Unexpected diversity and high abundance of putative nitric oxide dismutase (Nod) genes in contaminated aquifers and wastewater treatment systems

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Abstract

The oxygenic dismutation of NO into N_2 and O_2 has recently been suggested for the anaerobic methanotrophic *Candidatus* Methylomirabilis oxyfera and the alkane-oxidizing gammaproteobacterium HdN1. It represents a new pathway in microbial nitrogen cycling and is catalyzed by a putative NO dismutase (Nod). The formed O_2 enables microbes to employ aerobic catabolic pathways in anoxic habitats, suggesting an ecophysiological niche space of substantial appeal for bioremediation and water treatment. However, it is still unknown whether this physiology is limited to *M. oxyfera* and HdN1, and whether it can be coupled to the oxidation of electron donors other than alkanes. Here, we report first insights into an unexpected diversity and remarkable abundance of *nod* genes in natural and engineered water systems. Phylogenetically diverse *nod* genes were recovered from a range of contaminated aquifers and N-removing wastewater treatment systems. Together with *nod* genes from *M. oxyfera* and HdN1, the novel environmental *nod* sequences formed no less than 6 well-supported phylogenetic clusters, clearly distinct from canonical NO-reductase (qNor and cNor) genes. The abundance of *nod* genes in the investigated samples ranged from $1.6 * 10^7$ to $5.2 * 10^{10}$ copies g^{-1} wet sediment or sludge biomass, accounting for up to 10% of total bacterial 16S rRNA gene counts. In essence, NO dismutation could be a much more widespread physiology than currently perceived. Understanding the controls of this emergent microbial capacity could offer new routes for nitrogen elimination or pollutant remediation in natural and engineered water systems.

Importance statement

NO dismutation into N_2 and O_2 is a novel process, catalyzed by putative NO dismutase (Nod). To date only two bacteria, the anaerobic methane oxidizing bacterium *Methylomirabilis oxyfera* and the alkane-oxidizing gammaproteobacterium HdN1, are known to harbor *nod* genes. In this study, we report the first efficient molecular tools that can detect and quantify a wide diversity of *nod* genes in environmental samples. A surprising high diversity and abundance of *nod* genes was found in contaminated aquifers as well as wastewater treatment systems. This first evidence indicates that NO dismutation may be a much more widespread physiology in natural and man-made environments than currently perceived. The molecular tools presented here will facilitate further studies on these enigmatic microbes in the future.

Introduction

Microbial nitrogen cycling has been intensively investigated for over a century and was thought to be rather well understood. Yet recent discoveries of novel processes and microbes involved in the nitrogen cycle, e.g., methane-dependent nitrite- and nitrate-reduction (1-4), complete ammonia oxidation to nitrate by *Nitrospira spp.* (5, 6), and ammonia oxidizing archaea (7) have demonstrated that our understanding of microbial nitrogen cycling may still be incomplete. Recently, NO dismutation (NOD) to O_2 and N_2 (eq. 1) has been proposed for the anaerobic methanotrophic *Methylomirabilis oxyfera* (NC10 phylum), and may also occur in the alkane-oxidizing gammaproteobacterium HdN1 (3, 8).

$$2\,NO \rightarrow N_2 + O_2 \qquad \Delta G^{o'} = -173.1\ KJ\ (mol\ O_2)^{-1} \qquad \text{(eq. 1)}$$

M. oxyfera oxidizes methane to CO_2 and reduces nitrite via NO to N_2 under strictly anoxic conditions. Interestingly, *M. oxyfera* possesses and highly expresses a complete aerobic methane oxidation pathway, including particulate methane monooxygenase (pMMO; 3). Metagenomic and physiological evidence suggests that the bacterium forms O_2 to support the aerobic oxidation of methane under nitrite-reducing conditions (3). HdN1 grows on C6-C30 alkanes with oxygen as well as nitrate and nitrite as electron acceptors. However, HdN1

does not harbor any fumarate-adding enzymes or other catalysts for anaerobic hydrocarbon activation, and does not produce detectable alkyl-substituted succinates in anaerobically grown cultures (8). Instead, multiple copies of alkane monooxygenase genes were identified as the only means of alkane activation in HdN1. Therefore, HdN1 was suggested to utilize oxygen for its substrate activation when grown on alkanes with nitrate and nitrite as the electron acceptors (8).

In both *M. oxyfera* and HdN1, the O_2 used for substrate activation is thought to be generated via NO dismutation, catalyzed by putative NO dismutases (Nod), which belong to the quinol-dependent NO reductase (qNor) family (9). NO dismutases exhibit amino acid substitutions at positions that are essential for electron transfer in canonical qNor, suggesting an electron-neutral reaction to be catalyzed by Nod (9, 10). In an *M. oxyfera* culture, $^{18}O_2$ was indeed formed as an intermediate from ^{18}O-labelled nitrite during nitrite-dependent methane oxidation (3). However, direct biochemical evidence for this activity of the enzyme is not yet available.

As a next step in addressing the occurrence and potential relevance of Nod-harboring microbes in natural systems, targeted detection assays for the gene or respective transcripts are required. However, the development of such assays is hampered by the extremely low number of reference sequences available for primer design. Two copies of putative *nod* genes have been identified in the genome of *M. oxyfera*, and one in the HdN1 genome (3, 8). Recently, specific primer sets have been developed capable of detecting *M. oxyfera*-affiliated *nod* genes in a methane-oxidizing, nitrite-reducing laboratory reactor inoculated with river sediments (11). Respective transcripts have also been found in water samples taken directly from marine oxygen minimum zones (12). However, evidence for the occurrence of a potentially wider diversity of putative *nod* genes in environmental systems is still lacking. Also, it is still unclear whether NOD can be coupled to the oxidation of electron donors other than alkanes and how important NOD could be in different systems with intensive N-cycling. For example, such populations can be hypothesized to occur in

contaminated aquifers or in wastewater treatment systems. The latter especially offer a wealth of distinct niches for microbes involved in biological nitrogen removal (13).

Here, we provide a primary inquiry of the diversity and abundance of putative *nod* genes in such systems. A suite of primers capable of specifically detecting and quantifying a range of *nod* lineages was developed and highly diverse and abundant environmental *nod* gene pools were recovered. Our results provide first evidence for a wide-spread occurrence and high diversity of putative *nod* genes, suggesting that NOD could be an underestimated component of reductive nitrogen cycling in anthropogenically impacted and engineered water systems.

Materials and methods

Primer design

Since only limited *nod* sequences were available from public databases at the beginning of this study, an iterative improvement was applied during primer development. *M. oxyfera nod* DAMO_2434 (CBE69496), DAMO_2437 (CBE69502), HdN1 *nod* HDN1F_02620 (CBL43845) and two *nod* sequences (KX364454 and KX364455) assembled from the metagenome of a NC10-AAA enrichment culture (4) were aligned with selected *qnor* and *cnorB* sequences in MEGA6 by ClustalW algorithm. Based on this initial *nod* alignment, forward and reverse primers that covered all five *nod* sequences were developed (Table 1). These first primer pairs (combinations A, B, C) were tested using environmental DNA extracted from contaminated aquifer sediments (Table 2). The resulting aquifer *nod*-like sequences were then included in the *nod* alignment. Internal sequence information of the amplicons generated with the first primers was then used to further degenerate and optimize the specificity of a second generation of primers (combinations D, E). Of these, primer set D performed well and was used to recover putative *nod* sequences from different wastewater treatment plants and laboratory-scale reactor systems. The divergent *nod* sequences attained from engineered water systems further extended the *nod* alignment, based on

126 which a final forward primer (nod1446F) was designed to allow qPCR analysis in
127 combination with the reverse primer nod1706Rv2 (primer combination F).

128 **Sampling and sites**

129 The samples used in this study were collected from various sites as listed in Table 2. Siklós
130 sediment samples were collected in April 2015 from the bottom of monitoring well ST2 in the
131 center of a xylene plume in Siklós, Hungary (14, 15), while Flingern sediments were taken by
132 push-coring from the upper fringe of a toluene plume (6.4 m below ground) in Flingern,
133 Düsseldorf, Germany in September 2013 (16, 17). Sediments were transported to the lab in
134 cooling boxes and were then frozen at -20°C before DNA extraction.
135 Samples from wastewater treatment plants (WWTP) and laboratory-scale reactors were
136 collected in September 2015. Activated sludge samples from a WWTP in Garching,
137 Germany next to the Technical University of Munich (TUM) were collected from the
138 denitrifying basin, which receives clarified nitrate-rich effluent from an upstream trickling filter
139 (18). Biofilm carriers from the WWTP in Kempten, Germany were collected from the
140 deammonification basin (19).
141 Biomass samples were also collected from two single-stage deammonification bioreactors,
142 operating under alternating oxic/anoxic conditions (20). Here the deammonification reactor
143 that employed suspended biomass was designated as 'Suspension' and the other, which
144 employed granule biomass, was designated as 'Swing-redox' (Table 2). Biomass samples
145 from a two-stage sequencing batch cascade deammonification system were also analyzed.
146 The system comprises a reactor performing partial nitrification and a reactor performing
147 anammox, which were designated as '2-stage-nitritation' and '2-stage-AMX', respectively
148 (Table 2). Biomass samples were collected from a system implementing the CANDO
149 process (Coupled Aerobic-anoxic Nitrous Decomposition Operation), aiming for
150 simultaneous nitrogen removal, greenhouse gas mitigation and energy recovery (21). The
151 two reactors of the CANDO system, one performing partial nitrification to nitrite and one
152 nitrous denitritation to N_2O, were designated as 'CANDO-nitritation' and 'CANDO-N_2O'

153 respectively (Table 2). A comprehensive redefinition of the nomenclature for biological
154 processes contributing to nitrogen removal in such engineered water systems can be found
155 elsewhere (13).

156 **DNA isolation**

157 DNA was isolated from aquifer sediments as previously described (22) with a minor
158 modification, the final precipitation of DNA was done at 20,000 g and 4 °C for 30 min,
159 instead of 20 °C. For DNA isolation from WWTP and reactor samples, 0.5 - 1.0 ml
160 homogenized biomass or sludge were pipetted into 1.5 ml Eppendorf tubes, which were
161 spun at 13,000 rpm for 1 min. The supernatant was then removed and the remaining
162 biomass was weighed. For samples from the WWTP Kempten, biofilm from carriers was put
163 into 1.5 ml Eppendorf tube and weighed. DNA isolation was done as above (22), omitting the
164 second bead-beating step. DNA was extracted in triplicates from WWTP-TUM, other
165 samples were non-replicated. DNA concentration and quality were checked with by UV-
166 spectrophotometry (Nano-drop ND-1000; Isogen Life Science, The Netherlands) and
167 standard agarose gel electrophoresis.

168 **PCR and qPCR**

169 DNA samples diluted by 10- or 100-fold were used as template for *nod* gene PCR analysis.
170 Primer pairs used are listed in Table 1 and their positioning on the *M. oxyfera nod* gene and
171 the expected amplicon size is given in Fig. 1. To recover a potentially increased *nod* diversity,
172 gradient PCR with the following cycling conditions were performed: a 3 min initial
173 dissociation at 96 °C, followed by 35 cycles of amplification (45 s at 95 °C, 60 s at 52-62 °C,
174 90 s at 72 °C), and a final 5 min extension at 72 °C. All PCRs were performed in 25 µl
175 reactions containing nuclease-free H_2O, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.5
176 U Taq polymerase (all Fermentas GmBH, Basel, Switzerland), 5 µg BSA (Roche Diagnostics
177 GmbH, Basel, Switzerland), 0.5 µM of each primer and 1 µl template DNA. PCR products
178 were checked by standard agarose gel electrophoresis.

179 To quantify potential *nod*-harboring microorganisms and their relative abundance in each
180 sample, qPCR targeting *nod* as well as bacterial 16S rRNA gene was performed, using
181 primer pairs nod1446F / nod1706Rv2 and Ba519F / Ba907R (23), respectively. The
182 specificity of the primer pair nod1446F / nod1706Rv2 was verified by cloning and
183 sequencing its amplicons using Siklós DNA. All 8 sequenced clones were *nod*. Synthetic *nod*
184 gene (440 bp, *M. oxyfera* DAMO_2437) and *E. coli* 16S rRNA gene (980 bp) fragments,
185 covering the respective primer sites with > 60 bp flanking region at each end, were used as
186 respective standards for *nod* and 16S rRNA (gBlocks, Integrated DNA Technologies, Leuven,
187 Belgium). Sample DNA in 10- and 100-fold dilutions for *nod*, and in 100- and 1000-fold
188 dilutions for 16S rRNA was quantified. Standard and samples were quantified in triplicates,
189 repeated in at least two independent qPCR runs for each assay. qPCR experiments (25 µl
190 reaction volume) were carried out with MX3000p cycler (Agilent, Santa Clara, USA). 2x
191 GoTaq SYBR green master mix (Promega, Madison, USA) with Rox as the reference dye
192 was used. qPCR annealing temperature used for *nod* and 16S rRNA were 57 °C and 52 °C,
193 respectively. qPCR analysis with efficiency of 100±10 % were used for calculation. Absolute
194 *nod* and 16S rRNA gene counts of each sample were calculated per $g_{(wet\ weight)}$ of sediment or
195 biomass used in DNA extraction.

196 **Cloning, sequencing and phylogenetic analysis**

197 PCR products of different annealing temperatures from each sample were pooled and
198 purified with PCRextract spin columns (5Prime, Hamburg, Germany) according to the
199 manufacturer's protocol. Purified PCR products were cloned and sequenced as previously
200 described (23). High-quality sequences obtained were translated to amino acids in MEGA6
201 and then aligned with selected qNor and cNor sequences with the ClustralW algorithm with
202 default settings. A phylogenetic tree was constructed based on an amino acid alignment with
203 MEGA6 using the neighbor-joining method. The robustness of the tree topology was tested
204 by bootstrap analysis (1,000 replicates).
205

206 **Nucleotide sequence deposition**

207 Representative *nod* sequences as well as two putative *nor* sequences obtained in this study
208 were deposited at NCBI under the accession numbers KX364418 to KX364453, KX364416,
209 and KX364417 respectively. The two *nod* paralogs assembled from the metagenome of
210 NC10-AAA enrichment (4) can be found under the accession numbers KX364454 and
211 KX364455.

212

213 ## Results

214 **Primers targeting putative *nod* genes**

215 Nod belongs to the quinol-dependent nitric oxide reductase (qNor) family (9). Existing
216 general q*nor*-targeting primers, such as qnorB2F and qnorB5R (22), have critical multiple
217 mismatches to available *nod* sequences. Therefore, the development of suitable *nod*-specific
218 primers was the first objective of this study. Initially, two forward primers (nod631F and
219 nod684F) and four reverse primers (nod1465R, nod1706R, nod1896R and nod2015R)
220 designed to be selective for available *nod* sequences were developed (Fig. 1 and Table 1).
221 PCR with DNA extracted from an *M. oxyfera* enrichment culture yielded the expected
222 amplicon sizes, suggesting the primers to be functional. Based on the extended *nod*
223 alignment including sequences obtained from the two aquifers, adjusted forward (nod684Fv2)
224 and reverse (nod1706Rv2 and nod1896Rv2) primers were iteratively developed and applied
225 to the samples from water treatment systems.

226 **High diversity of environmental *nod* genes**

227 Amplicons of expected size were obtained from all analyzed samples by standard PCR, and
228 amplicons were cloned and sequenced. Altogether, 149 sequences were obtained, of which
229 147 sequences resulted in *M. oxyfera* and HdN1 *nod* genes as top BLASTN matches. Only
230 two sequences (KX364416, WWTP-Kempten clone R2-7 and KX364417, Siklós clone
231 Sik2DC09) were more similar to qNor sequences of microbes within the Fibrobactere-

232 Chlorobi-Bacteroidetes (FCB) superphylum (Fig. 2). This indicated a high specificity of the
233 developed *nod* primers. Together with Nod from NC10 bacteria and HdN1, the
234 environmental Nods (deduced amino acid sequences) formed a phylogenetic cluster distinct
235 from known qNor and cNor with robust bootstrapping support (97%, 1,000 replicates). Six
236 sub-clusters of putative *nod* genes were subsequently classified, tentatively named after the
237 organism or habitat where they were first discovered (Fig. 2).
238 Almost all *nod* sequences recovered from the Siklós aquifer (using initial primer
239 combinations A, B and C) showed high nucleotide similarity (84 - 99%) to one of the two *nod*
240 genes of *M. oxyfera*. The total number of clones that were either more closely related to
241 DAMO_2437 (18 clones) or to DAMO_2434 (17 clones) was nearly equal, suggesting that
242 those *nod* sequences could originate from *M. oxyfera*-related microbes with two *nod*
243 paralogs in their genomes. In contrast, all *nod* sequences retrieved from the Flingern aquifer
244 (primer pair A; ~835 bp) were nearly identical (>99%) and exhibited only low sequence
245 similarity to *nod* of both *M. oxyfera* (70 - 71% and 60 - 61%) and HdN1 (69% and 64%) on
246 nucleotide and amino acid level, respectively. This novel cluster of unidentified
247 environmental *nod* genes was tentatively named “aquifer cluster” (Fig. 2).
248 Most of the *nod* sequences obtained from the coupled two-stage cascade deammonification
249 reactors (2-stage-nitritation, 2-stage-AMX) as well as 2 clones from the ‘Swing-redox’ reactor
250 shared >98% nucleotide similarity with the *nod* gene of HdN1 (Figs. 2, 3). The other 11
251 clones from the ‘Swing-redox’ reactor system were surprisingly diverse (Fig. 3), comprising
252 not only the previously discovered “aquifer cluster”, but also two novel “reactor clusters”,
253 which were only distantly related to *nod* gene sequences of *M. oxyfera* (66% - 77%) and
254 HdN1 (65 - 69%). *Nod* gene pools within the ‘CANDO-N_2O’ and ‘Suspension’ reactors were
255 again mostly affiliated to the aquifer cluster, but also fell within the reactor clusters. Finally, a
256 novel “WWTP cluster” was identified to dominate the *nod* gene pool in the WWTP-TUM plant,
257 while samples from the WWTP in Kempten comprised the highest diversity of *nod* genes
258 amongst all samples analyzed, with no less than 5 *nod* clusters detected (Fig. 3).

259 **Nod gene abundance**

260 The abundance of *nod* genes in the investigated environments was notable (Fig. 4). In
261 aquifer samples, *nod* abundance was ~1.6 * 10^7 copies g^{-1} for Flingern sediments, and ~5 *
262 10^8 copies for Siklós well sludge. At both sites, *nod* genes accounted for ~2% of total
263 bacterial 16S rRNA gene counts. In the engineered water systems, absolute *nod* gene
264 abundance was up to 3 orders of magnitude higher, with a maximum of ~5 * 10^{10} copies g^{-1}
265 of sludge biomass in the CANDO-nitritation reactor (Fig. 4). However, the relative
266 abundance of *nod* vs. bacterial 16S rRNA genes was still in a low range (<5%) for most
267 samples. However, relative *nod* abundance was clearly elevated in the two-stage
268 deammonification reactors (2-stage-nitritation and 2-stage-AMX), accounting for up to 10%
269 of bacterial 16S rRNA genes (Fig. 4).

270 **Characteristics of environmental Nod sequences**

271 In canonical qNor, several functionally essential structures such as the quinol-binding site,
272 proton supply channel and catalytic center are constituted by highly conserved amino acids
273 (24-26), which are often substituted for variable amino acids in Nod. These amino acid
274 deviations arguably disfavor a role of Nod as NO reductase, and have been considered as
275 signatures for the function of NO disproportionation (9, 10). All these characteristic
276 substitutions were also consistent in the environmental Nod sequences recovered in this
277 study (Fig. 5, and the more extensive alignment of the environmental Nod sequences shown
278 in Supplementary Fig. S1).
279 Specifically, His328 and Asp746 (*G. stearothermophilus* qNor numbering) form hydrogen
280 bonds with NO and OH groups of quinol respectively, coordinated by Glu332 and Phe336
281 (25). In Nod, however, these highly conserved residues are replaced by variable amino acids
282 that are unlikely to provide proper quinol-binding capability (9, 10). Consistently, all
283 environmental Nod sequences covering the quinol-binding region had deviations at sites of
284 His328, Glu332 and Phe336 (Fig. 5). Information for site Asp746 is unfortunately not
285 covered by the length of our environmental Nod sequences. Furthermore, one of the three

non-heme metal (often Fe_B) coordinating His residues in qNor, His560, was consistently replaced by asparagine in Nod (Fig. 5), possibly leading to an altered active site configuration. One of the two highly conserved glutamate residues (Glu581) suggested as potential terminal proton donors in qNor (25) was exchanged for a glutamine in Nod, which was also observed for all environmental Nod sequences covering that region (Fig. 5 and Fig. S1). Intriguingly, the two more deeply-branching unknown Nor-related sequences retrieved in this study, as well as the genomic sequences discovered via BLAST (all of them were annotated as Nor, including genes of *Muricauda ruestringensis* and *Arenibacter algicola*) in the same phylogenetic cluster (Fig. 2) possessed the same substitutions as Nod at the catalytic site and partially also at the quinol-binding site (Fig. 5).

Discussion

Detecting environmental *nod* genes

Nitric oxide dismutation (NOD) to O_2 and N_2 (eq. 1) is an emergent process and a potential ecophysiology not well documented for biological systems to date. Genes of the putative NO dismutase (Nod) were first reported for *M. oxyfera* and HdN1 (3, 8). Although several other related laboratory enrichments have been described (e.g., 27-30), information on environmental *nod* gene occurrence remains scarce. Relatively short (329-426 bp) *nod* gene sequences closely related to that of *M. oxyfera* were recently reported from a nitrite-reducing, methane-oxidizing laboratory reactor inoculated with river sediments (11). Closely related *nod* gene transcripts have also been found directly in total transcriptome libraries from marine oxygen minimum zones (12). Still, a comparative survey of *nod* gene pools in different natural habitats has not been conducted to date.

In this study, we provide primary evidence for the existence of an extensive diversity of *nod* genes in a range of natural and engineered water systems (Table 1, Fig. 3). Although the employed *nod* primers were initially developed from a very limited number of reference sequences, environmental *nod* genes clearly distinct to those of *M. oxyfera* and HdN1 were

313 successfully detected from the contaminated aquifers, which were investigated with the first
314 generation of primers. Only the Siklós site was queried with the three initial primer pairs
315 (Table 2). However, differences in the affiliation of resulting *nod* libraries were not observed,
316 suggesting a similar performance of the assays. The improved primer pair D (nod684Fv2 /
317 nod1706Rv2) was then developed iteratively, based on actual environmental *nod* sequence
318 data obtained with the first assays. It covers a ~1000 bp region and several distinctive sites
319 of the *M. oxyfera nod* gene, and was capable of recovering a surprising diversity of putative
320 *nod* gene lineages from the engineered water systems (Fig. 3). Based on these results, the
321 use of primer pair D for recovering *nod* gene diversities from environmental samples is
322 recommended.

323 **Diversity and abundance of environmental *nod* gene lineages**

324 This study provides a proof-of-concept of the general detectability and diversity of putative
325 *nod* gene pools in terrestrial water systems. A 'classical' cloning-and-sequencing approach
326 was chosen, as it allows for more direct and less cost-intensive rounds in iterative primer
327 development compared to next-generation sequencing. The read length of Sanger
328 sequencing was clearly also beneficial to retrieve full sequence information from some of the
329 rather long amplicons (>1000 bp) generated. Still, we are aware that the small size of some
330 of our clone libraries prohibits more elaborate statistical comparison of the *nod* gene pools
331 recovered. Future studies should incorporate a NGS-strategy also for this gene marker.
332 Still, of all samples analyzed, the WWTP in Kempten and the 'Swing-redox' reactor samples
333 appeared most diverse, spanning 5 and 4 of the proposed 6 *nod* gene clusters, respectively
334 (Fig. 3). Both are biological nutrient removal systems removing nitrogen by partial nitritation
335 followed by anammox (19, 20, 31). Biomass samples were from thick biofilm carriers for
336 Kempten and large biomass granules from the 'Swing-redox' reactor. Thus, *nod* gene
337 diversity potentially reflected the typically high structural and physicochemical heterogeneity
338 of such habitats (32).

339 The *nod* gene pools recovered from the two BTEX-impacted aquifers were strikingly different,
340 possibly reflecting distinct contamination and redox scenarios at the sites (14-16). While the
341 absolute abundance of *nod* gene was one order of magnitude higher in Siklós than that in
342 Flingern (Fig. 4), relative *nod* gene abundance was comparable in both aquifers. This can
343 likely be explained by the distinct nature of the samples, with more organic well sump
344 material being taken in Siklós, while highly mineral sediments were taken in Flingern. The
345 absolute abundance of bacterial 16S rRNA gene counts at the Flingern site was consistent
346 with previous studies (16, 23), but the relative abundance of *nod* genes in both aquifers and
347 most of the wastewater samples was only a few percent. This is within the typical range of
348 the relative abundance of other nitrogen cycling genes that have been found in activated
349 sludge (33). In contrast, relative *nod* gene abundance was clearly elevated in the coupled '2-
350 stage-nitritation' and '2-stage-AMX' reactors, where *nod* genes accounted for up to 10% of
351 bacterial 16S rRNA gene counts. This was intriguing given that these reactors were
352 designed for partial nitrification and anammox, and a high abundance of anammox
353 organisms can be assumed. The affiliation of the *nod* genes detected in these systems
354 should be subject of further investigation.

355 **Functionality of environmental Nod**

356 Quinol-dependent NO reductase (qNor) reduces NO to N_2O with electrons accepted from
357 quinol (25). Although Nod belongs to the qNor family, it lacks proper quinol-binding sites and
358 has altered catalytic center configuration. This compromises an activity of Nod as a
359 conventional NO reductase and has been discussed as a possible signature of a role in NO
360 disproportionation (9, 10). All environmental Nod sequences recovered in this study
361 possessed similar substitutions as found in the genes of *M. oxyfera* and HdN1 (Figs. 4 and
362 S1), supporting that putative environmental *nod* genes were actually recovered, although we
363 cannot provide direct evidence for NOD activity or actual O_2 formation in this study.
364 Nevertheless, the primary detection of these genes in the investigated water systems is an

365 important prerequisite for follow-up studies on their potential expression and biochemical
366 activity in the future.
367 The two more deeply-branching *nod*-like sequences (KX364416 and KX364417) recovered
368 were in a phylogenetic cluster in-between known qNor and Nod genes, tentatively named as
369 "unknown Nor-related genes" in this study (Fig. 2). This cluster also included genomic
370 sequences of members of the *Fibrobacter-Chlorobi-Bacteroidetes* (FCB) superphylum,
371 recovered via BLAST, all of them annotated as Nor. Intriguingly, these sequences carry the
372 same residual substitutions as Nod at the catalytic site and partially at the quinol-binding site
373 (Fig. 5). Even more surprisingly, the Nor-related gene of *Arenibacter algicola*, an aerobic
374 degrader of polycyclic aromatic hydrocarbons (34), retained all the residual substitutions
375 characteristic for Nod (Fig. 5). To our best knowledge, direct biochemical evidence for the
376 physiological function of any member of this gene cluster is not available. Studying the
377 expression and functioning of these previously unknown Nor-related genes in members of
378 the FCB superphylum can provide valuable further cues on the potential role of the putative
379 *nod* genes detected in this study. Still, we cannot exclude at this stage that these could also
380 just be atypical qNors, using a different electron-supplying mechanisms for NO reduction.
381 Without direct biochemical evidence, clear functional connotations cannot be ascertained for
382 the novel gene clusters detected in the environment. More detailed studies on NO
383 respiration by pure cultures such as HdN1 and *A. algicola* can shed further light on this
384 enigmatic process.

385 **Potential environmental relevance of *nod*-harboring populations**

386 Nitrate/nitrite reduction via NO dismutation can be referred to as 'oxygenic denitrification' to
387 facilitate discussion. It can also be referred to as a potential new oxygenic route in 'nitrous
388 denitritation', in line with the nomenclature proposed for biological nitrogen removal systems
389 (13). It is interesting to consider whether the putative *nod* genes detected in the different
390 systems could be affiliated to microbes other than *M. oxyfera* and HdN1, and to which
391 physiologies they could be connected.

392 Although *M. oxyfera* possesses multiple NO reductases in addition to Nod, the N_2O
393 reductase is missing (3, 10). Therefore, the reduction of NO_2^- to N_2 seems to essentially
394 proceed via NO dismutation in this bacterium. In contrast, HdN1 contains a full canonical
395 denitrification pathway in addition to Nod (8), indicating that conventional and oxygenic
396 denitrification pathways could co-exist in a single microbe. In the environment, it can be
397 anticipated that microorganisms performing oxygenic denitrification will compete for
398 nitrate/nitrite with conventional denitrifiers as well as microbes mediating dissimilatory nitrate
399 reduction to ammonia (DNRA). The niche partitioning between the latter is driven by various
400 environmental controls such as the availability of organic carbon or the ratio of available
401 nitrite to nitrate (35). However, the environmental parameters defining the ecophysiological
402 niche of putative oxygenic denitrifiers are still far from clear.
403 Both *M. oxyfera* and HdN1 lack anaerobic catabolic pathways for their alkane substrates.
404 Thus, they rely on O_2 formed via NO dismutation to activate and oxidize hydrocarbons, when
405 growing under nitrate- and nitrite-reducing conditions. Methane and alkanes are among the
406 most stable compounds that require high energy for activating the first C-H bond (36).
407 Therefore, it can be speculated that O_2 formed via NO dismutation could possibly provide a
408 competitive advantage for oxygenic denitrifiers thriving on recalcitrant compounds in anoxic
409 environments.
410 It is also tempting to hypothesize that oxygenic denitrifiers could theoretically couple aerobic
411 catabolic processes other than alkane oxidation to oxygenesis. With an ability to rely on
412 aerobic catabolism under both aerobic and nitrate/nitrite reduction, oxygenic denitrifiers
413 could capitalize on ecologically advantages under hypoxic or fluctuating redox conditions
414 with transient availability of nitrate/nitrite. While *M. oxyfera* is a strict anaerobe and can be
415 inhibited by short exposure to low levels of O_2 (37), HdN1 is much more versatile, capable of
416 using nitrate, nitrite and O_2 as electron acceptor when growing on alkanes (8). Our finding of
417 a high abundance of *nod* genes nearly identical to HdN1 in several wastewater treatment
418 systems (Figs. 2-4), as well as the fact that HdN1 was initially isolated from activated sludge
419 (38) could suggest a potential relevance of HdN1-relatives in such systems. In the Flingern

aquifer, a previous study has revealed a surprising peak abundance of aerobic toluene monooxygenase (*tmoA*) genes in the highly reduced core of the anoxic BTEX plume (16). The *nod* genes recovered from the Flingern aquifer in the present study could potentially explain this unexpected high abundance of *tmoA* genes in highly reduced sediments with an at least transient supply of nitrate.

Conclusions

Our results reveal a hitherto unrecognized ubiquity and abundance of putative *nod* genes in terrestrial water systems. The wide phylogenetic diversity detected suggests that NOD capacity may exist in microbes other than *M. oxyfera* and HdN1. Although no direct evidence for an actual activity of the detected Nod-harboring populations is provided here, this primary study provides important molecular cues to follow up on this. Attempts to enrich and isolate putative oxygenic denitrifiers with a range of electron donors are currently ongoing, and essential to further substantiate our hypothesis. The fostering of microbes with a capacity for oxygenic denitrification, which may bypass N_2O as an intermediate of canonical NO reduction, could also be an attractive strategy in minimizing problematic N_2O emissions in wastewater treatment (39). A more detailed understanding of populations potentially carrying a capacity for NOD could be vital for a more comprehensive understanding of microbial N-cycling as well as for the development of novel bioremediation strategies and engineering solutions for biological nitrogen removal.

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References

1. **Haroon, M. F., S. Hu, Y. Shi, M. Imelfort, J. Keller, P. Hugenholtz, Z. Yuan, and G. W. Tyson.** 2013. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature **500:**567-570. 10.1038/nature12375.
2. **Raghoebarsing, A. A., A. Pol, K. T. van de Pas-Schoonen, A. J. Smolders, K. F. Ettwig, W. I. Rijpstra, S. Schouten, J. S. Sinninghe Damste, H. J. Op den Camp, M. S. Jetten, and M. Strous.** 2006. A microbial consortium couples anaerobic methane oxidation to denitrification. Nature **440:**918-921. 10.1038/nature04617.
3. **Ettwig, K. F., M. K. Butler, D. Le Paslier, E. Pelletier, S. Mangenot, M. M. Kuypers, F. Schreiber, B. E. Dutilh, J. Zedelius, D. de Beer, J. Gloerich, H. J. Wessels, T. van Alen, F. Luesken, M. L. Wu, K. T. van de Pas-Schoonen, H. J. Op den Camp, E. M. Janssen-Megens, K. J. Francoijs, H. Stunnenberg, J. Weissenbach, M. S. Jetten, and M. Strous.** 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature **464:**543-548. 10.1038/nature08883.
4. **Ettwig, K. F., B. Zhu, D. R. Speth, J. T. Keltjens, M. S. M. Jetten, and B. Kartal.** 2016. Archaea catalzye iron-dependent anaerobic oxidation of methane. Proc Natl Acad Sci USA **113:** 12792-12796..
5. **van Kessel, M. A. H. J., D. R. Speth, M. Albertsen, P. H. Nielsen, H. J. M. Op den Camp, B. Kartal, M. S. M. Jetten, and S. Lucker.** 2015. Complete nitrification by a single microorganism. Nature **528:**555-559. 10.1038/nature16459.
6. **Daims, H., E. V. Lebedeva, P. Pjevac, P. Han, C. Herbold, M. Albertsen, N. Jehmlich, M. Palatinszky, J. Vierheilig, A. Bulaev, R. H. Kirkegaard, M. von Bergen, T. Rattei, B. Bendinger, P. H. Nielsen, and M. Wagner.** 2015. Complete nitrification by Nitrospira bacteria. Nature **528:**504-509. 10.1038/nature16461.
7. **Francis, C. A., J. M. Beman, and M. M. M. Kuypers.** 2007. New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. ISME J **1:**19-27. 10.1038/ismej.2007.8.
8. **Zedelius, J., R. Rabus, O. Grundmann, I. Werner, D. Brodkorb, F. Schreiber, P. Ehrenreich, A. Behrends, H. Wilkes, M. Kube, R. Reinhardt, and F. Widdel.** 2011. Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation. Env Microbiol Rep **3:**125-135. DOI 10.1111/j.1758-2229.2010.00198.x.
9. **Ettwig, K. F., D. R. Speth, J. Reimann, M. L. Wu, M. S. Jetten, and J. T. Keltjens.** 2012. Bacterial oxygen production in the dark. Front Microbiol **3:**273. 10.3389/fmicb.2012.00273.
10. **Reimann, J., M. M. Jetten, and J. Keltjens.** 2015. Metal enzymes in "impossible" microorganisms catalyzing the anaerobic oxidation of ammonium and methane, p. 257-313. *In* P. M. H. Kroneck and M. E. Sosa Torres (ed.), Sustaining life on planet earth: Metalloenzymes mastering dioxygen and other chewy gases, vol. 15. Springer International Publishing. 10.1007/978-3-319-12415-5_7.

11. **Bhattacharjee, A. S., A. M. Motlagh, M. S. Jetten, and R. Goel.** 2016. Methane dependent denitrification- from ecosystem to laboratory-scale enrichment for engineering applications. Water Res **99:**244-252. 10.1016/j.watres.2016.04.070.
12. **Padilla, C. C., L. A. Bristow, N. Sarode, E. Garcia-Robledo, E. Gomez Ramirez, C. R. Benson, A. Bourbonnais, M. A. Altabet, P. R. Girguis, B. Thamdrup, and F. J. Stewart.** 2016. NC10 bacteria in marine oxygen minimum zones. ISME J **10:**2067-2071. 10.1038/ismej.2015.262.
13. **Weissbach, M., C. S. Criddle, J. E. Drewes, and K. Koch.** 2016. A proposed nomenclature for biological processes that remove nitrogen. Environ Sci: Water Res Technol. DOI: 10.1039/C6EW00216A.
14. **Tancsics, A., S. Szoboszlay, I. Szabo, M. Farkas, B. Kovacs, J. Kukolya, Z. Mayer, and B. Kriszt.** 2012. Quantification of subfamily I.2.C catechol 2,3-dioxygenase mRNA transcripts in groundwater samples of an oxygen-limited BTEX-contaminated site. Environ Sci Technol **46:**232-240. 10.1021/es201842h.
15. **Tancsics, A., M. Farkas, S. Szoboszlay, I. Szabo, J. Kukolya, B. Vajna, B. Kovacs, T. Benedek, and B. Kriszt.** 2013. One-year monitoring of meta-cleavage dioxygenase gene expression and microbial community dynamics reveals the relevance of subfamily I.2.C extradiol dioxygenases in hypoxic, BTEX-contaminated groundwater. Syst Appl Microbiol **36:**339-350. 10.1016/j.syapm.2013.03.008.
16. **Larentis, M., K. Hoermann, and T. Lueders.** 2013. Fine-scale degrader community profiling over an aerobic/anaerobic redox gradient in a toluene-contaminated aquifer. Env Microbiol Rep **5:**225-234. 10.1111/1758-2229.12004.
17. **Müller, H., J. Bosch, C. Griebler, L. R. Damgaard, L. P. Nielsen, T. Lueders, and R. U. Meckenstock.** 2016. Long-distance electron transfer by cable bacteria in aquifer sediments. ISME J **10:**2010-2019. 10.1038/ismej.2015.250.
18. **Koch, K., B. Helmreich, and J. E. Drewes.** 2015. Co-digestion of food waste in municipal wastewater treatment plants: Effect of different mixtures on methane yield and hydrolysis rate constant. Appl Energ **137:**250-255. 10.1016/j.apenergy.2014.10.025.
19. **Leix, C., J. E. Drewes, and K. Koch.** 2016. The role of residual quantities of suspended sludge on nitrogen removal efficiency in a deammonifying moving bed biofilm reactor. Bioresour Technol **219:**212-218. 10.1016/j.biortech.2016.07.134.
20. **Lackner, S., C. Lindenblatt, and H. Horn.** 2012. 'Swinging ORP' as operation strategy for stable reject water treatment by nitritation-anammox in sequencing batch reactors. Chem Eng J **180:**190-196. 10.1016/j.cej.2011.11.043.
21. **Scherson, Y. D., G. F. Wells, S. G. Woo, J. Lee, J. Park, B. J. Cantwell, and C. S. Criddle.** 2013. Nitrogen removal with energy recovery through N_2O decomposition. Energ Environ Sci **6:**241-248. 10.1039/c2ee22487a.
22. **Pilloni, G., M. S. Granitsiotis, M. Engel, and T. Lueders.** 2012. Testing the limits of 454 pyrotag sequencing: reproducibility, quantitative assessment and comparison to T-RFLP fingerprinting of aquifer microbes. PLoS One **7**. 10.1371/journal.pone.0040467.
23. **Winderl, C., B. Anneser, C. Griebler, R. U. Meckenstock, and T. Lueders.** 2008. Depth-resolved quantification of anaerobic toluene degraders and aquifer microbial community patterns in distinct redox zones of a tar oil contaminant plume. Appl Environ Microbiol **74:**792-801. 10.1128/Aem.01951-07.
24. **Shiro, Y.** 2012. Structure and function of bacterial nitric oxide reductases Nitric oxide reductase, anaerobic enzymes. BBA-Bioenergetics **1817:**1907-1913. 10.1016/j.bbabio.2012.03.001.
25. **Matsumoto, Y., T. Tosha, A. V. Pisliakov, T. Hino, H. Sugimoto, S. Nagano, Y. Sugita, and Y. Shiro.** 2012. Crystal structure of quinol-dependent nitric oxide reductase from *Geobacillus stearothermophilus*. Nat Struct Mol Biol **19:**238-245. 10.1038/nsmb.2213.
26. **Terasaka, E., N. Okada, N. Sato, Y. Sako, Y. Shiro, and T. Tosha.** 2014. Characterization of quinol-dependent nitric oxide reductase from *Geobacillus stearothermophilus*: Enzymatic

activity and active site structure. BBA-Bioenergetics **1837:**1019-1026. 10.1016/j.bbabio.2014.02.017.

27. **Zhu, B., G. van Dijk, C. Fritz, A. J. P. Smolders, A. Pol, M. S. M. Jetten, and K. F. Ettwig.** 2012. Anaerobic oxidization of methane in a minerotrophic peatland: enrichment of nitrite-dependent methane-oxidizing bacteria. Appl Environ Microbiol **78:**8657-8665. 10.1128/aem.02102-12.
28. **Hu, S., R. J. Zeng, L. C. Burow, P. Lant, J. Keller, and Z. Yuan.** 2009. Enrichment of denitrifying anaerobic methane oxidizing microorganisms. Env Microbiol Rep **1:**377-384.
29. **He, Z., S. Geng, C. Cai, S. Liu, Y. Liu, Y. Pan, L. Lou, P. Zheng, X. Xu, and B. Hu.** 2015. Anaerobic oxidation of methane coupled to nitrite reduction by halophilic marine NC10 bacteria. Appl Environ Microbiol **81:**5538-5545. 10.1128/AEM.00984-15.
30. **Luesken, F. A., T. A. van Alen, E. van der Biezen, C. Frijters, G. Toonen, C. Kampman, T. L. Hendrickx, G. Zeeman, H. Temmink, M. Strous, H. J. Op den Camp, and M. S. Jetten.** 2011. Diversity and enrichment of nitrite-dependent anaerobic methane oxidizing bacteria from wastewater sludge. Appl Microbiol Biotechnol. 10.1007/s00253-011-3361-9.
31. **Leix, C., Hartl R, Zeh C, Beer F, Drewes JE, Koch K** 2016. Performance and N2O formation of the deammonification process by suspended sludge and biofilm systems - A pilot-scale study. Water. (in press).
32. **Vlaeminck, S. E., A. Terada, B. F. Smets, H. De Clippeleir, T. Schaubroeck, S. Bolca, L. Demeestere, J. Mast, N. Boon, M. Carballa, and W. Verstraete.** 2010. Aggregate size and architecture determine microbial activity balance for one-stage partial nitritation and anammox. Appl Environ Microbiol **76:**900-909. 10.1128/Aem.02337-09.
33. **Geets, J., M. de Cooman, L. Wittebolle, K. Heylen, B. Vanparys, P. De Vos, W. Verstraete, and N. Boon.** 2007. Real-time PCR assay for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge. Appl Microbiol Biotechnol **75:**211-221. 10.1007/s00253-006-0805-8.
34. **Gutierrez, T., G. Rhodes, S. Mishamandani, D. Berry, W. B. Whitman, P. D. Nichols, K. T. Semple, and M. D. Aitken.** 2014. Polycyclic aromatic hydrocarbon degradation of phytoplankton-associated Arenibacter spp. and description of Arenibacter algicola sp. nov., an aromatic hydrocarbon-degrading bacterium. Appl Environ Microbiol **80:**618-628. 10.1128/AEM.03104-13.
35. **Kraft, B., H. E. Tegetmeyer, R. Sharma, M. G. Klotz, T. G. Ferdelman, R. L. Hettich, J. S. Geelhoed, and M. Strous.** 2014. The environmental controls that govern the end product of bacterial nitrate respiration. Science **345:**676-679. 10.1126/science.1254070.
36. **Rabus, R., M. Boll, J. Heider, R. U. Meckenstock, W. Buckel, O. Einsle, U. Ermler, B. T. Golding, R. P. Gunsalus, P. M. H. Kroneck, M. Kruger, T. Lueders, B. M. Martins, F. Musat, H. H. Richnow, B. Schink, J. Seifert, M. Szaleniec, T. Treude, G. M. Ullmann, C. Vogt, M. von Bergen, and H. Wilkes.** 2016. Anaerobic microbial degradation of hydrocarbons: from enzymatic reactions to the environment. J Mol Microb Biotech **26:**5-28. 10.1159/000443997.
37. **Luesken, F. A., M. L. Wu, H. J. Op den Camp, J. T. Keltjens, H. Stunnenberg, K. J. Francoijs, M. Strous, and M. S. Jetten.** 2012. Effect of oxygen on the anaerobic methanotroph *'Candidatus Methylomirabilis oxyfera'*: kinetic and transcriptional analysis. Environ Microbiol **14:**1024-1034. 10.1111/j.1462-2920.2011.02682.x.
38. **Ehrenreich, P., A. Behrends, J. Harder, and F. Widdel.** 2000. Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria. Arch Microbiol **173:**58-64. 10.1007/s002030050008.
39. **Law, Y. Y., L. Ye, Y. T. Pan, and Z. G. Yuan.** 2012. Nitrous oxide emissions from wastewater treatment processes. Philos T R Soc B **367:**1265-1277. 10.1098/rstb.2011.0317..

592

593 # Tables

594

595 **Table 1.** *nod*-targeted primers designed and applied in this study.

Designation	Sequence (5' to 3')	Position [a]
nod631F	TTCTTCTGGGGHGGYTGGG	631-649
nod684F	CTAYACHCACAACTGGCC	684-701
nod1465R	CGAAGAACAGGAACAGMACCATG	1465-1443
nod1706R	GGCTTGGCRATCCAGTAGAAG	1706-1686
nod1896R	GATGTTCCAGAAGTTRACGSC	1896-1876
nod2015R	ATGTTACCYTTKACACCGAAC	2015-1995
nod684Fv2	STAYACHCAYAACTGGCC	684-701
nod1706Rv2	GGCTTSGCRATCCAGTAGAAG	1706-1686
nod1896Rv2	GATRTTCCAGAAGTTRACGSC	1896-1876
nod1446F	GGTGBYBTTCCTGTTCTTYRG	1446-1466

596

597 [a] Position of target site according to the *M. oxyfera nod* DAMO_2437 gene sequence.

598

599

600 **Table 2.** Environmental samples investigated for *nod* genes in this study.

Environment	Designation	Main processes	Reference	Primer pair[a]	No. of clones	Nod-clusters[b]
BTEX-impacted[c] aquifer in Siklós	Siklós		(14, 15)	A	16	2
				C	9	
				B	13	
BTEX-impacted[c] aquifer in Flingern	Flingern		(16, 17)	A	5	1
Swing-Redox deammonification reactor	Swing-redox	nitritation and anammox	(20)	D	13	4
Two-stage sequencing batch deammonification reactor cascade	2-stage-nitritation	nitritation		D	16	1
	2-stage-AMX	anammox		D	14	2
CANDO reactor system	CANDO-nitritation	nitritation	(21)	D	7	1
	CANDO-N_2O	nitrous denitritation to N_2O	(21)	D	5	2
Suspension deammonification reactor	Suspension	nitritation and anammox	(20)	D	16	3
Wastewater treatment plant Garching	WWTP-TUM	nitritation and anammox	(18)	D	25	3
Wastewater treatment plant Kempten	WWTP-Kempten	nitritation and anammox	(19)	D	6	5
				E	4	

601

602 [a] Main N-removing process ongoing in the engineered water treatment systems according to
603 the nomenclature of (13).

604 [b] Primer combinations used as indicated in Fig. 1 and number of positive clones obtained
605 from each system.

606 [c] BTEX: benzene, toluene, ethylbenzene and xylene.

607

608 Figure Legends

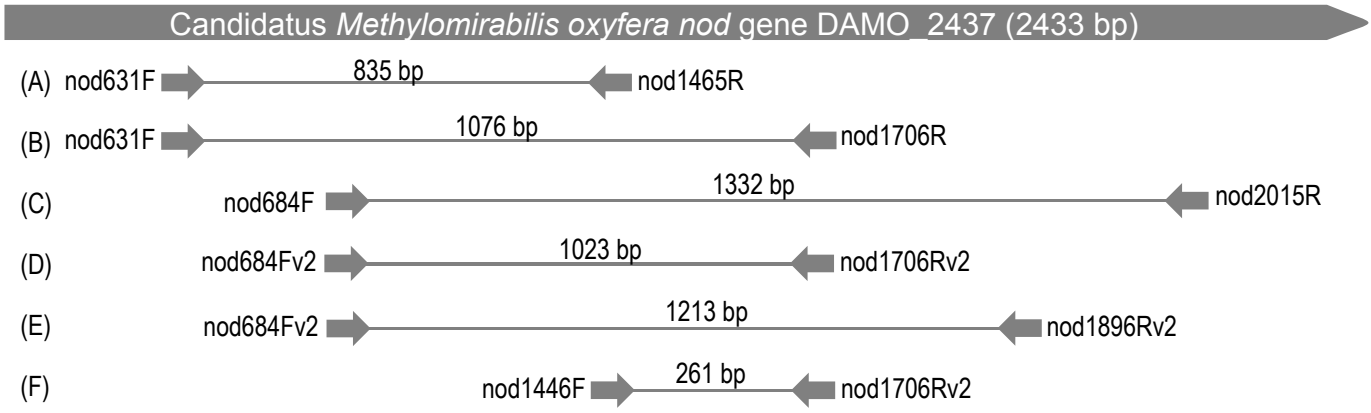
609 **Fig. 1.** Scheme of the localization of *nod*-targeted primers developed in this study and the
610 primer combinations used for clone library construction and qPCR. The positioning of *nod*
611 primers refers to the *M. oxyfera nod* gene DAMO_2437. Primer combinations A to E were
612 used for clone library construction, primer set F was used for qPCR. Expected amplicon
613 sizes are indicated, the scheme is not drawn to scale [bp].

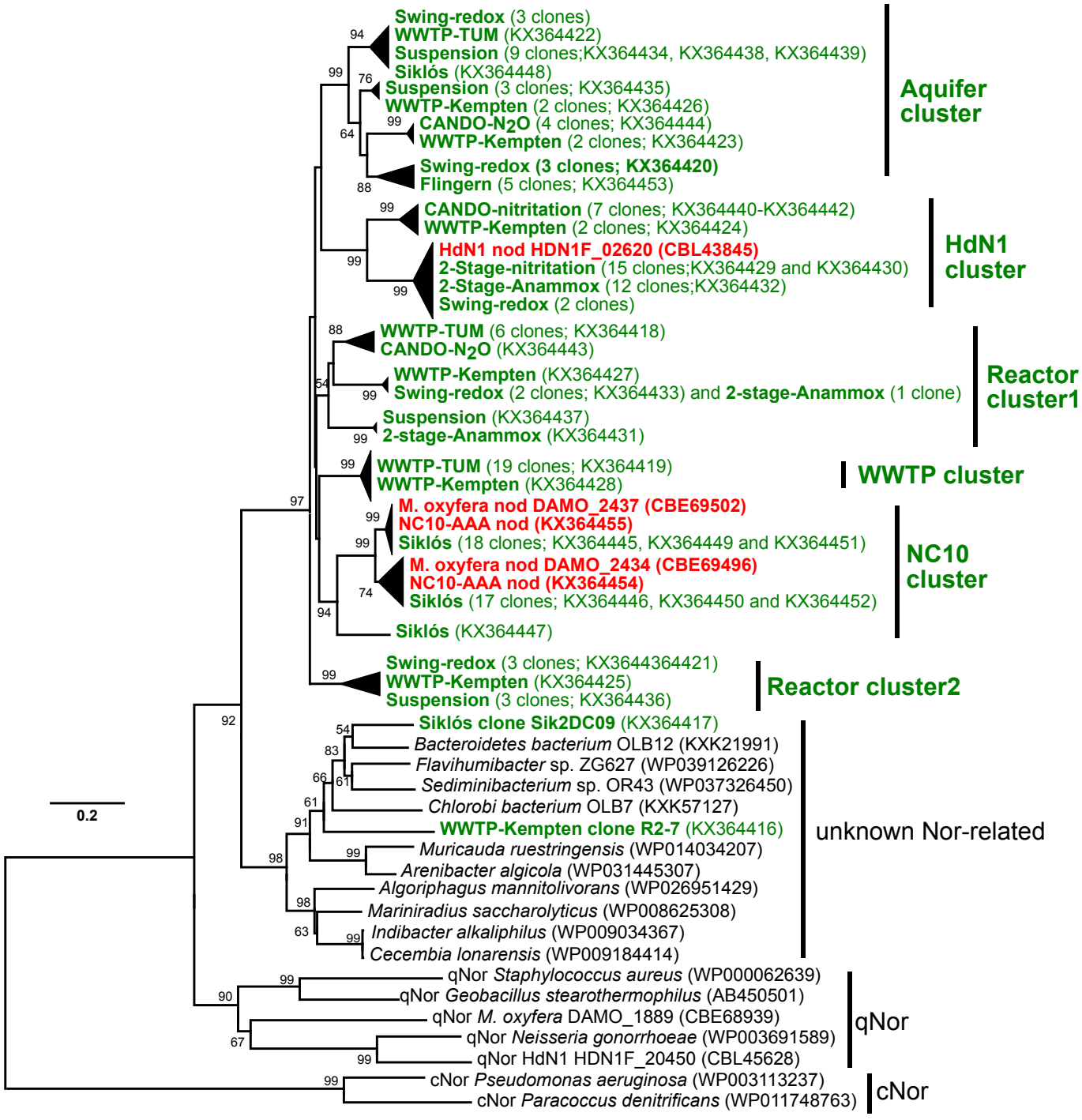
614 **Fig. 2.** Bootstrapped neighbor-joining phylogeny of putative Nod and selected qNor and
615 cNorB sequences. Nod clones generated in this study are shown in green, available
616 reference sequences are in red. The 6 sub-clusters of Nod identified in this study are
617 indicated. The accession numbers of reference sequences used and of selected *nod*
618 sequences generated in this study are shown in parenthesis. Bootstrap support (1,000
619 replicates) greater than 50% are indicated at the nodes. The scale bar represents 20%
620 amino acid sequence divergence.

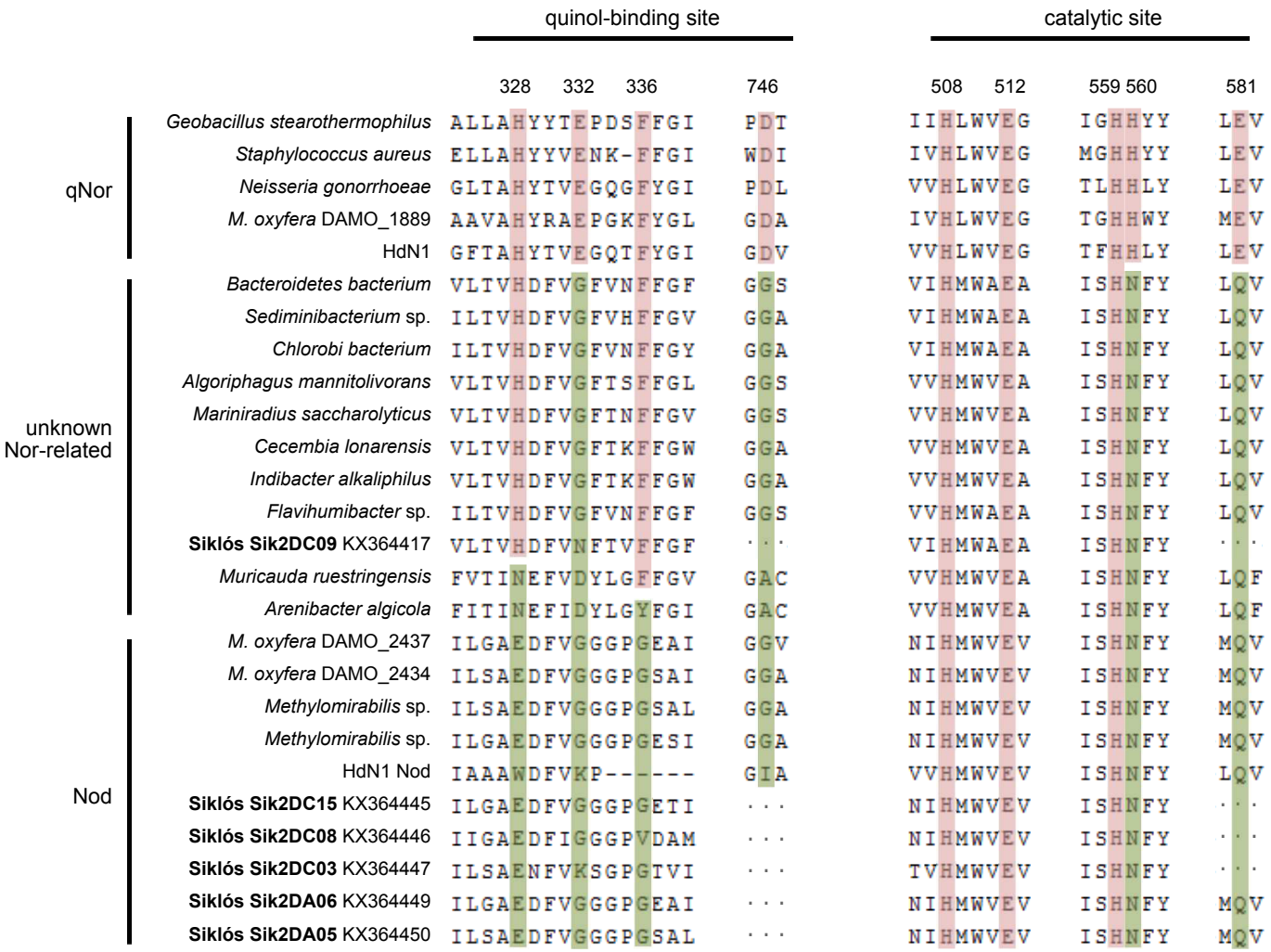
621 **Fig. 3.** Relative compostion of putative *nod* gene clone libraries generated from the
622 invesigated samples. The affiliation of *nod* clones to the identified sub-clusters is given as in
623 Fig. 2.

624 **Fig. 4.** Absolute abundance of *nod* (empty bars) and bacterial 16S rRNA (hatched bars) in
625 the investigated samples. Gene counts are averaged ± SD from at least three technical
626 qPCR replicates per sample, whicle WWTP-TUM is from biologically replicated DNA extracts.

627 **Fig. 5.** Multiple sequence alignment of selected putative Nod and qNor enzymes around the
628 quinol-binding site and the catalytic site of qNor. Representative environmental Nods were
629 deduced from the gene sequences generated in this study (in bold). Only five of the longest
630 environmental Nod sequences obtained in this study are included here (see supplementary
631 Fig. S1 for extended alignments including further environmental Nods). The conserved
632 residuals for quinol-binding and catalytic functioning in qNor are highlighted in red, whereas
633 substitutions at these sites in putative Nod and putative Nor are shown in green. Accession
634 numbers are the same as in Fig. 2. The alignment was generated with ClustalW in MEGA 6.







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