrifying community (5). Functions that
69 facilitate the processing of carbon compounds from chemolitho-autotrophic microorganisms should be present in
70 every ecosystem where these chemolitho-autotrophs are active as these microorganisms shape their own
71 chemical environment by producing organic compounds from carbon dioxide. However, due to deterministic
72 factors, such as species traits, interspecies interactions and environmental conditions, as well as stochastic
73 factors, such as colonization, extinction and speciation, each ecosystem with its own intrinsic environmental
74 characteristics will have its specific community of microbial species (6). Hence, it is hypothesized that the
75 assembly of a new community of heterotrophic, escorting or satellite microorganisms around a chemolitho-
76 autotrophic bacterium depends on the ecosystem from which the heterotrophs are picked.

77 To test this hypothesis, we inoculated pure cultures of *Nitrobacter winogradskyi* that are actively growing in
78 strictly nitrite-fed bioreactors with diluted sludge from a sewage treatment plant for domestic wastewater or with
79 a suspension from a calcareous grassland soil. 16S rRNA gene amplicon sequence analysis was applied to follow

80 the assembly of communities of heterotrophic, escorting microorganisms around *N. winogradskyi* during the
81 enrichment runs. Shotgun metagenomics was employed to establish the sets of functional genes that emerge in
82 the different enrichment cultures. Since actively growing and CO_2-assimilating *N. winogradskyi* is the only
83 source of carbon in the different enrichment runs, the pool of organic compounds produced should be
84 independent on the ecosystem from which the escorting communities were derived and so should be the sets of
85 emerging functional genes. Continuously fed bioreactors are an excellent choice for studying interspecies
86 interactions as non-participating species that are present in the inoculum will leave the bioreactor in the absence
87 of a suitable carbon and energy source. Only heterotrophic microorganisms that grow at the expense of organic
88 carbon produced by *N. winogradskyi* will survive the dilution in the bioreactor with freshly applied mineral
89 medium. At the same time, active *N. winogradskyi* cells will maintain the concentration of nitrite in the
90 bioreactors below the toxicity level of most bacteria (7). The carbon compounds originally present in the
91 inoculum will be diluted with the same washout rate. In our experiments, the enrichments were finished after 10
92 volume changes. At this time, 0.0005% of the microbial cells that were originally present in the slurries will still
93 be present, while the rest of the heterotrophic microbial community will consist of microbes growing at the
94 expense of carbon compounds derived from the chemolitho-autotrophs. The same percentage applies for the
95 carbon compounds that were originally present in the soil and in the sludge.

96 ## RESULTS

97 **Bioreactor performance.** In the first series of enrichments with runs Sewage 1, Sewage 2, and Soil 1, it took a
98 number of volume changes before the ammonium concentrations in the enrichment culture reached their
99 minimum values being on average 0.04 (± 0.01 s.d.) mM from the 6th volume change onward (Fig. 1). The nitrite
100 concentrations in the enrichments remained low from the start on, but accumulated occasionally for a short
101 period of time. Interrupting the supply of nitrite from the reservoir to the culture for a few hours was sufficient to
102 stimulate nitrite oxidation again, even in the case of run Soil 1 where nitrite once accumulated overnight until
103 1.92 mM.

104 In the second series of enrichments with runs Sewage 3, Soil 2 and Soil 3, the behavior of the mineral nitrogen
105 concentrations was as expected with an almost complete conversion of nitrite and ammonium to nitrate without
106 notable accumulation of nitrite (Fig. 1). The ammonium concentrations left in the cultures was on average 0.04
107 (± 0.01 s.d.) mM from the 6th volume change on.

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

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

117 **16S rRNA gene amplicons: *N. winogradskyi* versus escorting microorganisms.** The MiSeq analyses yielded
118 489,927 amplicons of the partial 16S rRNA gene (range 1055 – 27,438) from 53 different samples collected at
119 the start of each enrichment run or taken after each volume change during the runs. Whereas most DNA samples
120 yielded 16S rRNA gene amplicons, no amplicons were obtained from run Sewage 1 except from the sample
121 collected after 10 volumes changes. For this reason, run Sewage 1 has been omitted from further calculations
122 with the 16S rRNA gene amplicons. Based on 97% mutual similarity, 383 Operational Taxonomic Units (OTUs)
123 could be distinguished. The OTU, which was affiliated with *N. winogradskyi*, comprised on average 53.9% of
124 the total community of OTUs. The remaining OTUs belonged to taxa that had been introduced with the
125 inoculum and will be further referred as *N. winogradskyi*-escorting microorganisms or shortly as escorting
126 microorganisms. Of the OTUs belonging to the escorting microorganisms, 294 were affiliated with Bacteria, 2
127 with Archaea (both members of the family Halobacteriaceae), whereas 87 OTUs could not be assigned to
128 Archaea or Bacteria.

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

135 The percentage of escorting microorganisms increased during the enrichment runs. The median percentage of
136 amplicons affiliated with escorting bacteria at the start of the enrichments amounted to 8.1%, but quickly

137 increased to median percentages of 37.8 – 56.3% after two volume changes (Fig. S2 in the supplemental
138 material). However, due to the large variation in the percentages of amplicons affiliated with escorting
139 microorganisms, only the median values measured after 2, 4, 5, 6, and 8 volume changes were significant
140 different from those observed at the start ($p < 0.05$, Dunn's post hoc test). The nature of the inoculum, *i.e.,*
141 diluted sewage sludge or grassland soil suspension, had no significant effect on the median percentages of
142 escorting bacteria (Kruskal-Wallis test: H = 13.49, $p = 0.1971$).

143 **Diversity within assemblies of escorting microbes based on 16S rRNA gene amplicons.** The composition of
144 the original communities of escorting microbial communities at the start of the enrichment runs can be retrieved
145 from the 16S rRNA gene amplicons generated at time zero. In total, 66 and 47 different OTUs were detected at
146 the start of the various runs inoculated with sewage and soil, respectively. No significant effect of the origin of
147 the starting material, *i.e.*, sewage or soil, was observed on alpha diversity indices in the starting communities
148 (Table S1 in the supplemental material). With respect to beta diversity, no significant differences in escorting
149 community composition were observed between enrichment runs that started with diluted sewage sludge or with
150 a grassland soil suspension (one-way ANOSIM test: R = -0.4167, $p = 1.0000$). While 8 of the 25 OTUs that were
151 most dissimilar between sewage- and soil-based enrichment runs, were found in both the sewage- and the soil-
152 based communities, 5 were only found in sewage-based enrichment runs and 12 in soil-based runs (Table S2 in
153 the supplemental material). With 11 unassigned OTUs, the list of the 25 most dissimilar OTUs sewage- and soil-
154 based enrichment runs contained relatively a large number of non-classified OTUs (Table S3 in the supplemental
155 material). Of these 11 unassigned OTUs, 6 appeared only in the soil-based enrichment runs (*i.e.,* OTUs 075, 135,
156 279, 410, 730 and 804), 4 only in the sewage-based runs (*i.e.,* OTUs 123, 189, 535 and 793), while OTU402 was
157 found in both sewage- and soil-based runs.

158 The development of escorting communities was rather non-linear, although large differences in the direction and
159 the size of community changes were observed between the individual enrichment runs as can be seen in a biplot
160 of the results of a Principal Coordinates Analysis (PCoA) of the composition of the escorting communities
161 during the enrichment runs (Fig. 2). The variance explained by both axes in this biplot is 34.8% (20.4 and
162 14.4%, respectively) of the total variance observed between the escorting communities. The communities
163 detected after 2 or more volume changes were significantly different from the initial escorting communities
164 observed at the start (Dunn's post hoc test, $p < 0.05$), but they were not significantly different from each other.
165 After two volume changes, all enrichments reached apparently a less dynamic phase, in which the changes in
166 community structure were relatively small. In this phase, the runs were significantly different from each other

167 (one-way ANOIM test: R = 0.9957, p = 0.0001). The origin of the inoculums, *i.e.*, sludge or soil, had a
168 significant effect on the community composition of the escorting microorganisms in the less dynamic stage of
169 the runs (one-way ANOSIM test: R = 0.2485, p = 0.0003). However, any combination of 2 and 3 random runs
170 gave a significant effect on the community composition of the escorting microorganisms after two volume
171 changes in the reactors. Hence, likely due to the largely stochastic nature of the enrichment runs, we were not
172 able to show a definite effect of sample origin of their outcomes. Noteworthy in Figure 2, is the separation of the
173 enrichment runs in two groups being more or less affected by the X-axis. The group that was affected by the X-
174 axis all originate from runs in the first series of enrichments, while the second group comes from the second
175 series. The composition of the communities of escorting microorganisms enriched in the final phase of the
176 enrichment runs were significantly affected by these "PCoA groups" (one-way ANOSIM test: R = 0.4672, p =
177 0.001). But again, each combination of 2 and 3 random runs gave a significant difference in community
178 composition of the escorting microorganisms between the two groups. In addition, no significant difference in
179 community composition was found between the two series at the start of the enrichment runs (one-way
180 ANOSIM test: R = 0.6667, p = 0.2110).

181 In the period starting after 2 volume changes in the bioreactors, 250 and 310 different OTUs were detected in
182 total in the enrichment runs that were inoculated with sewage and soil, respectively. The 25 OTUs that were
183 most dissimilar between sewage- and soil-based enrichment runs, contributed 71.7% to the total dissimilarity
184 between both types of runs (Table 1). The most dissimilar OTU between sewage and soil was OTU444, which
185 mainly appeared in sewage-based runs and which is affiliated with the genus *Zoogloea* of the class
186 Betaproteobacteria (Table S4 in the supplemental material). This main dissimilar OTU was followed by
187 OTU384, which emerged predominantly in the soil-based runs and is affiliated with the genus *Acinetobacter* of
188 the class Gammaproteobacteria (Table S4 in the supplemental material). In comparison with the list of 25 most
189 dissimilar OTUs between sewage and soil-based runs from the start of the enrichment runs (Table S3 in the
190 supplemental material), the list of 25 most dissimilar OTUs from the period after two volume changes did not
191 contain unassigned microbial taxa any more (Table S4 in the supplemental material). Of the original list of 25
192 most dissimilar OTUs from the start, only 6 were recovered in the list of 25 most dissimilar OTUs after 2
193 volume changes in the bioreactors, *i.e.*, OTUs 039, 200, 204, 317, 614 and 705. These OTUs are affiliated with
194 an unassigned genus of the family Rhizobiaceae, the genus *Stenotrophomonas*, an unassigned genus of the
195 family Intrasporangiaceae, the genus *Rhodococcu*s, the genus *Deinococcus*, and the genus *Nitrosomonas*,
196 respectively (Table S4 in the supplemental material).

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

200 **Functional homologs.** Shotgun sequencing on the total DNA collected from each bioreactor after 10 volume
201 changes, *i.e.,* at the end of the enrichment runs enabled also the exploration of differences in functional
202 homologs between the enrichment runs. Overall, the sequencing yielded read lengths of 227 to 336 Mbp
203 distributed into 931,468 to 1,368,353 reads after quality control (Table S6 in the supplemental material). The
204 distribution of the different KEGG metabolism genes were similar between soil- and sewage-based samples (Fig.
205 S3 in the supplemental material). The analyses of Classical Univariate Statistical Comparisons using the Mann-
206 Whitney/Kruskal-Wallis test showed that 16 functional orthologous functions (KOs) seemed to be statistically
207 different ($p < 0.05$) between sewage- and soil-based samples. However, when adjusting the p value to FDR (*i.e.,*
208 false discovery rate) of 0.05, no statistical differences were observed between the two types of origin. The
209 similar patterns between soil- and sewage-based samples based on metagenome profile are shown in Principle
210 Component Analysis (Fig. 3) and Heatmap Analysis (Fig. 4). In the metagenome dataset, a total of 35 KOs were
211 only present in the *N. winogradskyi* reference genome (Table S7 in the supplemental material), while a total of
212 5,644 KOs were found that were specific for the escorting community (Fig. S4 in the supplemental material).

213 **Taxonomy based on metagenomic sequencing.** The shotgun metagenomes used for comparing functional
214 homologs between enrichment runs lend themselves also for a taxonomic analysis between the runs. In total,
215 5479 different taxa were detected, of which 384 were removed before the taxonomic analyses started because
216 they were classified as animals, diatoms, fungi, plants and protists or because they amounted to less than 10
217 copies in 1 or 2 enrichment runs. The remaining 5095 prokaryotic taxa belonged mainly to the Bacteria (95.8%),
218 while a smaller part belonged to the Archaea (4.2%). Of the alpha diversity indices based on shotgun libraries of
219 the individual enrichment runs, only the number of individuals was significantly affected by the origin of the
220 starting material with lower numbers in the sewage-based runs (Table S8 in the supplemental material). In
221 contrast, all alpha diversity indices obtained after 10 volume changes were significantly affected by the library of
222 choice, *i.e.*, 16S rRNA amplicons or shotgun metagenomics (Table S9 in the supplemental material). Except for
223 the Evenness index, all indices were significantly larger for the shotgun libraries.

224 The top-25 list of taxa that are most dissimilar between the enrichment runs that started with sewage or soil
225 represented 45.7% of the overall average dissimilarity between these series (Table 2). Remarkably, two genera

226 stand out in the top-25 list, *i.e.*, *Acidovorax* and *Sphingopyxis* belonging to the bacterial phyla Betaproteobacteria
227 and Alphaproteobacteria, respectively (Table S10 in the supplemental material). With the exception of
228 *Sphingopyxis* sp. QXT-31, *Sphingopyxis* species were most prominent in sewage-based runs. In contrast,
229 *Acidovorax* species were most noticeable in soil-based runs.

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

238 DISCUSSION

239 The outcome of the enrichment runs disproved the hypothesis that the assembly of a heterotrophic microbiome
240 of *N. winogradskyi* should be dependent on the origin of the heterotrophic microorganisms. The highly
241 stochastic nature of the individual enrichments makes it impossible to demonstrate a significant effect of the
242 origin of the starting material, which was either diluted sewage sludge or a grassland soil suspension, on the
243 outcome of the enrichments. However, despite the large taxonomic variation observed between the outcome of
244 the enrichment runs, we could demonstrate that the distribution of KEGG functional homologs or genes was
245 largely similar for all runs and hence apparently independent on the composition of the escorting community.
246 Although not triggered by the choice of starting material, dissimilar microbial communities do possess similar
247 functional genes.

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

252 **The role of substrate kinetics during community assembly.** The establishment of escorting communities of
253 microbes in an environment created by actively growing *N. winogradskyi* cells that was hitherto free of
254 heterotrophic microorganisms depends on deterministic and stochastic factors (6). It is likely that only those

255 microbes survive washout in the bioreactors that grow at the expense of organic carbon produced from CO_2 by
256 *N. winogradskyi*. Hence, deterministic factors as species traits and interspecies interactions will have played their
257 role in the establishment of new communities from diluted sewage sludge and grassland soil suspension.
258 However, stochastic factors such as species composition of the inoculum played also their role. The original
259 sewage and soil samples were 5 * 10^{-8} times diluted at the start of the enrichment runs, which may have caused
260 differences in species composition at the start. It is less likely that the conservation of samples of diluted sewage
261 sludge and soil suspensions in glycerol at -80° C would have caused the differences. Whenever this way of
262 conservation would have selected for certain species, it would have been the same for each enrichment run.
263 Hence, stochastic factors such as historical contingency or priority effects played also their role in the assembly
264 of new communities.

265 At the time of inoculation, the heterotrophic microorganisms from sewage and soil encountered an environment
266 largely created by the nitrite-oxidizing *N. winogradskyi*. This environment, which likely contained an excess of
267 organic compounds produced by this nitrite oxidizer, enables the selection of heterotrophic microorganisms
268 based on growth rates. In this first phase of the enrichments, the selection of fast-growing heterotrophic
269 microorganisms seems to be a stochastic process and relatively large changes in community composition can
270 occur during the first two volume changes in the bioreactors as can be observed from the PCoA analysis. After
271 this initial period changes in the compositions of the escorting communities became less dynamic. Bittleston and
272 colleagues (8) who studied the assembly of communities in microcosms containing bacterial communities
273 collected from wild pitchers of the carnivorous pitcher plant *Sarracenia purpurea*, observed that the diversity of
274 assembled communities is determined by the diversity at early pre-assembly stages. Only after one transfer in the
275 microcosms, the non-metabolically active species are pruned and the final diversity of the community is
276 determined. Hence, like in our experiment, long-lasting effects of early conditions and biota led to strong
277 differences in final community composition and ecosystem function emphasizing the importance of historical
278 contingency.

279 At the time of more stable escorting communities, the supply of organic compounds produced by the nitrite-
280 oxidizing *N. winogradskyi* became likely growth-limiting for the heterotrophic microorganisms and at the same
281 time dependent on the rate of release of organic compounds from the nitrite-oxidizing *N. winogradskyi*, that
282 grew at a growth rate similar to the dilution rate in the bioreactor. Such an imposed growth rate is likely much
283 lower than the (sub)maximum growth rates of the heterotrophic microorganisms reached in the first phase of the
284 enrichments. Under such conditions of constant low growth rates, *i.e.*, 0.0014 h^{-1}, as realized in the bioreactor by

285 pumping continuously fresh mineral medium, the affinity of heterotrophic microorganisms for organic
286 compounds becomes an important selection factor (9). However, according to the concept of specific affinity as
287 formulated by Healey (10), a low affinity for a compound is compensated by a high maximum substrate uptake
288 rate. Because of this, heterotrophic species with a high relative biomass remain competitive with respect to
289 scavenging low amounts of organic compounds released by the nitrite oxidizer and the community composition
290 will consequently hardly change.

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

311 Notwithstanding, the stochastic enrichment of heterotrophic communities in our strictly nitrite-fed bioreactors,
312 the composition of the enriched functions as represented by the enriched functional genes was always similar
313 and independent on the origin and the composition of the enriched heterotrophic community. Hence, the
314 different taxonomic guilds that had been enriched had the same functions regardless of its origin. Navarrete et al.

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

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

342 **Conclusions.** We demonstrated that despite the largely stochastic assembly of communities of escorting
343 microorganisms in continuously nitrite-fed bioreactors, the functional compositions of the communities were

344 statistically not different between soil- and sludge-based enrichments, suggesting a large redundancy in

345 functional characteristics among the enriched heterotrophic microorganisms.

346 MATERIALS AND METHODS

347 **Origin of the microorganisms.** The basis of the enrichment experiments consisted of a pure culture of

348 *Nitrobacter winogradskyi* strain ATCC 25391, which is growing in mineral medium with sodium nitrite as

349 energy source. The suspensions of heterotrophic microorganisms originated from a calcareous grassland soil

350 (upper 5 cm) near the village of Brummen in the floodplain of the River IJssel or from sewage sludge of a

351 municipal wastewater treatment plant near the village of Bennekom, respectively. Both locations are situated in

352 the center of the Netherlands. The Brummen soil had been used before by our group in nitrification related

353 studies (18)(19)(20). Immediately after sampling in July 2011, the soil and sludge samples were stepwise 10,000

354 times diluted in Milli-Q water and frozen in glycerol at a ratio of 1:1 (v/v) at -80 ^{o}C in Eppendorf cups of 1.5 ml.

355 Freezing and conservation in glycerol at -80 ^{o}C may have killed some species, but the composition of the

356 community of surviving species will be the same at every inoculation of the bioreactors over longer periods of

357 time. For each enrichment run, which may start several months after each other, one cup was defrosted and

358 added to the bioreactors as described below.

359 **Enrichment experiments.** The enrichment experiments were performed in 3-liter bioreactors (ADI 1010

360 Bioreactor, Applikon Biotechnology, UK) without any organic carbon source added. The mineral medium

361 consisted of 20 mM sodium nitrite, 0.1 mM ammonium chloride, 10 mM sodium chloride, 1 mM potassium

362 chloride, 0.2 mM magnesium sulfate heptahydrate, 1 mM calcium chloride dihydrate, and 1 ml L^{-1} of a trace

363 element solution (21), all in demineralized water. Ammonium was always added to prevent selection in favor of

364 nitrite- and nitrate-assimilating heterotrophic microorganisms. After autoclaving of the bulk of mineral medium,

365 sterile 0.4 mM mineral potassium dihydrogen phosphate was added. The complete mineral medium was

366 continuously pumped at a dilution rate of 0.014 h^{-1} into the bioreactor, which had a working volume of 1.5 L.

367 The pH was maintained at pH 7.5 with a combination of 1M hydrochloric acid and 1M sodium hydrogen

368 carbonate, while the temperature was set at 25 ^{0}C. The nitrite concentration in the culture was checked daily by a

369 colorimetric method and when the nitrite concentration exceeded 0.05 mM, the supply of nitrite from the

370 reservoir to the culture was interrupted for a few hours. In this time period, nitrite oxidation improved and the

371 level of nitrite in the bioreactor declined again to non-detectable concentrations.

372 The enrichment experiments started by inoculating a 1.5 L nitrite-fed, actively growing *N. winogradskyi* culture
373 with a 1.5 ml defrosted diluted sludge or soil suspension. Hence, the original sludge and soil samples were 5 *
374 10^{-8} times diluted at the start of the experiments. The enrichments were performed in 2 series of 3 runs each. The
375 first series with the runs Sewage 1, Sewage 2 and Soil 1 were completed in October – November 2013, and the
376 second series with the runs sewage 3, Soil 2 and Soil 3 in February – April 2015. Hence, each series consisted of
377 runs with diluted sewage and with soil suspensions to exclude a specific effect of time on the outcome of the
378 enrichment runs with sewage and soil samples, respectively. At the end, the enrichments series with diluted
379 sewage sludge and those with a grassland soil suspension, consisted both of triplicate runs. Immediately after
380 inoculation and again after each volume change, 15 ml samples were collected from the bioreactors, immediately
381 filtered through 0.22 µm nitrocellulose membrane filters (Merck Millipore Ltd, Tullagreen, Carrigtwohil, Cork,
382 Ireland) and stored at 4 °C in sterile 15 ml polypropylene tubes (Greiner Bio-One, Alphen aan de Rijn, the
383 Netherlands). The filters themselves were stored at -80 °C for DNA extraction. After 10 volume changes, a
384 larger sample was taken for DNA shotgun analysis.

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

388 **DNA extraction.** Total genomic DNA was extracted from the filtered material by homogenizing one broken
389 filter in 1 ml cetyltrimethylammonium bromide (CTAB) buffer in MP Lysing Matrix tubes (MP Biomedicals,
390 Santa Ana, California, USA), subjected twice to disruption by bead-beating at a 5.0 m/s rotation for 60 s, and
391 incubated at 37° C for 30 min in the presence of 5 µl proteinase K (20 mg/ml), while vortexing every 15 min.
392 The samples were supplemented with 150 µl of 20% sodium dodecyl sulfate (SDS) solution and incubated at 65
393 °C for 1 h in a thermoblock and vortexed every 15 to 20 min. After centrifugation at 10,000 X g for 10 min.,
394 approximately 500 µl the supernatant was collected and directly added to the lysis buffer of the Maxwell®16
395 DNA Tissue extraction kit (Promega, Fitchburg, Wisconsin USA). Automatic purification of genomic DNA was
396 performed using the Tissue DNA extraction program. DNA quality and quantity were good and yielding
397 high quality sequences (both amplicons and shotgun metagenomes). The DNA samples' A260/A280
398 ratios were between 1.7 – 1.9, and the quantity ranged from 15-25 ng/ul.

399 **Library preparation and analysis 16S rRNA gene amplicon sequences.** The primers 515F and 806R (22)
400 containing multiplex tags were used to amplify 16S rRNA partial gene fragment. PCR was carried out using 0.2

401 µl of 0.056 U fast StartExpTaq Polymerase (Roche Applied Sciences, Indianapolis, IN, USA), 2.5µl dNTP
402 (2mM each), 0.25µl of each primer and 1.0 µl of DNA template. Thermocycling conditions were: denaturing at
403 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30s, extension at
404 72 °C for 60 s followed by a final extension at 72 °C for 10 min. As negative control water was used instead of
405 DNA, and as positive control DNA of *Escherichia coli* was used. The PCR products were purified using
406 QIAquick PCR Purification Kit (Qiagen Technologies), checked for quality in agarose gel electrophoresis in
407 TBE buffer and quantified using Fragment analyser TM - Automated CE system (Advanced Analytical
408 Technologies, Inc). Equimolar PCR products were pooled and sequenced using Illumina HiSeq2000 platform
409 (2×150 bp) (BGI Inc. China). PANDASeq (23) was used to merge paired-end reads with a minimum overlap of
410 10 bp and at least a Phred score of 25. Sequences were converted to FASTA format and concatenated into a
411 single file for downstream analyses. MicrobiomeAnalyst (Dhariwal, Chong et al., 2017) was used to determine
412 differences in relative abundances between treatments. Statistical hypothesis tests were performed using the
413 Kruskal-Wallis test followed by Linear Discriminant Analysis to evaluate the relevance or effect size of
414 differential abundant features. The 16S rRNA datasets were rarefied to the same number of sequences per
415 sample and used to construct Bray Curtis dissimilarity matrixes generated using the "phyloseq" package in R.
416 The matrixes were ordinated by Principal Coordinate Analysis (PCoA) and adonis function was used to calculate
417 the Permutational Multivariate Analysis of Variance and verify the strength and statistical significance of groups
418 among soil and swage.

419 **Preparation and analysis shotgun libraries.** The shotgun sequencing of the total DNA was carried out in
420 samples at 10 volume changes to determine the functional potential of the escorting satellite bacteria growing
421 with *N. winogradskyi*. From each of the nine enrichment runs (n=5 grassland soil, n=4 sewage sludge), the total
422 DNA samples were prepared as TruSeq Nano libraries. These shotgun libraries were sequenced as paired-end
423 with 350bp inserts on the Illumina HiSeq 2000 sequencer (Macrogen, South Korea). The shotgun metagenomes
424 were analyzed on a Linux server with 64 nodes and 250 GB RAM. Read processing and analysis was carried out
425 in a snakemake pipeline with Snakemake version 3.7.1 (24), combined with bash and perl scripts (available upon
426 request). The metagenome reads were trimmed of sequencing adapters and trimmed on either end if the base
427 quality was below 20. Further, reads were kept if average quality was above 25, and if the read was longer than
428 50bp following trimming. Reads were separated based on 49-kmer similarity (*Nitrobacter winogradskyi* reads)
429 or not (satellite metagenome reads) to the *N. winogradskyi* genome downloaded from NCBI; the trimming,

430 filtering and separation steps were carried out using bbduk version 35.82 (available at
431 sourceforge.net/projects/bbmap). The separated *N. winogradskyi* reads were classified as 100% *N. winogradskyi*
432 using kraken version 0.10.5-beta (25). Taxonomic compositions of the satellite metagenomes were characterized
433 with diamond blastx version 0.8.20 against the NCBI-nr database (2016-10-04 release) with the Last Common
434 Ancestor algorithm from MEGAN v6.5.8 using the mapping from August 2016. To explore the functional
435 potential of the satellite metagenomes, the satellite reads were compared to the 2014-03-17 KEGG database
436 using the uproc-dna command with UPROC version 1.2.0. From the UPROC results the KEGG orthologous
437 groups present in each sample were visualized using iPath version 2. The lists of KO's were uploaded to the
438 EGGNOG server (26) on Aug-3-2019 using the EGGNOG-mapper v2 (available at http://eggnog-
439 mepper.embl.de/).

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

442 **Statistical analyses.** Significant effects of the origin of the inoculum (*i.e.,* soil or sludge) and time on the
443 composition of the enriched escorting satellite community were tested by one-way Analysis of Similarities
444 (ANOSIM) of the PAST version 4.02 statistical software package (27). The same PAST software package was
445 used to establish significant differences between median values by the Kruskal-Wallis and Dunn's post hoc tests
446 (the data were not normally distributed), to visualize the ordination of the enriched escorting assemblies by
447 Principal Coordination Analysis (PCoA) and to determine to establish explanatory independent variables in
448 relation to community assembly. The shotgun data analyses were carried out using the tools in
449 MicrobiomeAnalyst (28) platform to determine differences in functional profiles between treatments. The data
450 was normalized by cumulative sum scaling (CSS) and the clustering of the samples visualized by Principal
451 Component Analysis (PCA). The hierarchical clustering and heatmap visualization were determined by
452 Euclidean Distance using Ward clustering algorithm. The samples were compared by non-parametric Mann-
453 Whitney/Kruskal-Wallis method with P values adjusted using FDR method. The visualization of the specific
454 KEGGs of satellites and *N. winogradskyi* were carried out in iPath (29).

455

456 ## SUPPLEMENTAL MATERIAL

457 Supplemental material is available online only.

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463 We declare no conflict of interests.

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541

542 **Figure captures**

543 **Figure 1** Concentrations of ammonium (blue), nitrite (orange) and nitrate (grey) measured in samples collected
544 after each volume change during the enrichment runs in nitrite-fed bioreactors with *Nitrobacter winogradskyi*
545 that had been inoculated diluted sewage sludge (runs Sewage 1, Sewage 2 and Sewage 3) or with a grassland soil
546 suspension (runs Soil 1, Soil 2 and Soil 3). Some points are missing in the figure due to exceeding the scale of
547 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
548 missing data, *i.e.*, Run Soil 2, t = 8 and t = 10.

549

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

555

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

560

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

565

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

Taxon	Average dissimilarity[1]	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[3]
OTU650	2.89	3.37	38.30	0.03	0.06	Unassigned[4]
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[5]
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[6]
OTU674	0.90	1.05	66.76	<0.01	0.02	*Lacibacter*
OTU614	0.90	1.04	67.81	-[2]	0.02	*Deinococcus*
OTU217	0.88	1.03	68.83	0.01	0.01	Uncultured[7]
OTU204	0.87	1.01	69.84	0.01	0.02	Unassigned[8]
OTU784	0.82	0.95	70.80	0.01	0.01	*Pseudomonas*
OTU083	0.81	0.94	71.73	0.01	0.01	*Sphingopyxis*

569 [1] Overall average dissimilarity: 86.01; [2] not detected; [3] family Rhizobiaceae; [4] family Comamonadaceae; [5] family
570 Xanthomonadaceae; [6] family Comamonadaceae; [7] family env.OPS 17; [8] family Intrasporangiaceae;
571

572 **Table 2** Mean relative numbers of 16S rRNA amplicons observed in the top-25 of escorting taxa that are most

573 dissimilar between sewage- and soil-based enrichment runs as determined by SIMPER-based analyses in

574 samples collected at the end of the runs (*i.e.,* after 10 volume changes).

575

576

Taxon	Average dissimilarity[1]	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

577 [1] Overall average dissimilarity: 63.06