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Rapid succession of uncultured marine bacterial and archaeal populations in a denitrifying continuous culture

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Summary

Marine denitrification constitutes an important part of the global nitrogen cycle and the diversity, abundance and process rates of denitrifying microorganisms have been the focus of many studies. Still, there is little insight in the ecophysiology of marine denitrifying communities. In this study, a heterotrophic denitrifying community from sediments of a marine intertidal flat active in nitrogen cycling was selected in a chemostat and monitored over a period of 50 days. The chemostat enabled the maintenance of constant and well-defined experimental conditions over the time-course of the experiment. Analysis of the microbial community composition by automated ribosomal intergenic spacer analysis (ARISA), Illumina sequencing and catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) revealed strong dynamics in community composition over time, while overall denitrification by the enrichment culture was stable. Members of the genera *Arcobacter*, *Pseudomonas*, *Pseudovibrio*, *Rhodobacterales* and of the phylum *Bacteroidetes* were identified as the dominant denitrifiers. Among the fermenting organisms co-enriched with the denitrifiers was a novel archaeon affiliated with the recently proposed DPANN-superphylum. The pan-genome of populations affiliated to *Pseudovibrio* encoded a NirK as well as a NirS nitrite reductase, indicating the rare co-occurrence of both evolutionary unrelated nitrite reductases within coexisting subpopulations.

Introduction

Currently, anthropogenic nitrogen input to the environment exceeds natural nitrogen fixation (Fowler *et al.*, 2013). To understand the consequences of this aspect of global change, it is important to understand the fate of fixed nitrogen compounds such as nitrate and nitrite in the environment. Denitrification in marine coastal sediments contributes significantly to the removal of fixed nitrogen from the marine environment (Rao *et al.*, 2007; Gao *et al.*, 2012) and may be responsible for up to half of the marine export of fixed nitrogen to the atmosphere (Gruber, 2004). Especially, sandy coastal sediments are characterized by high potential denitrification rates (Gao *et al.*, 2010). Despite the important contribution of denitrifying microorganisms to biogeochemical cycling of nitrogen and carbon, the microbial community responsible for organic carbon turnover coupled to denitrification in coastal sediments remains understudied compared with sulfate reduction in deeper sediment layers (Llobet-Brossa *et al.*, 2002; Gittel *et al.*, 2008). The community composition of denitrifiers is often determined via molecular methods targeting the functional genes of denitrification, which encode the nitrite reductases NirS or NirK, nitric oxide reductase NorB and nitrous oxide reductase NosZ (for review, see Kraft *et al.*, 2011), but incongruence between the functional genes and 16S rRNA gene phylogenies makes it hard to link functional gene diversity to the microbial community structure (Jones *et al.*, 2008).

Next to denitrification and aerobic respiration, fermentation is the main organic carbon degradation pathway in the upper layers of coastal tidal-flat sediments; fermenting organisms may even constitute the largest part of the anaerobic microbial community in marine sediments (Wilms *et al.*, 2006, Köpke *et al.*, 2005). They take part in the initial degradation of organic carbon substrates and provide a variety of fermentation products, which serve as substrate for denitrification and other terminal degradation processes (Schink, 2002).

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Fermenting organisms are mostly facultative anaerobes; they are also able to respire oxygen, when it is available. Therefore, *in situ* detection of active fermenters is difficult and little is known about the ecophysiology of fermentative bacteria *in situ* and their potential interactions with respiratory heterotrophs such as denitrifiers.

These potential interactions also play a role in the stability of ecosystem functions such as denitrification. It has been hypothesized that in communities with significant cross feeding, a high (background) diversity along with a dynamic population structure maintains functional stability (Briones and Raskin, 2003). However, it is difficult to directly observe syntrophic interactions between microbes in nature. Indeed, by direct determination of *in situ* microbial community composition, it can already be difficult to link specific phylotypes to specific processes or to correlate the abundance of specific phylotypes to specific environmental factors (Leser *et al*., 2002; Curtis and Sloan, 2004), because changes in microbial activity, abundance and community composition are generally caused by a multitude of interconnected environmental factors (Wallenstein *et al*., 2006).

Cultivation of natural microbial communities in continuous culture (e.g. chemostats) offers the possibility to monitor microbial community composition as well as the stability of specific processes (such as denitrification) under constant, environmentally relevant conditions. Continuous cultivation selects for a simplified microbial community that is optimally adapted to the applied conditions and may thus bridge the gap between direct ecosystem observations and pure culture studies.

In the present study, we cultivated a natural microbial community sampled from a sandy coastal sediment in continuous culture under stable, denitrifying conditions for 50 days. We observed that while denitrification was stable, rapid microbial community succession occurred as shown by metagenomics and other methods.

Results

A chemostat was inoculated with biomass from a coastal intertidal-flat sediment and fed with a medium containing nitrite, nitrate and organic carbon in order to select for a nitrate-respiring microbial community. The organic carbon mixture (consisting of glucose, amino acids and acetate) represented the composition (monomers) of decaying biomass, the main carbon and energy source *in situ*. After an initial phase (8 days) in which the concentrations in the inflowing medium were gradually increased, nitrate and nitrite were completely depleted in the culture liquid (Fig. 1A). From this day onwards, the nitrate and nitrite concentrations in the culture remained in the low micromolar range, close to the conditions *in situ* (< 0.5 μM for nitrite, < 10 μM for nitrate). Dinitrogen was identified as the main product and thereby denitrification as the main nitrate-reducing process. The ammonium concentrations were between 0.7 mM and 2 mM. Because a complete degradation of the provided amino acids in the medium would lead to an ammonium concentration of 3.5 mM, the produced ammonium most likely originated from the degradation of amino acids. Even if most of the ammonium produced resulted from the reduction of nitrite to ammonium, this pathway would have been responsible for less than 5% of the nitrite reduction. The concentration of dissolved organic carbon remaining in the culture was constant at 5.3 ± 1 mM (Fig. 1B). The protein content of cell biomass increased until day 24 and then stabilized at 180 ± 3 mg protein l^{-1}. This corresponded to a biomass yield of 0.36 ± 0.035 C-mol biomass/C-mol substrate. Overall, the continuous culture showed stable conversion of substrates.

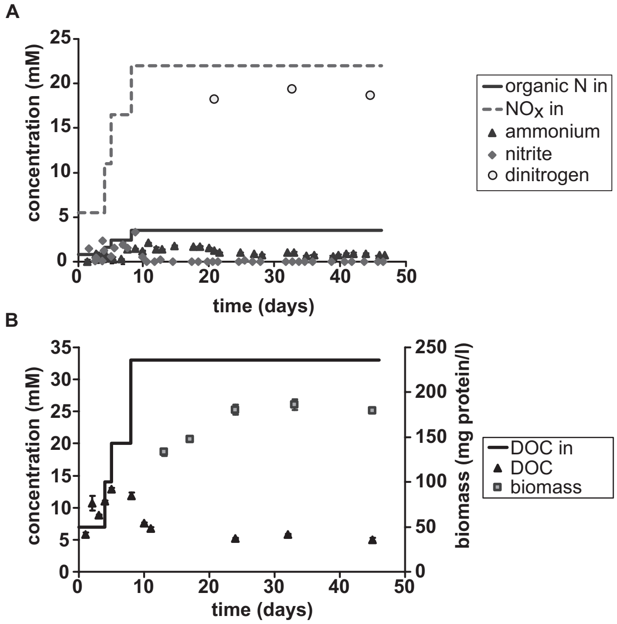

Fig. 1. Conversion of (A) nitrogen and (B) carbon compounds during continuous culture enrichment. The solid lines represent the concentration of compounds in the medium supplied. The symbols represent the concentrations measured in the culture. DOC, dissolved organic carbon; N, nitrogen. Error bars indicate the standard deviation. For nitrite concentrations, error margins are within the sizes of the symbols.

Microbial community dynamics were monitored with automated ribosomal intergenic spacer analysis (ARISA). The number of total operational taxonomic units (OTU) per sample remained constant (150 ± 12) over time. In contrast, the OTU composition and the relative abundance of specific OTUs changed considerably. Only 10% of all OTUs were present at all time points sampled. Figure 2A shows the dynamics of the 15 OTUs with the highest overall abundance. Some populations were lost from the community (e.g. OTU 384 and 396) within the

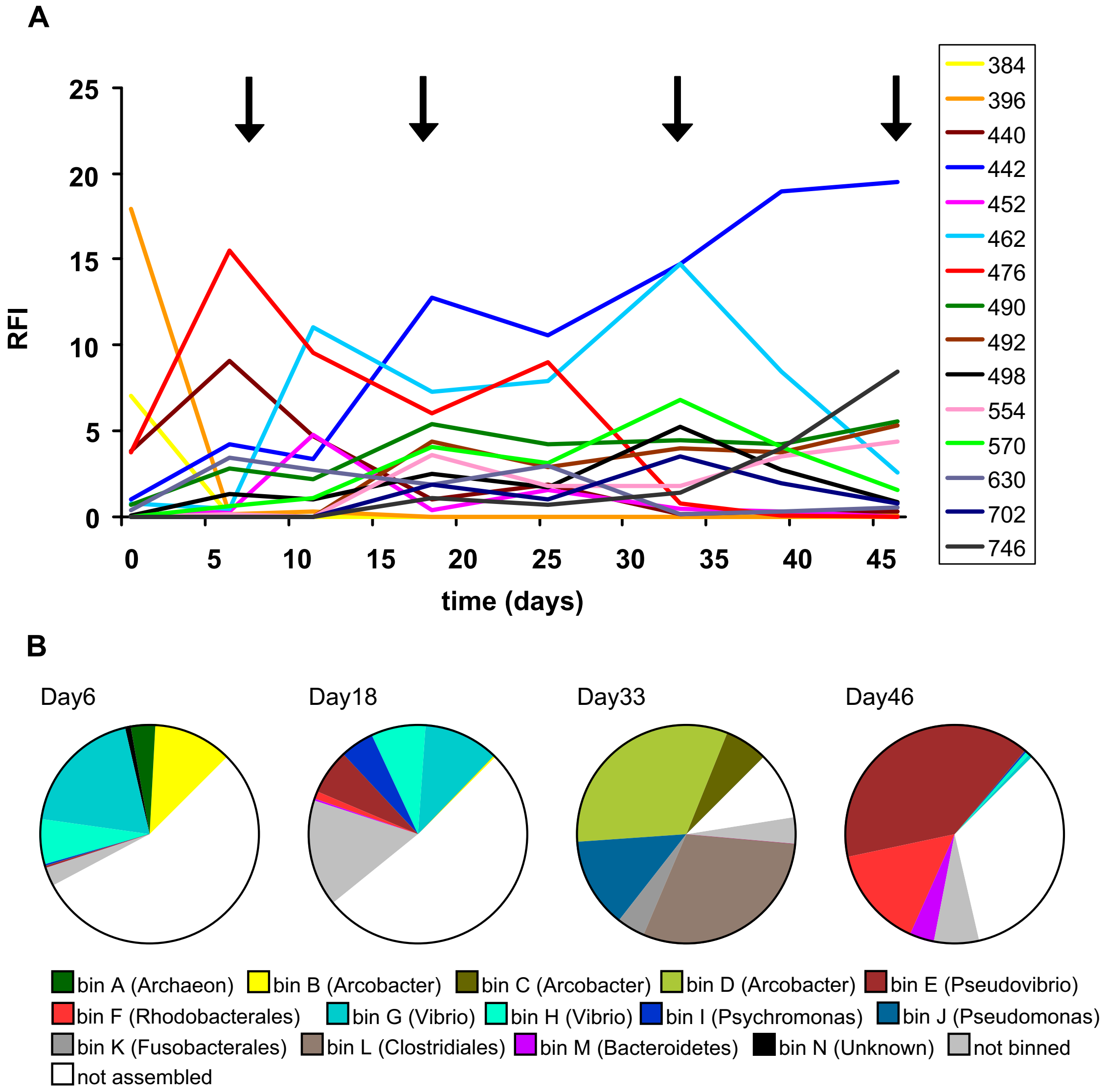

Fig. 2. Dynamics of the microbial community.
A. Automated ribosomal intergenic spacer analysis (ARISA) pattern over time of the 15 most abundant OTUs. OTUs with the highest overall abundance are depicted. Arrows indicate time points of metagenomic sequencing and CARD-FISH. RFI, relative fluorescence intensity.
B. Relative abundance estimates of metagenomic bins obtained by read mapping at different time points.

first week of the experiment, while others showed a transient build-up before they collapsed again (e.g. OTUs 440, 476 and 462). Only OTU 442 was clearly enriched over time.

Four time points (day 6, 18, 33 and 46) were selected for metagenomic sequencing with an Illumina GA IIx. Between 3.2 and 8.3 million reads per sample were generated (Supporting Information Table S1). Binning of assembled contigs of the four metagenomic samples resulted in the identification of 14 different bins, each associated with a different population. The position of each bin on a coverage versus GC content plot of the assembled contigs as well as the taxonomic profiles of the bins are shown in Supporting Information Fig. S1. For almost all bins, corresponding full-length 16S rRNA genes were obtained by *de novo* assembly and iterative read mapping. The phylogenetic affiliation of the 16S rRNA sequences is shown in Supporting Information Table S2 and Fig. S2. 16S rRNA sequences could be linked to the corresponding bins based on consistent phylogeny between the 16S rRNA gene and the sequence data of each bin and consistent squared Pearson product-moment correlation coefficients between bin coverages and 16S rRNA gene coverages over the four samples (Supporting Information Table S3). Generally, high correlations between the coverage of a bin and the

Table 1. Characteristics and inferred metabolism of the metagenomic bins.

Bin	Phylogenetic affiliation	CSCGs[a] (duplicates)[b]	Bin size (Mb)	N50 contig length	Denitrification genes					Fermentation genes	Inferred lifestyle
					napA	*narG*	*nirS/nirK*	*norB*	*nosZ*		
A	Archaeon	43 (0)	1.54	28074	–	–	–	–	–	*aadh*, *dld*	FERM
B	Arcobacter	65 (1)	3.08	5643	+	–	*nirS*	+	+	*adh*, *aadh*, *pta*, *ackA*	DEN
C	Arcobacter	111 (0)	2.51	51624	+	+	*nirS*	+	+	*pta*, *ackA*	DEN
D	Arcobacter	85 (1)	2.89	8992	+	–	–	–	–	*adh*, *aadh*, *pta*, *ackA*	FERM
E	Pseudovibrio	117 (84)	5.18	719	+	+	*nirS, nirK*	+	+	*pfl*, *adh*, *aadh*, *ackA*, *dld*	DEN
F	Rhodobacterales	21 (7)	1.45	543	+	–	*nirS*	+	+	*aadh*, *pta*, *dld*	DEN
G	Vibrio	131 (15)	4.26	1594	+	–	–	–	–	*pfl*, *adh*, *aadh*, *pta*, *ackA*, *dld*	FERM, NO_3-RED
H	Vibrio	119 (57)	3.20	622	+	–	–	–	–	*pfl*, *adh*, *aadh*, *pta*, *ackA*, *dld*	FERM, NO_3-RED
I	Psychromonas	39 (3)	1.78	685	–	–	–	–	–	*pfl*, *adh*, *aadh*, *ackA*, *pta*, *dld*	FERM
J	Pseudomonas	125 (3)	2.63	1171	–	+	*nirS*	+	+	*adh*, *aadh*, *pta*, *ackA*	DEN
K	Fusobacterales	130 (4)	2.63	4592	–	–	–	–	–	*pfl*, *adh*, *aadh*, *pta*, *ackA*, *dld*	FERM
L	Clostridiales	132 (4)	3.45	37879	–	–	–	–	–	*pfl*, *adh*, *aadh*, *pta*, *ackA*, *dld*	FERM
M	Bacteroidetes	72 (6)	2.26	536	–	+	*nirS*	–	–	*aadh*, *pta*	Partial DEN

a. Number of different CSCGs present in the bin, 44 = 100% for archaea, 139 = 100% for bacteria.
b. Number of the CSCGs that have duplicates.
DEN, denitrification; FERM, fermentation; NO_3-RED, nitrate reduction to nitrite.

corresponding 16S rRNA gene were observed. Only bin F and I had low correlation coefficients. Together with the taxonomic profiles of the bins (Supporting Information Fig. S1), this indicated misbinning of contigs from bin E into F [a rather incomplete bin estimated based on the presence of conserved single copy genes (CSCGs), Table 1] and of bins G and H into I.

The abundance of each of the bins in every sample was estimated by mapping the reads of every sample to the contigs of every bin. Fifty to 85% of the sequence data could be mapped to the assembled contigs and 35–80% was mapped against contigs that were part of one of the bins. Bin sizes and estimated genome completeness are shown in Table 1. The differences in relative abundances between samples indicated a highly dynamic community with drastic changes from one time point to the next (Fig. 2B).

The main bins present in the first sample were affiliated with *Arcobacter* (bin B), *Vibrionales* (bin G and bin H) and *Archaea* (bin A). The closest relatives based on the 16S rRNA gene are given in Supporting Information Table S2. The bins G and H (*Vibrionales)* were also recovered in the second sample (day 18) together with two bins affiliated with *Alphaproteobacteria* (bin E and bin F) and a bin affiliated with *Fusobacter* (bin K). On day 33, none of the bins of the previous sample was detected. Bins C and D (*Arcobacter*) predominated, accompanied by bin I (affiliated with *Psychromonas*), bin J (affiliated with *Pseudomonas*) and bin L (affiliated with *Clostridiales*). On day 46, bins E and F (*Rhodobacterales*) were detected again, accounting together for more than 50% of the sequenced reads. Furthermore, bins affiliated with *Vibrio* and *Bacteroidetes* (bins H and M) were obtained.

To independently validate the composition of the microbial community, catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) was performed for the most abundant populations (class and genus level) at the same time points as metagenomic sequencing was performed (Table 2). An increase in relative abundance of *Rhodobacterales* and a decrease in the fraction of *Gammaproteobacteria*, particularly *Vibrios*, and *Epsilonproteobacteria* (*Arcobacter*) during the course of the experiment supported the metagenomic results. Only for day 33, a major difference between the community composition of the CARD-FISH and metagenome data was observed. Generally, the relative abundance of *Gammaproteobacteria*, particularly *Vibrio* and *Epsilonproteobacteria*, was overestimated and the relative abundance of *Alphaproteobacteria* was underestimated by metagenomic abundance estimates based on coverage. However, the overall community dynamics detected by CARD-FISH followed the same trend as the metagenomic data.

Five bins (B, C, E, F and J) contained the genes necessary for complete denitrification (Table 1). In addition, bin M contained the genes for a part of the denitrification pathway. Interestingly, in bin E (*Pseudovibrio*) genes for the two types of nitrite reductases, *nirK* and *nirS*, were present. The *nirS* gene had 95% identity to *nirS* from *Pseudovibrio* sp. FO-BEG1. The *nirK* gene was most

Table 2. Relative abundance of different phylogenetic groups determined by CARD-FISH with class and genus specific probes.

	Day 6	Day 18	Day 33	Day 46
Gammaproteobacteria	33	30	22	12
Vibrio	27	13	10	3
Alphaproteobacteria	46	60	67	87
Roseobacter	49	55	58	84
Epsilonproteobacteria	19	1	12	0
Arcobacter	18	1	1	0

closely related to *nirK* genes of other *Rhodobacterales* (63%, *Roseobacter* sp. SK209-2-6; 61%, *Phaeobacter gallaeciensis*). On the contig with the *Roseobacter*-related *nirK* gene, we also detected a gene coding for a transposase that had 93% identity to *Pseudovibrio* sp. FO-BEG1. The consistent affiliation of the whole contig with the order *Rhodobacterales* independently supported that it belonged to bin E. Phylogenetic analysis showed clustering of the *nirS* sequence together with other alphaproteobacterial *nirS* gene sequences (Supporting Information Fig. S4). The presence of a gene encoding a transposase suggested that *nirK* might possibly have been acquired by horizontal gene transfer. The phylogenetic analysis of the *nirK* sequence is depicted in Supporting Information Fig. S5.

Two of the three bins affiliated with the genus *Arcobacter* (bin B and C) contained the complete denitrification pathway, whereas bin D did not (Table 1). Instead, it contained genes for dissimilatory nitrate reduction to ammonium (*napA* and *nrfA*). However, the low concentration of produced ammonium indicated that the activity of this pathway was low compared with denitrification (at most < 5%). Bins B and D both contained a *soxCDXYZAB* operon and thus showed the potential for the complete oxidation of sulfide to sulfate. Bin E (*Pseudovibrio*) also contained genes for sulfide oxidation (*soxB*, *D*, *X*, *Z* and *W*), but these were distributed over different contigs.

In bins D, G, H, I, K and L (affiliating with *Arcobacter*, *Vibrio*, *Psychromonas*, *Fusobacter* and *Clostridiales*), the presence of different sets of genes being associated with a fermentative lifestyle (Table 1) suggested mixed acid fermentation as the metabolism. Bin K (*Fusobacter*) also contained genes encoding capacity for sulfite reduction (*dsrABC*). The abundance of populations associated with denitrification and fermentation appeared to be relatively constant during the first three time points (days 6, 18 and 33). Only at day 46, denitrifiers seemed to be more abundant.

At the beginning of the experiment, we observed the transient enrichment of a bin related to uncultured archaea (bin A). Bin A (1.54 Mb, 10× coverage) consisted of 107 contigs and constituted 4% of the assembled sequence data of the first sample. The archaeal 16S rRNA gene shared 90% sequence identity with environmental clones and 74% with members of the *Methanobacteria*, the most closely related cultured *Archaea*. Because the phylogeny of the archaeon was not well resolved based on the 16S rRNA gene (Supporting Information Fig. S3), a more in-depth phylogenetic analysis was based on 44 concatenated CSCGs, according to Lloyd and colleagues (2013). This showed that the archaeal population clustered within the deeply branching newly proposed DPANN superphylum (Rinke *et al.*, 2013), with *Nanoarchaeum equitans* being the closest relative (Fig. 3). The genome size was estimated to be approximately 1.6 Mbp and the genome completeness to be 98% (Table 1).

The relative abundance of the archaeon estimated by metagenomics was confirmed by CARD-FISH. Cells hybridizing to the newly designed probe specific for this

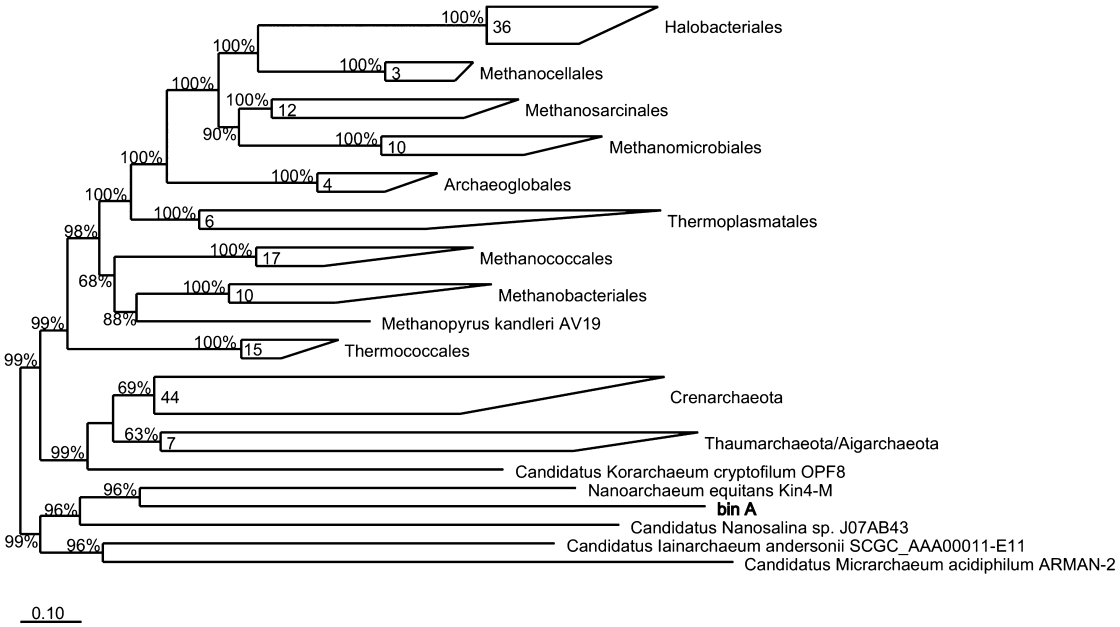
100%
36
Halobacteriales
100%
100%
3
Methanocellales
100%
100%
12
Methanosarcinales
90%
100%
10
Methanomicrobiales
100%
100%
4
Archaeoglobales
100%
100%
6
Thermoplasmatales
98%
100%
17
Methanococcales
68%
100%
10
Methanobacteriales
88%
Methanopyrus kandleri AV19
99%
100%
15
Thermococcales
99%
69%
44
Crenarchaeota
63%
7
Thaumarchaeota/Aigarchaeota
99%
Candidatus Korarchaeum cryptofilum OPF8
96%
Nanoarchaeum equitans Kin4-M
bin A
96%
Candidatus Nanosalina sp. J07AB43
99%
96%
Candidatus Iainarchaeum andersonii SCGC_AAA00011-E11
Candidatus Micrarchaeum acidiphilum ARMAN-2
0.10

Fig. 3. Phylogenetic placement of bin A within the archaeal DPANN superphylum. Maximum likelihood tree of concatenated conserved single copy genes (RAxML). Bin A is in bold font. Bootstrap values higher than 50% are given.

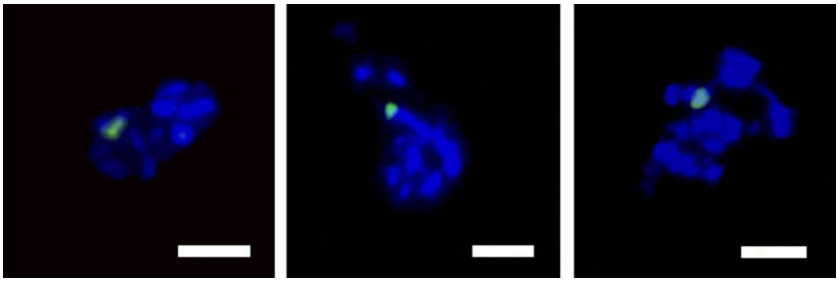

Fig. 4. Epifluorescence microscopy image (CARD-FISH) of the archaeal population present in the enrichment culture at day 6. Green: archaeal cells hybridized with probe Darch335; blue = 4,6-diamidino-2-phenylindole-stained cells. The scale bar corresponds to 2 μm.

group of archaea (Supporting Information Table S4) accounted for 5% of all 4,6-diamidino-2-phenylindole counts at day 6. Cells had a coccoid shape with a diameter of 0.5–0.8 μm (Fig. 4).

For bin A, we observed that the genes for RNA polymerase A and B were both split into two parts. This was also observed previously for *N. equitans* (Spang *et al*., 2010). A split RNA polymerase subunit B has so far been considered to be a characteristic only present in euryarchaea (Brochier *et al*., 2004; Spang *et al*., 2010). Furthermore, topoisomerase type IIa subunits A and B were present, which also have been assumed to exist within the *Archaea* exclusively in *Euryarchaea* (Spang *et al*., 2010).

In bin A, genes were present that encode a complete glycolysis pathway and a lactate dehydrogenase gene. Bin A also contained an *arcABCD* gene cluster encoding the arginine deiminase pathway. The presence of these genes suggests a fermentative lifestyle by sugar and amino acid fermentation. Consistently, the F-type (proton pumping) ATPase and respiratory complexes were not encoded in this bin and the tricarboxylic acid cycle was incomplete. The *arcABCD* genes were most closely related to homologues previously found in *Clostridia* and not to archaeal homologues. Also, the gene encoding a putative D-lactate dehydrogenase was most closely related to bacterial genes (*Sphaerobacter thermophilus*). The contigs that contained these genes generally also contained conserved genes that were most closely related to homologues of archaeal origin. Genes encoding the complete glycolysis pathway and an incomplete pentose phosphate pathway were related to homologues of either bacterial or archaeal origin. Because of the large phylogenetic distances, a more detailed phylogenetic analysis would be required to trace the origins of these genes more robustly. Finally, a muramoyltetrapeptide carboxypeptidase, which functions in bacterial cell wall lysis, is present. A RelA- and SpoT-like ppGpp synthetase (signalling molecules involved in bacterial stringent response) was also found in the archaeal bin. This is the third report of an archaeon that possesses complete multi-domain alarmones, with all of them being members of the DPANN superphylum (Rinke *et al*., 2013).

Discussion

Rapid succession of microbial populations was observed despite the maintenance of stable conditions in the continuous culture. These populations represented populations also observed *in situ.* For example, *Rhodobacterales* were found to make up 10%, Bacteroidetes 5–25% and *Arcobacter* ~ 1% of the microbial community on the sampled tidal flat (Llobet-Brossa *et al*., 1998; Stevens *et al*., 2008; Lenk *et al*., 2011). Members of the genus *Psychromonas* have previously been identified as important glucose fermenters in sediments from the sampling site by RNA-based stable isotope probing (Graue *et al*., 2011). Based on different sets of genes involved in fermentation that were detected in the different bins, we inferred the potential production of fermentation products such as lactate, hydrogen, formate and acetate. Such compounds would serve as suitable substrates for the part of the community that coupled denitrification to heterotrophic oxidation of monomeric carbon substrates.

The enriched community had the potential for cryptic sulfur cycling with the involvement of *Epsilonproteobactria* in the sulfide oxidation step. Members of the genus *Arcobacter* have frequently been associated with autotrophic denitrification coupled to sulfide oxidation in marine environments (Lavik *et al*., 2009; Fuchsman *et al*., 2012).

Generally, in marine habitats, the nitrite reductase NirS is considered to be more common than NirK (Abell *et al*., 2010; Jones and Hallin, 2010). This was supported by our study. With our metagenomic approach, we could exclude that the overrepresentation of *nirS* with regard to *nirK* results from a bias based on the available primers, which have been shown to fail to detect the nitrite reductases of a great proportion of cultivated denitrifiers (Heylen *et al*., 2006). Interestingly, the bin E (*Pseudovibrio*) not only contained a *nirS* gene but also a *nirK* gene. The presence of genes for both types of nitrite reductases within one organism is very rare (Jones and Hallin, 2010). Bin E (*Pseudovibrio*) contained a relatively large amount of unique sequence data in relatively small contigs and a high number of duplicated CSCGs (Table 1). Together, these observations suggested the presence of multiple subpopulations and that the different nitrite reductase genes might be present in different, closely related populations. The co-enrichment of two closely related subpopulations with different types of nitrite reductases is remarkable as bacteria carrying different Nir types have been suggested to occupy different ecological niches (Jones and Hallin, 2010).

Interestingly, the applied selective pressure did not lead to the enrichment of one single dominant denitrifier but to the co-enrichment of different denitrifying microbes and their succession over time. With different independent methods, we showed that strong shifts in the community composition occurred while the overall performance of the culture was stable. A similar continuous cultivation experiment carried out in parallel (at the same time and with the same inoculum) showed similar ongoing dynamics over an even longer time scale (350 days): strong fluctuations in populations affiliated with *Fusobacter*, *Clostridia* and *Vibrio* and succession of populations affiliated with *Pseudomonas*, *Arcobacter* and *Rhodobacter* (Strous *et al.*, 2012). This experiment was performed with a higher ratio of inflowing organic carbon to nitrate and nitrite, which suggests that the observed population dynamics are reproducible even when different conditions are applied. Furthermore, similar dynamics of a functional stable methanogenic community have been described by Fernández and colleagues (1999).

In the present study, functional stability was demonstrated not only in the conversion of substrates, but also in the presence of different populations that possessed the same metabolic potential at all time points. Based on basic kinetic considerations, one could expect the constant build-up of the most successful population under the given conditions (Veldkamp and Jannasch, 1972). Once the fittest population established the highest cell density, it is hard to outcompete and we would expect the stabilization of one dominant population. Because in our experiments we supplied a variety of organic carbon substrates supporting different metabolisms, we potentially created multiple niches for several coexisting populations. Nevertheless, stability of the community composition would still be expected unless other processes controlling population abundance come into play (Waters *et al.*, 2013).

Complex mutualism such as cross feeding or antagonistic behaviour such as competition and production of toxins (Huisman and Weissing, 1999; Czárán *et al.*, 2002; Dethlefsen *et al.*, 2006; Cordero *et al.*, 2012) are likely drivers of population dynamics. If the community structures observed at different time points were characterized by specific interactions between specific populations (e.g. obligate cross feeding of specific fermentation products), a change in the abundance of one population would have consequences for other interacting populations. Functional stability could then only be maintained if the community composition would change substantially, as observed in this experiment. In fact, chaotic behaviour of community composition has been hypothesized to be important for maintaining functional stability of complex food webs (Graham *et al.*, 2007; Benincà *et al.*, 2008). Finally, viruses could also be important drivers causing turnover of dominant populations according to the 'killing the winner' hypothesis (Rodriguez-Brito *et al.*, 2010; Shapiro *et al.*, 2010).

The strength of the applied selective force can influence the reproducibility of the final community (Pagaling *et al.*, 2013). Thus, the nature of the selected metabolism could define the strength of dynamics that occur. For example, specialized aromatic hydrocarbon-degrading communities have been shown to produce stable communities (Massol-Deya *et al.*, 1997). Furthermore, already pre-adapted communities (communities originating from a habitat that better resembles the conditions in the continuous culture) may show a different community development compared with not adapted communities (Pagaling *et al.*, 2013). Thus, we cannot exclude that different source communities might exhibit different dynamics. Multiple repetitions of continuous culture experiments with inocula from multiple sampling sites may help to identify such patterns.

It appears that community composition was governed by niche-based selection as well as neutral processes. Niche-based species sorting led to the selection of denitrifying and fermenting populations, while it seems that neutral processes were responsible for the ongoing dynamics among these populations because stable ecosystem functioning throughout the experiment suggests that the changes in community composition can be regarded as neutral.

Although the general trend in changes over time was consistent for metagenomic and CARD-FISH data, the different approaches sometimes yielded different estimates of community composition. Data obtained by ARISA also showed ongoing dynamics, but it was not possible to link the ARISA results to the other results because the intergenic spacer regions were not assembled properly. However, the results obtained by ARISA and FISH both suggested that some populations only increased in abundance with time whereas the metagenome suggested more complex population dynamics. This might be explained by a variable degree of micro-diversity leading to poor assembly for some samples (Albertsen *et al.*, 2012). Furthermore, differences could be explained by different potential biases of the respective methods such as cell lysis and DNA extraction efficiency (Zhou *et al.*, 1996) or cell permeabilization efficiency for different microbial populations (Amann and Fuchs, 2008). For ARISA, different rRNA gene copy numbers and polymerase chain reaction (PCR) amplification biases may also lead to artefacts in population-abundance estimates (Crosby and Criddle, 2003).

Among the fermenting organisms was a transiently enriched newly discovered archaeal population. We hypothesize a fermentative lifestyle based on the presence of genes for glycolysis, lactate dehydrogenase and the arginine deiminase pathway, and the absence of genes that could support respiration. This so far unknown archaeon falls into the newly proposed DPANN superphylum (Rinke *et al.*, 2013). So far, this superphylum consisted of a few very distantly related extremophiles, which mostly have been recently discovered by metagenomics or single-cell sequencing. *Nanoarchaeum equitans* is the only cultured member so far (Huber *et al.*, 2002) and Archaeal Richmond Mine acidophilic nanoorganisms archaea have been recently enriched (Ziegler *et al.*, 2013). With its origin from coastal sediments of the North Sea, the archaeal population from this study is the only member of this superphylum that does not originate from an extreme habitat. After the addition of the enriched archaeon to a multi-protein tree, the monophyly of the DPANN superphylum was reproduced, even though different sets of CSCGs were used for the phylogenetic analysis (approximately 50% overlap). This finding supported the status of DPANN as a superphylum. The topology within the superphylum was not consistent with Rinke and colleagues (2013). This is probably due to the different selections of CSCG used for the analysis in combination with the very distant relationships between different members and the fact that only few representatives were available for the analysis. All the members of the DPANN superphylum so far are characterized by small genome size and this was proposed to be a defining trait for these archaea (Baker *et al.*, 2010; Narasingarao *et al.*, 2012; Rinke *et al.*, 2013). With an estimated genome size of 1.6 Mbp, our novel archaeum sets a new upper limit for this archaeal group. A split of polymerase subunit B has so far been assumed to have occurred only once in the archaeal domain, after the divergence of the *Thermococcales* (Brochier *et al.*, 2004; Spang *et al.*, 2010). The observed split RNA polymerase subunit B and the presence of topoisomerase type IIa subunit A and B show that members of the DPANN superphylum possess characteristics that have so far been believed to be exclusively euryarchaeal. A systematic study of the presence of such features in members of the DPANN superphylum in comparison with other archaeal phyla may give new insights into phylogeny and evolutionary history of *Archaea*.

In conclusion, continuous cultivation allowed us to select for environmentally relevant denitrifying and fermentative populations, among them a novel member of the archaeal DPANN superphylum. The community was highly dynamic despite stable conditions, indicating that for functional stability, only the presence of the overall metabolic potential is important, independent of the microbial community composition.

Materials and methods

Sediment collection

Sediment was collected from the back barrier tidal-flat Janssand (N53°44.151′ and E07°41.945′) located in the German Wadden Sea, in January 2011 at low tide. The upper two centimetres of the tidal flat were sampled with a flat trowel.

Continuous cultivation

The sediment was mixed vigorously in a ratio of 1:1 with red sea salt medium (33.4 g l^{-1}; http://www.Aquaristic.net). After the sediment grains had settled, the overlying suspension was used as inoculum for the continuous culture. Cycloheximide (50 mg l^{-1}) was added to prevent eukaryotic growth and thus protect bacteria from being grazed. Continuous cultivation was performed in a 3 l glass vessel at controlled temperature (20 ± 1°C). Electron donors and acceptors were separated in an acidic and an alkaline medium, which were pumped into the vessel at a ratio of 1:1 and a pump speed of 1.5 ml min^{-1} each (overall dilution rate 1.6 per day). This dilution rate was chosen according to an estimate of the average growth rate of the microbial community of the sampling site based on codon usage bias in the sampling site's metagenome (Accession number: SRP015924; H.K. Marchant, M. Holtappels, G. Lavik, F. Schreiber, R. Vahrenhorst, M. Strous, M.M.M. Kuypers and H.E. Tegetmeyer, submitted) according to Vieira-Silva and Rocha (2010).

The pH of the two media were adjusted in such a way that the resulting pH was 8 ± 0.2 in the culture. Substrate concentrations in the media were gradually increased. The final composition of the acidic medium (pH=2.2) was (g l^{-1}): Red Sea Salt (http://www.Aquaristic.net) 66.8, $NaH_2PO_4.1H_2O$ 0.276, glucose 0.870, acetate 0.204, glutamic acid 0.207, aspartic acid 0.254, alanine 0.166, serine 0.107, tyrosine 0.118, histidine 0.023, methionine 0.048 and trace element solution 0.5 ml l^{-1}. The trace element solution contained (g l^{-1}): $FeSO_4.7H_20$ 0.21, H_3BO_3 0.03, $MnCl_2.2H_20$ 0.1, $CoCl_2.6H_20$ 0.12, $NiCl_2.6H_20$ 0.024, $ZnCl_2$ 0.07, $Na_2MoO_4.2H_20$ 0.036, $CuSO_4.5H_2O$ 0.015. The alkaline medium (pH = 12.2 ± 0.2) contained (g l^{-1}): $NaNO_2$ 2.76 and $NaNO_3$ 0.34. Medium pH was adjusted with HCl or NaOH. The liquid volume in the culture vessel was 2.8 l. The pH and oxidation/reduction potential were monitored by gel-filled electrodes with an Ag/AgCl reference system (Mettler-Toledo, Giessen, Germany). To maintain anaerobiosis, the culture was continuously flushed with argon (purity > 99 998%, Air Liquide, Germany) at a flow rate of 10 ml min^{-1} dosed by a mass flow controller (Alicat Scientific, USA). The culture was mixed by pumping (1.2 l min^{-1}; pump: Watson Marlow 620S, USA) gas from the headspace through a sintered glass membrane inserted into the bottom of the culture vessel, from where it bubbled through the culture. To prevent growth of photosynthetic organisms, the culture vessel was covered with a neoprene cover.

Analytical procedures

Nitrite, nitrate, ammonium and dissolved organic carbon concentrations and protein content in the enrichment culture as well as dinitrogen production were measured as described in the supplementary information.

CARD-FISH

CARD-FISH was performed as described in the Supporting Information.

ARISA

DNA for ARISA was extracted using the protocol of Martín-Platero and colleagues (2007). The quantity of DNA was set to 10 ng per PCR amplification (quantified by a ND-1000 Nanodrop). The ARISA, including evaluation of the raw fingerprint profiles and binning into OTUs, was performed as described by Ramette (2009). Primers (ITSF/ITSReub) are listed in Supporting Information Table S4.

Sequencing, assembly, binning and annotation

DNA extraction and sequencing was performed as described in the supplementary information. Assembly was performed with METAVELVET-V0.3 (Supporting Information Table S2). Binning of assembled contigs was performed with the METAWATT BINNER according to Strous and colleagues (2012). More detailed information about the assembly and binning and information about full-length 16S rRNA reconstruction and phylogenetic analyses are described in the Supporting information.

Data submission

Illumina sequencing data sets were submitted to the Short Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/), and the assembled contigs were submitted to the Whole Genome Shotgun Submission Portal (https://submit.ncbi.nlm.nih.gov/subs/wgs/). Both reads and contigs are accessible under the BioProject PRJNA226580 and the BioSamples: SAMN02391470 / SRS498469 (day 6), SAMN02391471 / SRS498472 (day 18), SAMN02391472 / SRS498471 (day 33), SAMN02391473 / SRS498488 (day 46). This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accessions AYRE00000000, AYRF00000000, AYRG00000000, AYRH00000000. The version described in this paper is version AYR(E-H)01000000.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Note S1. Supplementary methods.

Fig. S1. Separation of contigs belonging to the most abundant populations in the samples of four different time points by combined tetranucleotide and interpolated Markov Model binning. Left panel: The distribution of the contigs on a sequencing coverage versus GC plot shows clouds each corresponding to a different bin and population. The contigs of the different populations were clearly separated from each other except for bin G and H, two *Vibrionales* subpopulations, and bin I, as well as bin D and K. They only show a small difference in GC content. The two separate clouds differing in coverage for bin E at day 46 may result from different subpopulations. The squares indicate the contigs, that encode the nitrite reductases of bin E (blue: nirS; red: nirK). Right panel: The pie charts show the taxonomic distribution of blast hits of fragmented contigs to reference genomes. The distance of each slice from the centre of the pie is a measure for the median e-value of the associated hits (larger e-value correspond to larger distances from the centre).The low number, relatively high e-values and scattering of blast hits obtained for bin A (Archaeon) is caused by the distant relationship of the bin to any available reference.

Fig. S2. Phylogenetic tree of bacterial 16S rRNA sequences. Phylogenetic affiliations were determined by maximum likelihood (RAxML). Bootstrap values were generated from 100 replicates. Bootstrap values higher than 50% are given. Sequences from this study are written in bold.

Fig. S3. Phylogenetic analysis of archaeal 16S rRNA sequences. Phylogenetic affiliations were determined by maximum likelihood (RAxML). Bootstrap values were generated from 100 replicates. Bootstrap values higher than 50% are given. The sequence from this study is written in bold.

Fig. S4. Phylogenetic analysis of nirS sequences (aa). Phylogenetic affiliations were determined by maximum likelihood (RAxML). Bootstrap values were generated from 100 replicates. NirN was used as outgroup. Bootstrap values higher than 50% are given. Sequences from this study are written in bold.

Fig. S5. Phylogenetic analysis of nirK sequences (aa). Phylogenetic affiliations were determined by maximum likelihood (RAxML). Bootstrap values were generated from 100 replicates. Bootstrap values higher than 50% are given. The sequence from this study is written in bold.

Table S1. Assembly results of the four sequenced metagenomes.

Table S2. Closest relatives (based on BLAST) to reconstructed 16S rRNA gene sequences.

Table S3. Squared Pearson product-moment correlation coefficients between bin coverages and 16S rRNA gene coverages over four samples. Rows: Bin coverages; Columns: 16S rRNA coverages.

Table S4. List of oligonucleotide probes and primers used.